ECOLOGICAL, BEHAVIORAL AND GENOMIC CONSEQUENCES IN THE RODENT FAMILY SCIURIDAE: WHY ARE SQUIRRELS SO DIVERSE?

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ECOLOGICAL, BEHAVIORAL AND GENOMIC CONSEQUENCES IN THE RODENT FAMILY SCIURIDAE: WHY ARE SQUIRRELS SO DIVERSE?

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EDITORIAL
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Editorial: Ecological, Behavioral and Genomic Consequences in the Rodent Family Sciuridae: Why Are Squirrels So Diverse?

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Editorial on the Research Topic

Ecological, Behavioral and Genomic Consequences in the Rodent Family Sciuridae: Why Are Squirrels So Diverse?

Rodents form the most diversified mammalian clade, but our knowledge about this intriguing animal group is centered around a few taxa, particularly the Muridae. However, another exceptional example of rodent diversification is the family Sciuridae (squirrels), the most speciose family after Muridae (murids). With >300 species living across the globe in a range of habitats such as deserts, high altitudes and rainforests, their diversity in morphology, ecology, and behavior is broad. Squirrels' lifestyles are extremely diverse—there are climbing, flying, and burrowing forms. Distinct species within a single genus can live in extremely different conditions, such as marmots or ground squirrels that range from arid to permafrost regions. Along with their outstanding diversity, sciurids play an important role in the formation and function of ecosystems. Many are considered keystone species and ecosystem engineers for their ability to influence the environment and create new habitat niches. Despite their importance, many populations have already experienced severe fragmentation and decline, mainly due to habitat loss, overhunting, and persecution as pests. Some squirrel species, however, are successfully surviving and even thriving in anthropogenic biotopes.

The underlying mechanisms and consequences of the diversification of squirrels remain poorly understood. To understand the selective forces influencing their evolution, it is crucial to broadly examine their behavioral, ecological, and morphological adaptations. As many members of the Sciuridae possess extreme features in relation to sociality, behavior, metabolism, and ecosystems, they are, in fact, model species for the functional and evolutionary underpinnings of unusual and extreme traits. This Research Topic explores the link between ecosystem adaptation and species diversification, in particular for squirrels that have gained little attention in the literature thus far.

Ecologically, squirrels live in habitats that vary in altitude, latitude, and extremes of environmental conditions from arid-adapted to arctic-adapted. For many species, resource availability is a driver for social systems and home range dynamics, and differences in the timing of these resources suggest that extrinsic factors rather than body size, influenced home range, and territoriality in two closely related tree squirrels (Mazzamuto et al.). Changes in habitat, particularly changes caused by human impacts, can lead to changes in species interactions, as Sovie et al. found using occupancy models to examine habitat use in North American and European red squirrels. Local adaptation to food resources is a form of ecological plasticity that may help species to cope with rapid habitat changes, such as anthropogenic transformation, as demonstrated for

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steppe marmots inhabiting the dry grasslands of Eurasia (Ronkin et al.). The high food selectivity of the European subspecies of the steppe marmot makes it very vulnerable to degradation of these grasslands with the cessation of grazing cattle. In contrast, the Kazakh subspecies has lower dietary selectivity and therefore allows it to respond more successfully to anthropogenic changes of grasslands.

While habitat change impacts steppe marmots, in North America, the opposite pattern of squirrels affecting habitat is seen in a study of the ecosystem engineering black-tailed prairie dog. This species is well-known as a keystone species in many prairie grasslands, but Hale et al. demonstrate they can also restore ecosystem functions, by limiting the expansion and proliferation of woody plants. Such tight-knit interactions between squirrels and their habitats are also reflected in a paper focusing on squirrels as predators and seed dispersers (Steele and Yi). While squirrels can negatively impact the seeds of many tree species, their scatter-hoarding behaviors are also critical in the dispersal of many trees and many tree species have co-evolved with squirrels in areas of high squirrel density to reduce the costs of squirrel predation.

For almost a decade the available genomic resources for squirrel species were limited to the thirteen-lined ground squirrel genome. However, recently several marmot genomes have been published (Alioto et al., 2019; Bai et al., 2019; Gossmann et al., 2019; Puiu et al., 2020). The study by Junker and Gossmann uses the available genomic resources of marmots to study the molecular signatures of a key aging protein family, Sirtuins. They find that SIRT1, a key player in human aging research, shows a molecular signature of adaptation which they argue might be linked to the extreme lifestyle of marmot species. The relevance of the genomic resources also becomes apparent in a natural hybrid zone of two marmot species, *Marmota baibacina* and *M. sibirica* (Brandler et al.). Although the study has only a limited marker number, it is the first evidence of hybridization of marmot species in the wild. Understanding the genomic underpinnings of natural hybridization is a major task in evolutionary and conservation biology and is particularly important for squirrels because of their role as keystone species. While hybridization is a key mechanism of increasing genetic complexity, it is predated by how species diverge. Two studies look at particular examples in squirrels. First, the study by Fiedler de Abreu-Jr et al. looks at diversification in South American tree squirrels, a potential textbook example of rapid species diversification. They find that the South American invasion was not as recent as previously inferred, but that the species diversification there was indeed quite fast and impactful. Second, Hinckley et al. investigate species diversification in Sundaland squirrels and identify allopatry as the main driver of diversification but also show that ecomorphological and behavioral adaptations were important and suggest this diversification as evidence for niche divergence. The diversifications in South American and Sundaland squirrels as well as hybridization in a secondary contact zone and adaptive extension of lifespan in marmots illustrate responses to global paleoclimatic changes. They are powerful examples that indicate the potential of squirrels to adapt and thereby not only maintain but also increase their species richness.

The need for clade-wide genome sequencing of squirrel species is not only fostering our knowledge on how this remarkable rodent family diversified but could also shed light on how important traits have contributed to it. The study of Sheets and Chavez investigates whether Gloger's rule, the prediction that darker colored animals are associated with environments that are warmer and more moist, is supported among squirrel species. Based on data from 137 species using phylogenetic and univariate regression methods they find that the rule generally applies. Identifying the genomic regions underlying Gloger's rule using genomic approaches would be the natural next step. However, establishing the molecular underpinnings of trait diversification on macroevolutionary scales is only feasible when large-scale genomic and phenotypic data are available (Yusuf et al., 2020). There is also an urgent need to complement large scale comparative studies with microevolutionary approaches, such as the study by Potash et al.. They investigate, using a non-genomic approach, pelage polymorphism in Easter fox squirrels and its relation to environmental heterogeneity—ultimately concluding that anthropomorphic influences will likely lead to a loss of pelage diversity. Newar and Bowman examine another important trait in their study—vocalization—and review how vocalization has been studied in squirrels and investigate possible explanatory variables of the evolution of vocalization. Indeed they find that open habitat and social complexity are associated with high frequency sounds, but body mass is associated with low frequencies.

A key question in evolutionary biology considers whether diversification of particular traits is associated with fitness benefits. Showing this in empirical data is often difficult, because many confounding factors may obscure a signal of fitness advantage. The study by Dobson et al. investigates different measures in Columbian ground squirrels and highlights that population dynamics and specific features such as longevity have to be accounted for when estimating fitness.

The diversity and breadth of adaptations in the Family Sciuridae support this group's potential to gain insight into our natural world. These large rodents (relative to the size of most rodents) that are primarily diurnal, may allow us to address genetic, behavioral, and ecological questions that are more difficult in their smaller more cryptic cousins. The studies in this special topic point the way to new questions and highlight the potential of this group as excellent models for studies in evolutionary biology and ecology.

AUTHOR CONTRIBUTIONS

JW and TG drafted the manuscript. All authors contributed to revising and finalizing the manuscript.

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Ecological Drivers of Eastern Fox Squirrel Pelage Polymorphism

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The color patterns of an animal's pelage, feather, or skin serve a variety of adaptive functions; importantly, one function is concealment through background matching. In spatially and temporally heterogeneous environments, some species exhibit multiple distinct color patterns within a population (i.e., color polymorphism). The environmental drivers of color polymorphism are poorly understood. We used the polymorphic eastern fox squirrel (*Sciurus niger* ssp.; hereafter, fox squirrel) as a model species to investigate the role of environmental factors on pelage coloration. Building upon previous research that investigated the drivers of melanism, we measured fox squirrel pelage coloration across the visible light spectrum. Agouti-colored squirrels were positively associated with increased proportion of burned area in a fox squirrel dispersal buffer. Light-colored (less melanistic) squirrels were positively associated with increasing proportion of cropland in a fox squirrel dispersal buffer. We posit that agouti pelage is broadly adapted to a range of heterogeneous conditions created by fire. Conversely, croplands, once established, are relatively stable ecosystems which promote a consistently adaptive light-colored pelage morph. We suggest that in an increasingly human-dominated environment, spatially and temporally homogeneous processes, such as prescribed burning, may not sufficiently recreate environmental heterogeneity, which could result in lost pelage diversity.

Keywords: adaptation, color, fire, fox squirrel, pelage, polymorphism

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INTRODUCTION

One of the most distinct forms of animal diversity is the variety of color patterns animals exhibit. The color patterns of an animal's pelage, feather, or skin serve a variety of adaptive functions such as concealment, communication, and thermoregulation (Caro, 2005; Ancillotto and Mori, 2017; Cuthill et al., 2017). Animal coloration patterns are an evolutionary response to selective forces that promote fitness-maximizing color patterns (Cloudsley-Thompson, 1999; Caro, 2005). In prey species, pelage coloration can reduce predation risk, often through concealment, thereby reducing an animal's likelihood of being identified by a predator (Krupa and Geluso, 2000; Boratyński et al., 2014). In heterogeneous or changing environments, a prey species with a consistent color pattern may be maladapted (Kettlewell, 1955; Mather, 1955). Accordingly, color polymorphism is often associated with spatial and temporal environmental heterogeneity that promotes multiple strong selective forces on coloration (Ford, 1945; Mather, 1955; Merilaita et al., 1999;

Bond and Kamil, 2006; Gray and McKinnon, 2007). Understanding the specific environmental factors that influence pelage coloration provides insight into the adaptations of polymorphic species and the ecological factors that promote the persistence of polymorphism.

Eastern fox squirrels (*Sciurus niger* ssp.; hereafter, fox squirrel) found in the southeastern United States are a model species for polymorphism research in heterogeneous environments (i.e., fire-prone pine [*Pinus* spp.] forests). The fox squirrel is the most variably colored mammal in North America (Hall, 1981), such that individuals are identifiable using their unique pelage patterns (Tye et al., 2015). In the southeastern United States, fox squirrels typically have gray or agouti dorsal pelage, with varying amounts of melanism (Moore, 1956). Agouti pelage has multiple colors on individual hair follicles, and often appears as a mottled brown color. Fox squirrels show greater variation in ventral coloration, which can be cream, red, beige, or black (Moore, 1956). Broadly, fox squirrels in Florida can be classified into one of six color morphs: gray, buff/tan, agouti with partial dorsal melanism, black-bellied, dorsal melanism, and melanistic (**Figure 1**; Florida Fish and Wildlife Conservation Commission, 2013). Fox squirrels commonly exhibit countershading, a pelage pattern where an animal is dorsally dark and ventrally light (Steele and Koprowski, 2001). Countershading may conceal animals from above and below, especially in well-lit environments (Caro, 2005), though evidence for countershading as an adaptive coloration is mixed (Kiltie, 1988; Ruxton et al., 2004; Yahner, 2012). Countershading is thought to be a more effective antipredator adaptation in marine environments, where predators swim above and below prey (Kiltie, 1988). Similarly, arboreal animals, such as fox squirrels, face predation threats from above and below (Koprowski, 1994), and may therefore benefit from countershading.

Previous research on fox squirrel color polymorphism in the southeastern United States suggests that pale-agouti dorsal pelage, defined as agouti pelage with no non-agouti black pelage, is most cryptic when static against a background of bark from multiple species of tree (Kiltie, 1992b) and in unburned environments (Kiltie, 1992a). Dark and intermediate dorsal coloration is more cryptic against burned backgrounds, and moving squirrels with partial dark dorsal pelage may be more difficult for avian predators to identify (Kiltie, 1992b). Melanistic fox squirrels are most concealed against fire-blackened substrates, but trees, other vegetation, and bare soil only remain blackened after a fire for approximately 2 weeks (Kiltie, 1992b). Two weeks is likely too brief for concealment to be the adaptive benefit that allows melanistic fox squirrels to persist (Kiltie, 1992b).

FIGURE 1 | Exemplars of the six fox squirrel color morphs found in Florida. From left to right, gray, agouti with partial dorsal melanism, buff/tan, black-bellied, dorsal melanism, melanistic.

Research on the ecological drivers of fox squirrel coloration show a positive correlation between dorsal melanism and historic lightning-caused wildfire frequency and temperature, and a negative correlation with elevation (Kiltie, 1989). Kiltie (1992b) suggested that dark coloration persists because dark squirrels are more difficult for aerial predators to identify, not because dark pelage is more cryptic in a fire-dominated environment. Historic fire patterns are only one possible ecological driver of squirrel polymorphism. Other ecological factors such as landcover (Kettlewell, 1955; Sumasgutner et al., 2018), precipitation/moisture (da Silva et al., 2016), and soil type (Krupa and Geluso, 2000) drive polymorphism in other species, and may therefore shape fox squirrel polymorphism. Furthermore, previous work on fox squirrels focused on the ecological drivers of dorsal melanism (Kiltie, 1989, 1992a), while explaining variation in fox squirrel pelage across the full color spectrum is likely to provide a more complete understanding of the evolutionary links between coloration and the environment. Color vision is important for hunting in some species of fox squirrel predators, including canids (Kasparson et al., 2013) and raptors (Håstad et al., 2005), suggesting that pelage darkness alone may not be enough to describe an animal's cryptic pelage adaptation.

The aim of this study was to identify environmental variables that drive fox squirrel pelage polymorphism. We hypothesized that fox squirrel pelage variation would correlate with the background color of the environment as an antipredator adaptation. We predicted that squirrels with agouti-colored pelage would positively correlate with fire frequency and burned area (Kiltie, 1989). Dark-colored pelage in rodents, including sciurids, is often associated with low-light environments, including closed-canopy forests and increased tree density (Wauters et al., 2004; Ancillotto and Mori, 2017). Thus, we predicted that squirrels with more melanistic pelage would positively correlate with closed-canopy forests where decreased light penetration through the canopy makes dark pelage more cryptic (Wauters et al., 2004; Ancillotto and Mori, 2017). Lastly, we predicted that countershading would negatively correlate with canopy cover as countershading is an antipredator adaptation in well-lit environments (Caro, 2005).

MATERIALS AND METHODS

Between 2012 and 2015, we collected roadkill fox squirrel specimens across Florida (**Figure 2**). To increase sample sizes and coverage throughout Florida, we enlisted the help of various wildlife agencies and citizen scientists to collect roadkill

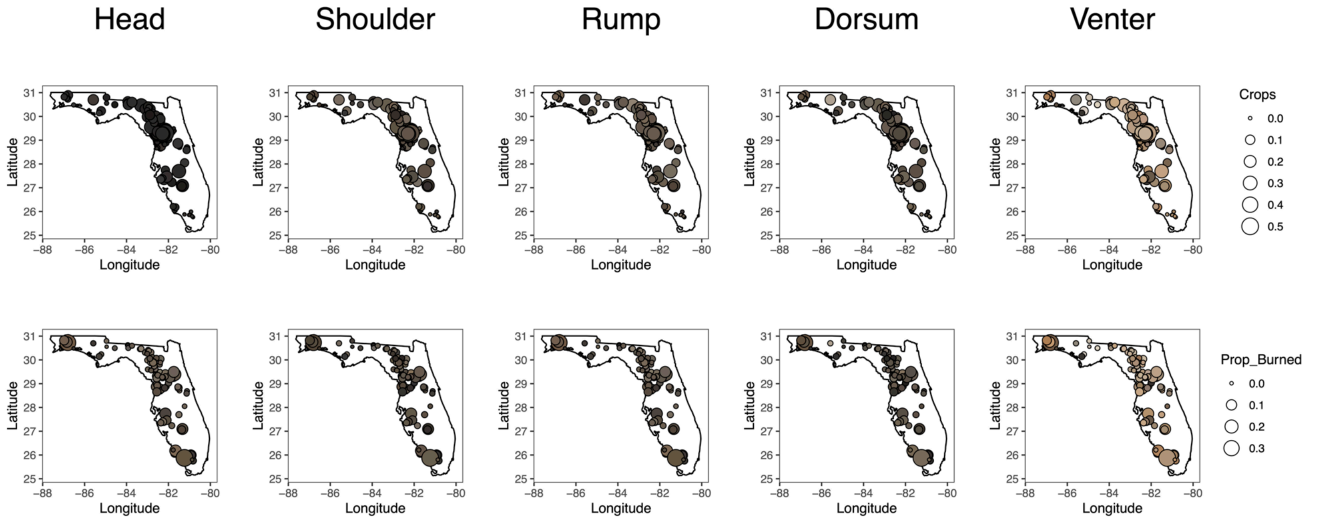

FIGURE 2 | Maps showing the locations of 135 roadkill fox squirrels collected throughout Florida used to investigate the role of environmental drivers of polymorphism. The fill color of each circle corresponds with the color measured at each location on an individual squirrel. The size of the circle corresponds with the proportion of cropland **(top)** and burned area **(bottom)** within a fox squirrel dispersal buffer.

fox squirrel specimens. Although our sampling design was opportunistic, we managed to collect fox squirrels throughout most of their range within Florida. We recorded the location of each roadkill specimen using a handheld GPS. To prevent the pelage color from fading, we kept all specimens frozen until they were prepared as flat skins and dried. We stored all specimens in a dark cabinet at the Florida Museum of Natural History (FLMNH).

We used a digital color sensor (Nix^{TM} Pro Color Sensor, Nix Sensor Ltd., Hamilton, ON, Canada) to quantify squirrel pelage color composition (Hodgen, 2016; Stiglitz et al., 2016; Holman et al., 2018). In a separate study, we found that the sensor gives highly correlated measures of fox squirrel pelage compared to traditional photography methods (Potash et al., 2020). The sensor measures the average color within a 15 mm diameter round aperture. We described and recorded our color readings using the Commission Internationale de L'Eclairages (CIELab) color space which uses three axes, L*, a*, and b* to define colors (Schanda, 2007). The L* axis measures brightness and ranges from 0 to 100, with higher values indicating a lighter color (i.e., L* = 0 is black and L* = 100 is white). Values on the a* and b* axes can be positive or negative, where at a* = 0 and b* = 0 corresponds to the color gray. The a* axis describes a continuous gradient where negative values indicate more of the color green, while positive values indicate more of the color red. The b* axis describes a continuous gradient where negative values indicate more of the color blue, while positive values indicate more of the color yellow.

We measured L*, a*, and b* values at five locations on each squirrel specimen. These locations were between the ears (head), centered between the front legs (shoulder), at the base of the tail (rump), midway between the shoulder and rump (dorsum), and laterally perpendicular to the dorsum along the edge of the specimen (venter; **Figure 3**). To increase the accuracy of our measurements, we averaged 7 repeated measurements at each location, removing and replacing the sensor at each location between each recording (Holman et al., 2018).

Environmental Data

We measured environmental characteristics that previous studies show influence pelage variation within a 3.7 km radius around each specimen (Greene and McCleery, 2017). The 3.7 km radius (hereafter, buffer) is the average dispersal distance of southeastern fox squirrels (Wooding, 1997). We measured seven environmental variables, the proportion of burned area between 1992–2015 (*Prop_Burned*), the number of fires that occurred between 1992–2015 (*Count_Fire*), the mean percent canopy cover (*Canopy*), the mean elevation in meters (*Mean_Elevation*), the mean annual precipitation from 1981–2014 in cm (*Precip*), the dominant soil order (*Soil*), and proportion of landcover type.

We chose proportion of landcover as a metric to describe the available habitat to each squirrel, and to test how much variation in pelage could be ascribed to habitat. We calculated the proportion of landcover type in each squirrel buffer using the 2011 National Land Cover Database (NLCD; Homer et al., 2015). To reduce the number of landcover types, we binned the original classifications into 19 classes based on the Florida Natural Areas Inventory (Florida Natural Areas Inventory, 2010; Tye et al., 2017). To further reduce the number of landcover types in our analysis, we only included landcover classes where fox squirrels have been observed in Florida, which were *Hardwood and Hammock*, *Pinelands*, *Crops*, *Coniferous Wetlands*, *Parks Cemeteries and Golf Courses*, and *Tree Plantations* (Greene and McCleery, 2017).

We selected *Prop_Burned* and *Count_Fire* as explanatory variables because previous studies have shown correlations between fox squirrel dorsal melanism and fire-blackened substrates (Kiltie, 1992b) as well as the number of historic wildfires (Kiltie, 1989). We calculated *Prop_Burned* and

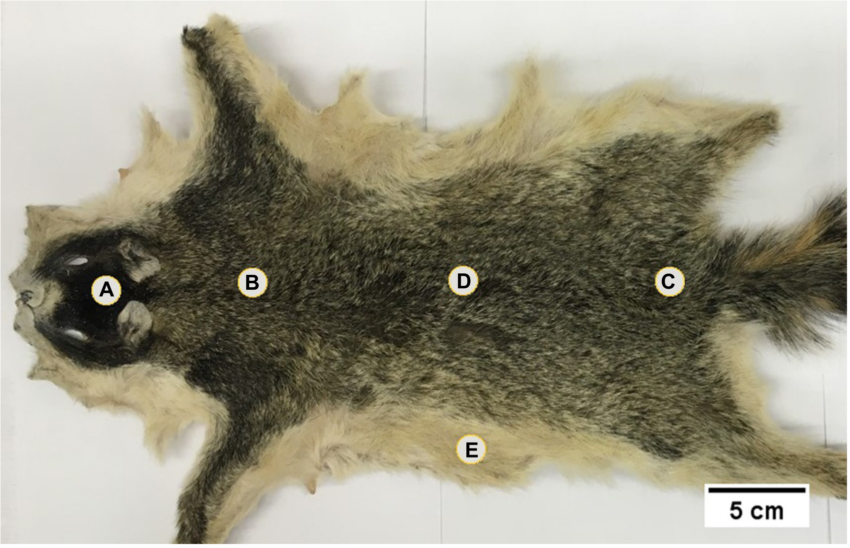

FIGURE 3 | An example of an eastern fox squirrel specimen used to investigate the environmental drivers of color polymorphism. Each circle, scaled to 15 mm diameter, shows the location where we measured coloration using a handheld digital color sensor (**(A)** = Head, **(B)** = Shoulder, **(C)** = Rump, **(D)** = Dorsum, **(E)** = Venter).

Count_Fire in each buffer using the spatial wildfire occurrence dataset from 1992–2015 (Short, 2017). The spatial wildfire occurrence dataset consists of ignition points for wildfires, each of which has an associated attribute for the final fire size. We created circular polygons around each ignition point such that the area of the circle equaled the fire size of the associated point and measured the amount of burned overlap in each squirrel buffer. In each buffer, we measured a burned area only once even if the same area burned on multiple occasions.

We included *Canopy* as an explanatory variable to describe the openness of the habitat, which influences dorsal pelage in rodents (Ancillotto and Mori, 2017). We calculated mean percent canopy in each squirrel buffer from the 2011 NLCD Tree Canopy Cartographic dataset (Homer et al., 2015). Each 30 m^2 pixel in this raster layer contains a single value for percent canopy cover, which we averaged for each squirrel buffer. We included *Precip* and *Elevation* as explanatory variables in our model because both variables have been correlated with fox squirrel pelage variation in previous studies (Kiltie, 1989). We used a digital elevation model (DEM; Gesch et al., 2018) with 30 m^2 resolution to calculate *Mean_Elevation* within a squirrel buffer. To calculate *Precip*, we averaged the annual precipitation from 1981–2010 (PRISM Climate Group, 2020) within a squirrel buffer. We retrieved soil data from the Web Soil Survey (USDA Natural Resources Conservation Service, 2020), and calculated the proportion of all soil orders in a fox squirrel dispersal buffer. To reduce the number of parameters in our model, we included only the most common (greatest proportion in a buffer) soil order for each squirrel in our analysis.

Statistical Analysis

To better understand the environmental variables that influence fox squirrel polymorphism, we subset the pelage color data into four non-exclusive groups. The first subset (Specimen, Full Color [SFC]) contained the L*, a*, and b* color data from all the locations we measured on each specimen. The second subset (Specimen, Brightness [SB]) included only the L* axis from each of the measured body components. This allowed us to explicitly test our prediction that darker pelage would positively correlate with increasing canopy cover in a fox squirrel's dispersal buffer. The third (Dorsum, Full Color [DFC]) and fourth (Dorsum, Brightness [DB]) data subsets also included the L*, a*, and b*, and the L* measurements only, respectively, but only used the three measurements from dorsal locations (shoulder, dorsum, rump). These subsets allowed us to compare our results to past research that focused only on fox squirrel dorsal pelage variation (Kiltie, 1989, 1992a,b).

To identify the environmental drivers of pelage variation, we conducted a redundancy analysis (RDA) for each subset of squirrel pelage color values. An RDA extends multiple regression to test relationships between a multivariate response matrix and multiple predictor variables. For analysis the response matrix was a squirrel-by-color measurement matrix for the particular group (i.e., SFC SB, DFC, or DB) and the response variables were the seven environmental variables we detailed above (van den Wollenberg, 1977). We standardized all continuous response and predictor variables to have a mean of 0 and standard deviation (SD) of 1. For each RDA, we used a forward selection approach to identify the model terms that create the most parsimonious model of predictor variables (Blanchet et al., 2008). Forward selection iteratively models individual predictor variables to the response variables, and additively combines significant predictor variables until additional predictors do not significantly improve model fit (R^2). We used $\alpha \leq 0.05$ for determining significant variables. Using both α and R^2 as stopping criteria in forward selection reduces type I error, a common problem with forward selection approaches (Blanchet et al., 2008). If forward selection identified more than one significant predictor variable, we used variance partitioning to quantify the unique amount of variation explained by each term (Peres-Neto et al., 2006).

For all RDAs we used a permutation test with 999 permutations to test for significance of the model terms and canonical axes (Legendre et al., 2011). We calculated an adjusted R^2 (R^2adj) using Ezekiel's formula (Legendre et al., 2011) to determine the overall amount of variation explained by each model. To ensure model results were not influenced by spatial autocorrelation (Tobler, 1970) we plotted residuals from each model as a function of spatial distance between specimens (Wagner, 2004). We found that model residuals showed no dependency on distance and model results were not affected by spatial autocorrelation (Wagner, 2004). We therefore did not include any spatial covariates in our model. We performed all multivariate statistical analysis in R Version 3.5.0 (R Core Team, 2018) using the package Vegan (Oksanen et al., 2017).

RESULTS

Pelage Values

We measured pelage coloration from 135 fox squirrel specimens. Fox squirrels showed the greatest variation along the L* axis (brightness), followed by the b* axis (blue to yellow coloration), and the least amount of variation along the a* axis (green to red coloration). Fox squirrels exhibited the least amount of variation overall in head coloration as indicated by the narrowest range and lowest SD along the L* (mean = 18.8 ± 3.10 SD), a* (mean = 0.50 ± 0.47 SD), and b* (mean = 1.00 ± 1.00 SD) axes (**Figure 4**). Fox squirrel venters had the widest range and largest SD for all color axes (L*: mean = 62.90 ± 11.40 SD; a*: mean = 5.40 ± 2.69 SD; b*: mean = 16.5 ± 5.57 SD). The three dorsal metrics, shoulder, dorsum, and rump were similar along the a* and b* axis (a^*_{Shoulder}: mean = 2.17 ± 1.09 SD; b^*_{Shoulder}: mean = 7.72 ± 3.20 SD; a^*_{Dorsum}: mean = 1.94 ± 1.06 SD; b^*_{Dorsum}: mean = 7.55 ± 3.41 SD; a^*_{Rump}: mean = 2.13 ± 0.98 SD; b^*_{Rump}: mean = 8.66 ± 2.84 SD). The L* value was similar for shoulder and dorsum (L^*_{Shoulder}: mean = 35.30 ± 8.34 SD; L^*_{Dorsum}: mean = 36.20 ± 9.58 SD), but was greater and had a lower SD for rump (L^*_{Rump}: mean = 40.40 ± 7.59 SD). Greater L* values for the rump compared to the dorsum and shoulder indicate that fox squirrel dorsal coloration lightens (decreased melanism) between the central portion of the dorsum and the base of the tail. Qualitatively, increased a* and b* dorsal values represent agouti-colored pelage, while lower L* values represent melanistic pelage (**Figure 4**).

RDAs

Using the SFC data subset, the forward selection identified the proportion of burned area (*Prop_Burned*) as the only significant model term ($p < 0.001$) in the RDA. Since there was only one predictor variable, there was only one RDA axis that explained pelage variation ($p = 0.003$). The model adjusted $R^2 = 0.03$. In general, a greater proportioned of burned area positively correlated with greater values of a* (increased red) and b* (increased yellow), and negatively correlated with increasing L* (lighter colors) for the venter (**Figure 5A**).

Using the SB data subset, the forward selection identified an additive model with the predictor variables *Crops* ($p = 0.01$), *Precip* ($p = 0.03$), and *Canopy* ($p = 0.014$) as the most parsimonious model. Using variance partitioning we found that *Crops*, *Precip*, and *Canopy* accounted for 3.8, 1.9, and 1.9% of the variation in pelage brightness, respectively. Only the first RDA axis explained a significant amount of variation in the pelage data (RDA1, $p = 0.002$). *Crops* loaded most on RDA1 (0.67) followed by *Precip* (−0.46) and *Canopy* (0.15). *Precip* loaded most heavily (0.81) on RDA2, but RDA2 did not explain a significant amount of variation in the data ($p = 0.31$). The model adjusted $R^2 = 0.05$. Along RDA1, increasing proportion of row crops was positively associated with increasing L* values for all body components except head (**Figure 5B**).

The forward selection for the DFC subset identified only *Prop_Burned* as a significant model term ($p < 0.001$). Since there was only one predictor variable, there was only one RDA axis that explained pelage variation ($p < 0.001$) with model adjusted $R^2 = 0.05$. Increasing *Prop_Burned* was most associated with increased rump a* coloration, as well as increased rump b* and dorsum a* (**Figure 5C**).

The forward selection for the DB subset identified only *Crops* as a significant model term ($p = 0.016$). Since there was only one predictor variable, there was only one RDA axis that explained pelage variation ($p = 0.009$) with model adjusted $R^2 = 0.03$. Increasing proportion of row crops was associated with increasing L* in shoulder, dorsum, and rump coloration (**Figure 5D**).

DISCUSSION

We found that variation in fox squirrel pelage coloration was associated with differences in spatial and temporal environmental heterogeneity. Fires can increase heterogeneity in the environment at a variety of scales (McKenzie et al., 1995), while cropland remains comparatively stable. Fire may therefore promote pelage variation while crops promote a more uniform light-colored pelage morph. Our results support previous findings that environmental heterogeneity shapes pelage polymorphism by manifesting a variety of different colored backgrounds for prey to match (Kettlewell, 1955; Merilaita et al., 1999; Bond and Kamil, 2006).

We found support for our prediction that agouti pelage was positively associated with fire (**Figures 3**, **5**). Previous research found that dorsally pale-agouti and intermediate-agouti (partial melanism) colored fox squirrels were superiorly camouflaged against a variety of backgrounds in fire-prone ecosystems (Kiltie, 1992a,b). Compared to other fox squirrel pelage morphs, agouti pelage is the best overall at background matching against the ground and common trees in the southeast (Kiltie, 1992b). The large amount of variation within the agouti morph may make it well-adapted for concealment in the spatially and temporally heterogeneous habitats found in fire-driven ecosystems (McKenzie et al., 1995). For example, in the open

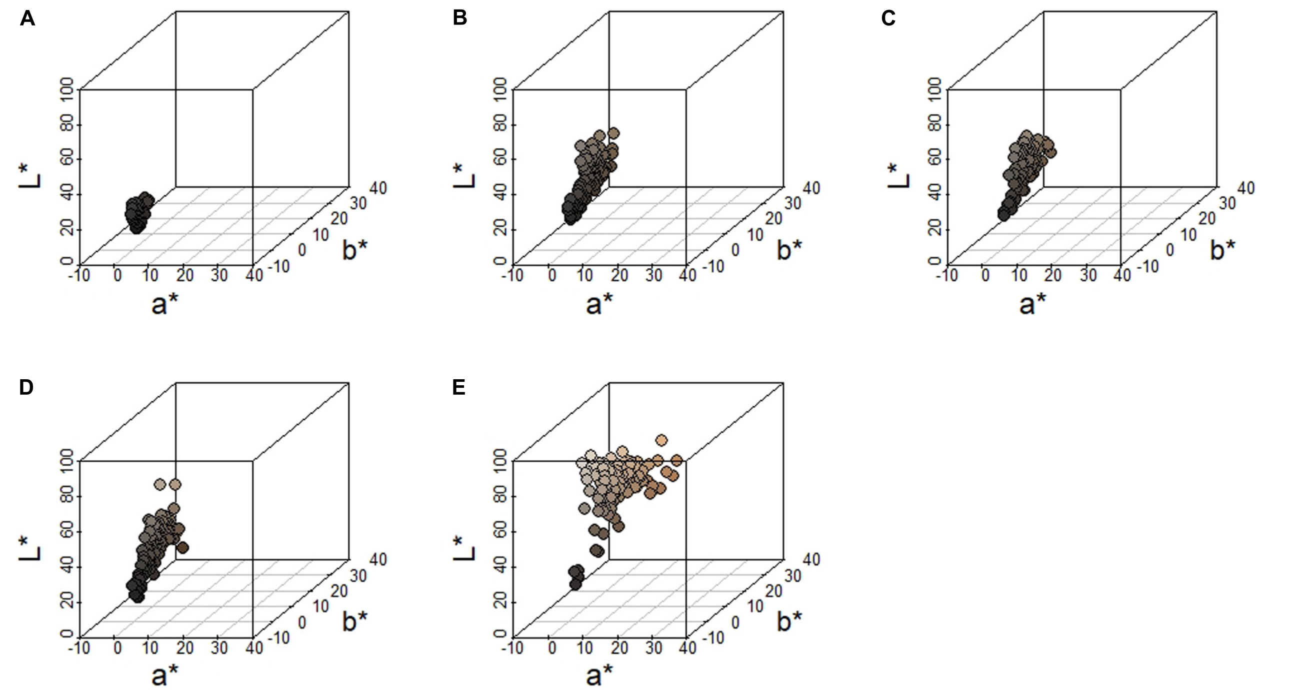

FIGURE 4 | Distribution of color measurements along the L* (dark to light), a* (green to red) and b* (blue to yellow) axes from eastern fox squirrels **(A)** head, **(B)** shoulder, **(C)** rump, **(D)** dorsum, and **(E)** venter. Each circle represents an individual squirrel and the fill color of each circle matches the color recorded.

pine forests of the southeastern United States, frequent fire can create a bare understory (Darracq et al., 2016), in which pale agouti rodents are most cryptic (Ancillotto and Mori, 2017). Fox squirrels in open pine forests rely on interspersed hardwoods for food and nest sites (Perkins et al., 2008), and hardwoods can create fire shadows which prevent complete burning (Jack and Pecot, 2017), increasing heterogeneity at a fine spatial scale. In contrast with burned areas, croplands in the Southeast are temporally and spatially stable, likely favoring a more consistent, uniformly colored population. We found that cropland was associated with decreased melanism in fox squirrels, possibly indicating that fox squirrels in human-engineered landscapes converge toward a uniform light-colored morph.

Dark-colored rodents are more cryptic in darker environments (Wauters et al., 2004; Ancillotto and Mori, 2017). However, we found no support for our prediction, that dark-colored pelage would be positively associated with closed canopy ecosystems. Fox squirrels are primarily found in open canopy forests (Moore, 1957; Weigl et al., 1989). Although fox squirrels use closed canopy hammocks occasionally (Weigl et al., 1989; Conner et al., 1999), periodic usage of closed canopy areas may not be enough to influence pelage coloration. Thus, fox squirrels may not use poorly lit closed canopy forests enough for dark pelage to have a cryptic advantage in these areas.

We did not find that countershading was associated with open canopy forest. Although forward selection identified canopy cover as a significant term in the SB model, canopy cover explained relatively little variation along the only significant RDA axis (RDA1). Furthermore, increased canopy cover was associated with lighter colored pelage in fox squirrel dorsal and ventral coloration (**Figure 4**). Countershading may not be an effective antipredator adaptation in terrestrial environments (Kiltie, 1988; Ruxton et al., 2004) and thus, countershading is unlikely to convey an antipredator advantage to fox squirrels.

Our analysis was limited by measuring pelage coloration in CIELab color space, which is based on human vision. Most animals have different visual acuity and color perception than humans (Endler, 1978; Cuthill et al., 2017), and therefore may not respond to the colors as we measured them; however, it was beyond the scope of this study to incorporate predator vision and behavior into our analysis. Our results explained only a small amount of pelage variation, suggesting that additional environmental factors not included in our analysis are likely driving pelage variation. Animal coloration is driven by additional factors other than predation risk (Smith et al., 1972), such as communication and thermoregulation (Stoner et al., 2003; Caro, 2005), hair strength (Bonser, 1995), or a combination of multiple factors (Cloudsley-Thompson, 1999). Fox squirrels are capable of frequent long-distance movements (Potash et al., 2018) and may therefore encounter a variety of ecological conditions that influence pelage coloration. Thus, there are likely many factors driving pelage coloration, preventing any individual variable we included in our analysis from explaining large amounts of pelage variation.

In this study we showed clear links exist between environmental heterogeneity and color polymorphism.

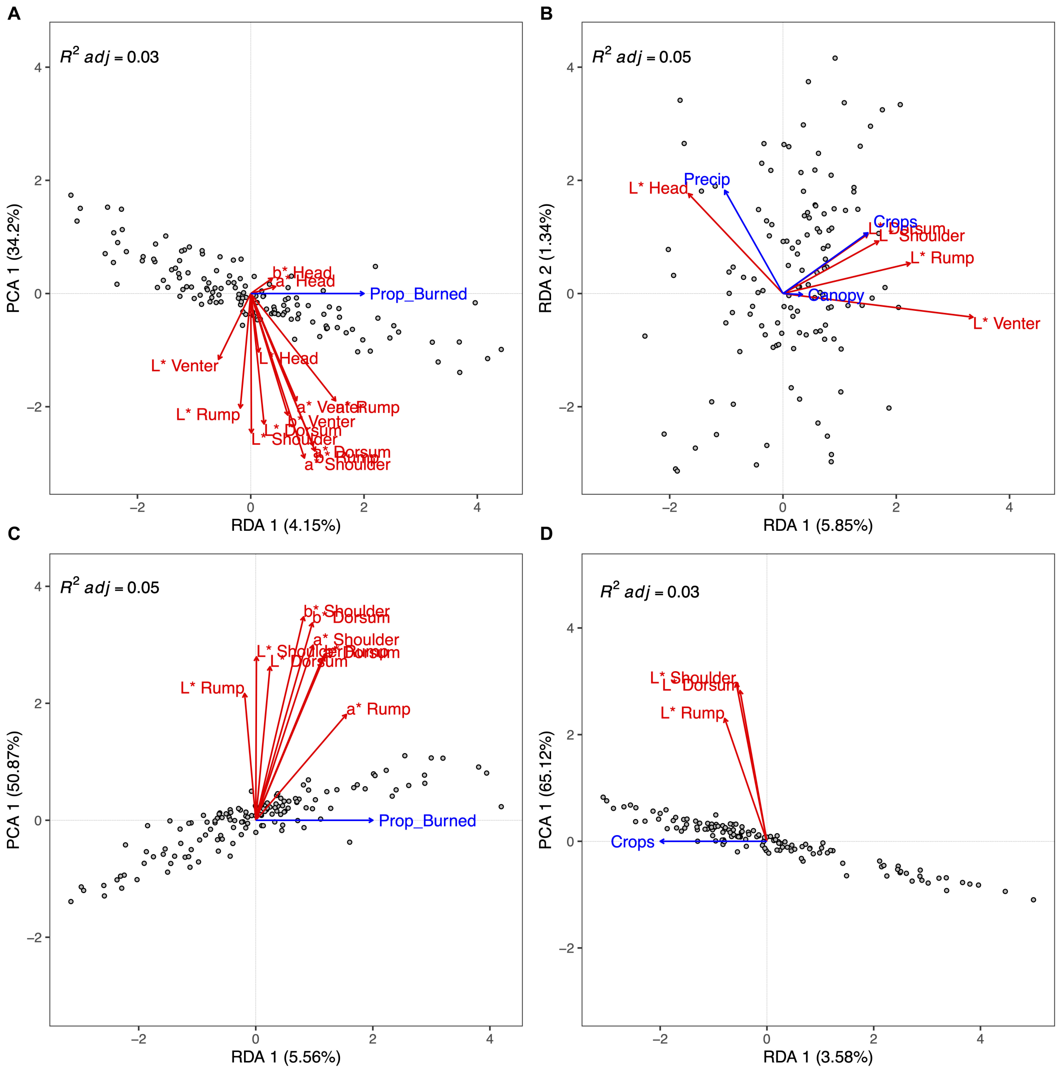

FIGURE 5 | Results of forward selection redundancy analysis (RDA) showing associations between ecological factors and eastern fox squirrel pelage color measurements at multiple locations on each specimen. Each plot shows a different, non-exclusive data subset (**(A)** = full specimen [head, shoulder, dorsum, rump, venter]), full color [L*, a*, b*]; **(B)** = Full specimen, brightness only [L*]; **(C)** = dorsal [shoulder, dorsum, rump], full color; **(D)** = dorsal, brightness only). The length of each environmental variable (blue arrow) along each axis correlates to the amount of variation that variable accounts for. Pelage measurements (red arrows) positively correlate with an environmental variable if both arrows stretch in the same direction. For plots **(A)**, **(C)**, and **(D)**, the *x*-axis shows the only significant RDA axis from forward selection, while the y-axis shows the first principal components (PCA). The PCA axis represents the residuals of the constrained ordination.

To preserve diverse animal color patterns, management and conservation efforts should consider the spatial and temporal scale of all management actions. Pelage adaptations to heterogeneity may be ineffective and lost in regularly managed ecosystems. Managing ecological processes, such as prescribed fire to promote heterogeneity rather than uniformity (Darracq et al., 2016) may be key for maintaining animal color diversity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the animals used in this study were previously collected opportunistically as roadkill and were part of the Florida Museum of Natural History's mammal collections.

AUTHOR CONTRIBUTIONS

AP, DG, LC, and RM conceived the original idea. AP, DG, and VM collected the data and developed data collection protocols. AP, BB, and RM developed the statistical analyses. All authors contributed to the final manuscript.

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ORIGINAL RESEARCH
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Timing of Resource Availability Drives Divergent Social Systems and Home Range Dynamics in Ecologically Similar Tree Squirrels

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Intraspecific variation in home range size has important implications for the distribution of animals across landscapes and the spatial structuring of population, community, and ecosystem processes. Among species of similar trophic guild and body mass, differences in home range size can reflect extrinsic variables that exert divergent selective forces upon spacing behavior and social organization. We tested predictions about how resource availability and timing influence social system, home range size, and territoriality in two tree squirrel species of similar size and ecological niches but that differ in foraging strategy and social organization. We estimated home range size and intraspecific home range core overlap in the Mt. Graham red squirrel (*Tamiasciurus fremonti grahamensis*; Arizona USA; MGRS) and the Eurasian red squirrel (*Sciurus vulgaris*; Alps, Italy; ERS) as functions of species, sex, season, and individual's body mass. However, body mass did not explain differences found between the two species. We found MGRS home ranges being three times smaller with higher core area exclusivity compared to ERS in all seasons. In fact, territorial MGRS evolved in a system of brief resource pulses and are larder hoarders, whereas ERS experience prolonged resource availability and are non-territorial. Only male MGRSs increased their home range during the breeding season, reflecting interspecific differences in social organization and mating behavior. Male ERS home ranges always overlap with several females to enhance mating success although male and female MGRS maintain nearly exclusive territories throughout the year. Only during spring and summer do males temporarily leave their food-based territory to increase mating opportunities with neighboring estrus females. Home range comparisons between ecologically similar species emphasize the importance of divergent extrinsic factors in shaping variability in body size–home range size scaling relationships. Timing in resource availability influenced the social structure and space use in tree squirrels of similar body size, highlighting how the coevolution of arboreal squirrels with conifer tree species has shaped their natural history.

Keywords: conifer-squirrel coevolution, home range size variation, Eurasian red squirrel, Mt. Graham red squirrel, foraging strategy, producer–consumer dynamics

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INTRODUCTION

Individuals may travel across a discrete area, or home range, on a daily basis or over longer intervals of time in search of limited resources that include food, shelter, nesting sites, and potential mates (Burt, 1943; Moorcroft, 2012). Home range is dynamic, and its size represents a key trait for individuals in a population with important implications for the distribution of animals across landscapes and ultimately the spatial structuring of community and ecosystem processes (Stamps, 1995; Van Beest et al., 2011; Tamburello et al., 2015).

At the species level, average home range size scales positively with body mass (Jetz et al., 2004; Tamburello et al., 2015), yet tremendous variability in home range size exists among species of similar body mass and among populations of the same species (Tucker et al., 2014; Ofstad et al., 2016). There is feedback among species-, population-, and individual-level drivers such that mean home range size for a species is a product of top-down and bottom-up processes (McLoughlin and Ferguson, 2000). At the species level, home range size is a product of slow, evolutionary processes (e.g., energetics and natural history in response to climate, the physical environment). Among populations, home range size is a product of moderate-to-slow processes that occur over generations (e.g., climate on local food availability and distribution of resources); among individuals in a population, home range size is a product of rapidly changing intrinsic and extrinsic drivers (e.g., resource availability, age, sex, and body condition) (McLoughlin and Ferguson, 2000).

Species-level extrinsic drivers of home range size also influence spacing behavior and social organization and are reflected in home range exclusivity and overlap (Maher and Lott, 2000). Jetz et al. (2004) developed a mechanistic model that demonstrates how animal space use reflects both energy requirements (body size) and degree of intraspecific competition (home range overlap). Larger animals have higher energy requirements and, consequently, need larger home ranges to find sufficient (food) energy (Jetz et al., 2004; Tamburello et al., 2015). Because larger home ranges and their resources are more difficult to defend against conspecific intruders, exclusivity of home range use (hence, territoriality) decreases with increasing body size (Jetz et al., 2004) or resource distribution, availability, and predictability (McLoughlin and Ferguson, 2000; McLoughlin et al., 2001). Hence, species of similar size and occupying comparable ecological niches can have different average home range sizes and home range exclusivity due to divergent extrinsic factors, such as the co-evolutionary history within a community, distribution and predictability of limited resources, and subsequent evolution of different spacing behaviors (Maher and Lott, 2000; McLoughlin and Ferguson, 2000; McLoughlin et al., 2001).

Herein, we sought to understand the role of important species-, population-, and individual-level drivers of home range size and overlap by comparing space use of two ecologically similar tree squirrel species: an endangered population endemic to southeastern Arizona in the United States, the Mt. Graham red squirrel (*Tamiasciurus fremonti grahamensis*; Hope et al., 2016), and the Eurasian red squirrel (*Sciurus vulgaris*) in the Italian Alps (Loy et al., 2019). Tree squirrels (genus *Sciurus* and *Tamiasciurus*) have remained virtually unchanged in form and function over the last 38 million years, a testament to their successful co-evolutionary history with forest ecosystems of the world (Smith, 1970; Steele, 2008). Both species occupy similar high-elevation montane coniferous forests that produce seed crops of comparable size but that are highly variable over time, typical for pulsed resource systems. The species differ slightly in body mass with adult Mt. Graham red squirrels in our study areas weighing 160–298 g (mean 234 g) and most adult Eurasian red squirrels in our study areas weighing 270–330 g (mean 304 g; Wauters et al., 2007a). Eurasian red squirrels have overlapping home ranges with more intensively used core areas, whose size differs between the sexes and in relation to season and habitat quality (e.g., Lurz et al., 2000; Wauters et al., 2001; Thomas et al., 2018). Males have larger home ranges than females and overlap with several females to increase their probability of reproducing. Females tend to defend exclusive core areas against other females (Wauters and Dhondt, 1992; Wauters et al., 2001; Di Pierro et al., 2008; Romeo et al., 2010). The mating system is promiscuous although most females only mate with a dominant male of high body mass (Wauters et al., 1990). In North American red squirrels (*T. hudsonicus* and *T. fremonti*), territoriality is the most common social system, particularly in coniferous forests in which individuals defend a central larderhoard or midden (Kemp and Keith, 1970; Rusch and Reeder, 1978; Steele, 1998). The Mt. Graham red squirrel maintains nearly exclusive territory core areas with considerable overlap at the home range periphery, primarily driven by males overlapping female home ranges in the spring–summer breeding season (Koprowski et al., 2008). In contrast, females minimize home range overlap with other females during all seasons. Home range size is 3–10 times greater in the Mt. Graham red squirrel compared to other ecologically similar populations of North American red squirrel (*T. hudsonicus*), and home range size varies seasonally for both sexes with the largest home ranges documented in summer (June–August) (Koprowski et al., 2008; Munroe et al., 2009).

Because both species share similar body size and food resources, we tested for the relative importance of season, sex, resource availability, and resource predictability on individual home range size and exclusivity across multiple years. At the species level, we predicted that (1) Mt. Graham red squirrels, being highly territorial, have smaller home ranges and core areas that are defended against conspecifics than non-territorial Eurasian red squirrels, and these patterns are true for both sexes. This difference between the species should hold true when taking individual variation in body mass into account. (2) Home range core area overlap is higher in Eurasian red squirrels than Mt. Graham red squirrels because the latter can better protect a smaller territory from intruders. (3) Home range size is larger in males of both species given that, in rodents with promiscuous or polygynous mating systems, males have larger home ranges than females. At the population and individual levels, we predicted that (4) home range size varies seasonally in both species using larger ranges in spring – a time of low food availability and increased breeding activity – than in autumn when the new seed crop becomes available. Finally, we predicted that (5) seasonal differences are largest in male Mt. Graham red squirrels as males often temporarily leave their food-based territory in the spring mating season to increase mating opportunities with neighboring females; because male Eurasian red squirrels already overlap with ranges of neighboring females, we do not expect to see big seasonal differences in their range size (Wauters and Dhondt, 1992; Koprowski et al., 2008).

MATERIALS AND METHODS

Study Areas

Eurasian Red Squirrel (ERS)

We conducted the study in four sites within mature, secondary montane and subalpine mixed conifer forests in the Italian Alps with elevations ranging from 1100 to 2150 m (the upper timberline; **Figure 1**). All areas are in the upper Valtellina Valley in the Central Alps. Specific locations and distances between study sites are presented in Trizio et al. (2005). Study site Oga (OGA, 47 ha) is part of a mature mixed montane conifer forest dominated by Scots pine (*Pinus sylvestris* L.) with some Norway spruce (*Picea abies* Karst) and larch (*Larix decidua* Miller). Bormio (BOR, 93 ha) is mature secondary forest dominated by Arolla pine (*Pinus cembra* L.). Cedrasco (CED, 76 ha) is a mixed montane conifer forest on the northern slope of the Orobic Alps with large areas of even-age stands dominated by silver fir (*Abies alba* Miller) and Norway spruce with sparse Scots pine, larch, and dead trees and some beech (*Fagus sylvatica* L.) at lower elevations (Salmaso et al., 2009). Site Cancano (CAN, 60 ha), located in a high-elevation valley, is almost entirely composed of a dense and homogeneous (dwarf) mountain pine (*Pinus mugo* Turra) subalpine woodland (Romeo et al., 2010). Seeds of all conifer species mature inside cones in September–October, and cones remain closed until the first warm and dry days of the following spring. The only exception is silver fir, which sheds mature seeds in autumn of the same year.

Mt. Graham Red Squirrel (MGRS)

MGRS are restricted to mature spruce–fir and mesic mixed-conifer forest in the Pinaleño Mountains of southeastern Arizona, the United States, 32.7017°N, 109.8714°W at elevations above 2400 m (**Figure 1**). The Pinaleño Mountains are part of the Madrean Archipelago, a series of high-elevation peaks rising from desert grassland that are considered biodiversity hot spots (Spector, 2002). In the Pinaleño Mountains, mixed-conifer forest is dominated by Douglas fir (*Pseudotsuga menzesii*), southwestern white pine (*Pinus strobiformis reflexis*), white fir (*Abies concolor*), corkbark fir (*Abies lasiocarpa* var. arizonica), Engelmann spruce (*Picea engelmannii*), and aspen (*Populus tremuloides*). Spruce–fir forest occurs at the highest elevations and is dominated by corkbark fir and Engelmann spruce (Smith and Mannan, 1994; O'Connor et al., 2014). We examined MGRS space use and food availability in both spruce–fir and mixed-conifer forest. Our spruce–fir study areas (529 ha, 3048–3267 m) were severely damaged by infestations of western balsambark beetle (*Drycoetes confusus*), spruce beetle (*Dendroctonus rufipennis*), and introduced spruce aphid (*Elatobium abietinum*) followed by the stand-replacing Nuttal–Gibson fire in 2004 (O'Connor et al., 2014), and conifer seed production was reduced (Zugmeyer and Koprowski, 2009). Our mixed-conifer study areas (190 ha, 2647–2979 m) were not as heavily impacted by insect infestations and fire during the study period (2002–2016).

Three dominant conifer species in the Pinaleño Mountain study areas have winged, wind-dispersed seeds that can be collected in seedfall traps: Douglas fir, Engelmann spruce, and corkbark fir. Each of these species has cones that ripen and shed seeds in the fall of the same year. Douglas fir cones ripen in late July–early August and shed seeds mid-August–mid-September. Engelmann spruce cones ripen August–early September and shed seeds September–late October although seed dispersal continues through the winter. Corkbark fir cones open in mid-August–mid-October and shed seeds from mid-September–late October, and cones disintegrate when ripe (Burns and Honkala, 1990).

Seed and cone count methods and estimated seed (cone) energy content of the conifer species in the Alps and Mt. Graham study sites are given in **Supplementary Material 1** (**SM 1.1**, **SM 1.2**). Estimated average seed energy availability over all study sites each year for the Alps and for Mt. Graham is presented in **Figure 2** and **SM 1.3**.

Trapping, Handling, and Radio-Tracking

Eurasian Red Squirrel

We conducted trapping in the Italian Alps during three periods per year (April–May, June–July, September–October) between 2000 and 2016. A trapping session involved the use of 20 (CED, BOR, CAN) or 25 (OGA) ground-placed Tomahawk live traps [Tomahawk Live Trap, WI, United States: models #201 (40.64 × 12.7 × 12.7 cm) and #202 (48.26 × 15.24 × 15.24 cm)]. We placed traps on a grid with distances of 100–150 m between traps and average trap density of 0.6–0.7 traps ha^{-1} except for BOR, in which traps were placed along a transect due to constraints of slope and forest composition. We pre-baited traps with sunflower seeds and hazelnuts four to six times over a 30-day period and then baited and set for 5–8 days until no new, unmarked squirrels were trapped for at least 2 consecutive days (Wauters et al., 2008). We checked traps two to three times per day.

Each trapped squirrel was flushed into a light cotton handling bag with a zipper or a wire-mesh "handling cone" to minimize stress during handling and individually marked using numbered metal ear tags (type 1003 S, 10 by 2 mm, National Band and Tag, Newport, KY, United States). We weighed squirrels to the nearest 5 g using a spring balance (Pesola AG, Baar, Switzerland) and determined sex and age class on the basis of external genitalia and body mass with juveniles weighing less than 250 g (Wauters and Dhondt, 1995). We fitted adult squirrels with radio collars with adjustable necklace transmitters (PD-2C transmitters, Holohil Systems Ltd., Carp, Ontario, Canada or TW-4 transmitters, Biotrack Ltd., Wareham,

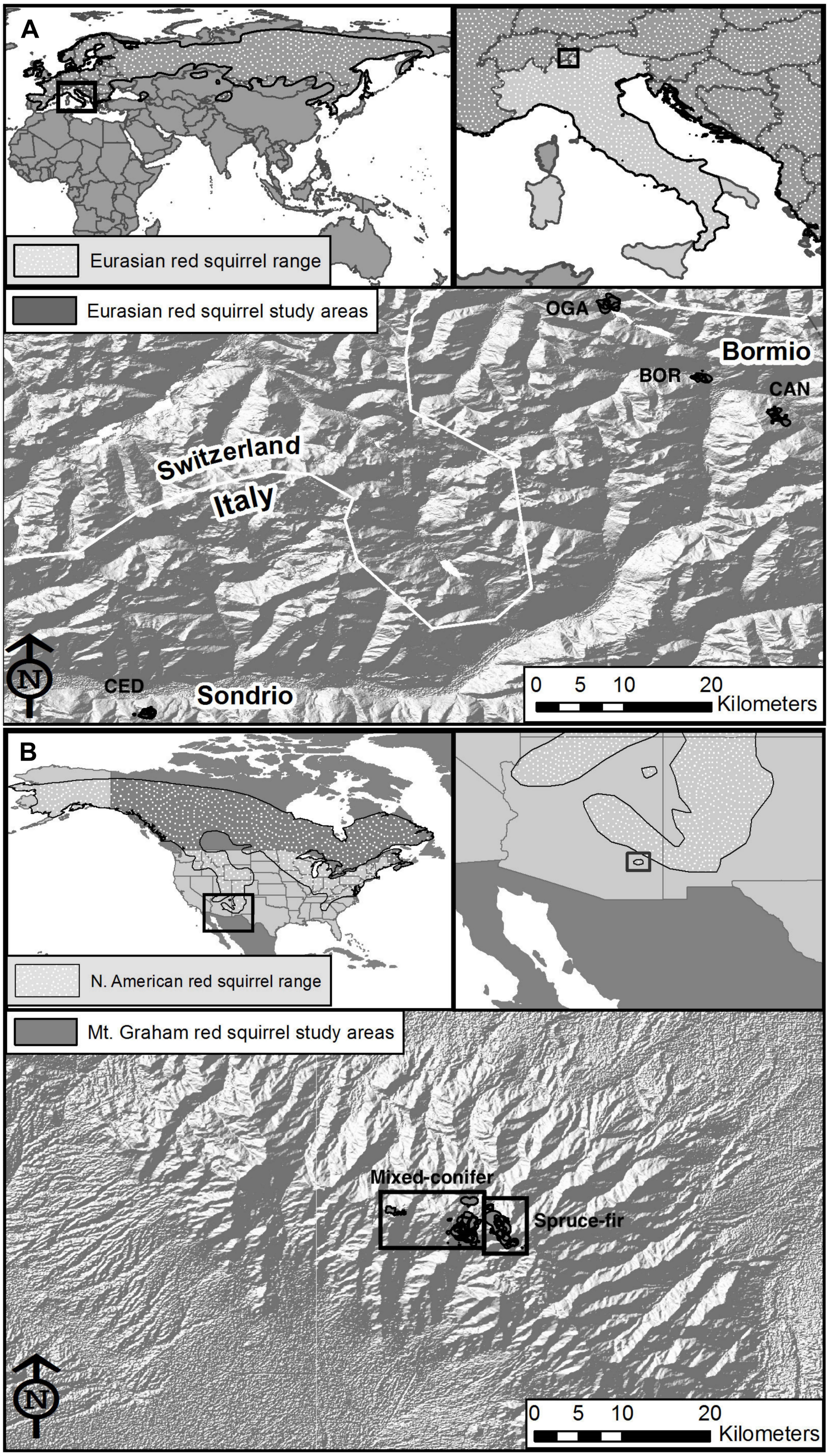

FIGURE 1 | Overview of study areas for Eurasian red squirrels (*Sciurus vulgaris*) in the central Italian Alps of Lombardy, Italy (**A**, top) and Mt. Graham red squirrels (*Tamiasciurus fremonti grahamensis*) in the Pinaleño Mountains of southeastern Arizona, the United States (**B**, bottom). Study areas are shown in relation to the geographic range of the species or, in the case of North American red squirrels, species group.

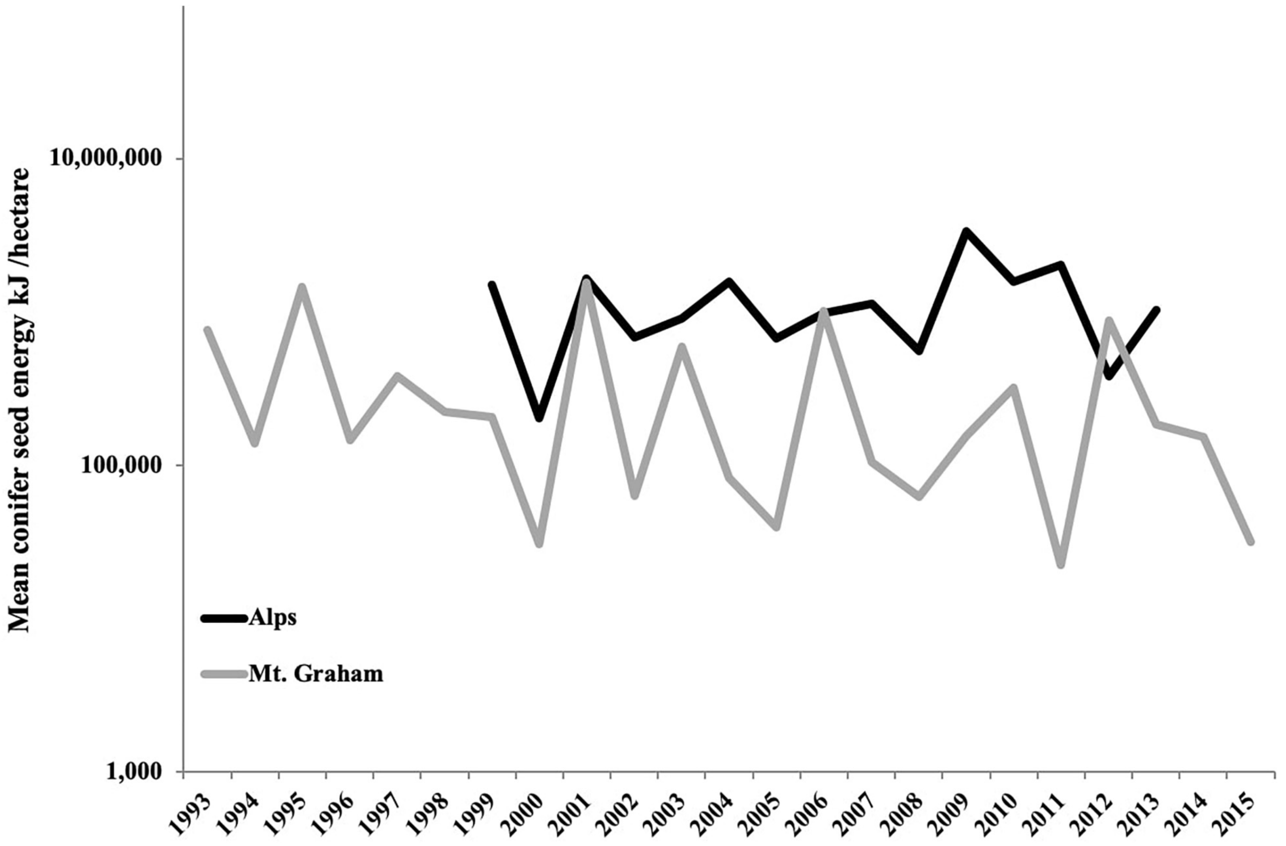

FIGURE 2 | Mean annual conifer seed production across study areas in the Alps (Lombardy, Italy: black line) and on Mt. Graham (Pinaleño Mountains, Arizona, the United States: gray line) expressed in kJ/ha. Seed energy is a measure of food resources available to Eurasian red squirrels (*Sciurus vulgaris*) and Mt. Graham red squirrels (*Tamiasciurus fremonti grahamensis*), respectively each year. More details provided in **SM 1.2–1.3**.

Dorset, United Kingdom), and we used homing-in to relocate all radio-collared animals. Tracking periods and radio-tracking materials are described in detail elsewhere (Di Pierro et al., 2008; Romeo et al., 2010).

Mt. Graham Red Squirrel

Between June 2002 and May 2016, we trapped, radio-collared, and tracked MGRS as part of a long-term study of MGRS space use (Koprowski et al., 2008). Systematic surveys within our Pinaleño Mountain study areas over the years and seasons (March, June, September, and December) allowed us to map all occupied middens (territorial larderhoards). We used Tomahawk live traps (model #201: 40.64 × 12.7 × 12.7 cm), baited with peanuts and peanut butter and placed at an animal's midden between 06:00 and 18:00 to capture MGRS. We checked traps every 2 h. Upon capture, we transferred each individual to a cloth handling cone (Koprowski, 2002) to measure morphological traits, apply colored ear tags, and fit radio collars (SOM 2190, Wildlife Materials International; Koprowski et al., 2008). We recaptured individuals at least every 3 months to assess body condition, reproductive status, and check radio collar fit. Methods for estimating age and reproductive condition were similar to those for ERS (Koprowski, 2005). Individuals $\geq$ 190 g were considered adults. We used homing and biangulation to relocate all radio-collared animals at least 12 times each month (Koprowski et al., 2008).

Statistical Analyses

All analyses were performed using R software 3.0.3 (R Development Core Team 2017). We used the 95% fixed kernel density estimator (KDE) with adjusted bandwidth (KDE_{adj} proposed by Wauters et al., 2007b) to calculate home ranges within the R package HRTools (Preatoni and Bisi, 2013). Use of the 95% isopleth when estimating home range size excludes outlying locations and avoids an overestimation of home range size. We used the 85% KDE_{adj} to estimate core area size because the utilization distribution curve of range size on percentage of fixes used showed a clear inflection point between the 85 and 90% isopleths (Wauters et al., 2007b). Although this estimator could potentially overestimate the size of core areas, it has the advantage that it can be used for both mono- and multinuclear core areas (Wauters et al., 2007b).

Per year, we estimated total averaged and seasonal home ranges: spring–early summer (March–July), which included mating and the main breeding season for both species (gestation, birth, and lactation for spring litters), and late summer–autumn

(August–November), the period when animals start feeding on maturing (August) and fully mature (September–November) seeds of the current year's cone crop.

Because the two species slightly differed in average body mass (ERS: 319 g; MGRS: 233 g), to follow the mammalian body mass–space use scaling rules, we applied the equation calculated by Tucker et al. (2014) for omnivores [Log (home range size) = −0.94 + 1.12 Log (body mass)] to our two study species so that we could predict their home range size based on their body mass and diet. We ln transformed home range size (ha) and core area size (ha) to meet the assumption of normality (Shapiro–Wilk's test W = 0.99). To discern the species–home range size relationship from the body mass–home range size relationship, we ran two competing LMM with ln home range size as the dependent variable. The first had species (ERS vs. MGRS), sex (male, female). and season (breeding season, seed season) as fixed effects and included the species by sex, species by season and sex by season interactions. The second had body mass, sex, and season as fixed effects and included the body mass by sex, body mass by season, and sex by season interactions. We ln transformed body mass following Ofstad et al. (2016). In both linear mixed effect models (LMMs), individual was added as a random factor to account for repeated measures of the range of a given animal. Because it was not our aim to explore variation in home range size among years and not all years had data for both species, year was added as a random factor and not as a fixed effect. Model fit was based on the difference in log likelihood between the two models. The same approach was used for the ln of the core area size.

We ran the models with the best fit for the home range size and core area size and used the differences of least square means (hereafter DLSM) with Tukey *p*-value adjustment for multiple comparisons to analyze pairwise comparisons of two-factor interactions and obtain effect-size estimates.

We estimated overlap of core areas (85% KDE_{adj}) as the percentage of overlap of a squirrel's core area with the core area of all other radio-collared squirrels in the population (Wauters and Dhondt, 1992). We used the square root transformation of total proportion of core-area overlap of each individual squirrel within a given season and year as dependent variables to meet the assumption of normality (Shapiro–Wilk's test W = 0.98). We tested our hypothesis of differences in core-area overlap between the species with an LMM with species (ERS vs. MGRS), sex of the individual, sex of the overlapper [four categories: male by males (MM), male by females (MF), female by males (FM), and female by females (FF)], and season (breeding season, seed season) as fixed effects and all the two-factor and the three-factor interactions. Individual was added as a random factor to account for repeated measures of the overlap of a given animal. Because it was not our aim to explore variation in core-area overlap among years and not all years had data for both species, year was added as a random factor. We used the DLSM with Tukey *p*-value adjustment for multiple comparisons to analyze pairwise comparisons of two-factor interactions.

RESULTS

Home Range and Core Area Size

We obtained 139 (87 squirrels: 51 males, 36 females) home range size estimates for ERS and 720 (260 squirrels: 128 males, 132 females) for MGRS (see **Supplementary Material 2** for detailed sample size per area, year, and season). On average, ERS home range size was three times larger than MGRS (mean ± *SD*: ERS = 20.92 ± 20.77 ha; MGRS 6.45 ± 11.20 ha). The formula by Tucker et al. (2014) predicted home range size of 3.18 ha for ERS and 2.25 ha for MGRS. Hence, based on their average body mass, ERS should have a 41% larger home range than MGRS. However, the model with the species as independent variable showed a better fit than the model with body mass for the home range size (difference in LogLikelihood = 32.26; $df = 1$; $p < 0.0001$).

Home ranges were larger during the breeding season than in the season of tree-seed maturation and consumption (**Tables 1**, **2**). Several pairwise comparisons were significant. Both male and female ERS had larger home ranges than male and female MGRS, respectively (DLSM: males ERS – MGRS = 1.19 ± 0.12, $t_{393} = 9.70$, $p < 0.0001$; females ERS – MGRS = 0.91 ± 0.14, $t_{426} = 6.69$, $p < 0.0001$; **Table 1**). The difference in home range size between the two species was consistent over both seasons (DLSM: ERS – MGRS breeding season = 0.98 ± 0.11, $t_{586} = 8.68$, $p < 0.0001$; ERS – MGRS seed season = 1.12 ± 0.11, $t_{607} = 10.3$, $p < 0.0001$; **Table 1**). Moreover, the difference in home range size between the sexes was significant only for ERS with male ERS occupying larger ranges than females and no difference between male and female home ranges in MGRS (DLSM: ERS males – females = 0.35 ± 0.15, $t_{417} = 2.30$, one-tailed $p = 0.049$; MGRS males – females = 0.07 ± 0.08, $t_{299} = 0.83$, $p = 0.84$).

Home range size was influenced by season in MGRS but not in ERS. Within-species comparisons of seasonal variation in home range size showed that ERS did not use larger breeding ranges than autumn (seed season) ranges (DLSM = 0.18 ± 0.10, $t_{612} = 1.80$, $p = 0.27$), but in MGRS, breeding ranges were much larger than autumn ranges (DLSM = 0.33 ± 0.11, $t_{562} = 7.71$, $p < 0.0001$) (**Table 1**). MGRS males and females respond differently to the breeding period as indicated by a significant sex by season interaction. Male MGRS enlarge their home ranges in the breeding season compared to seed season (DLSM = 0.45 ± 0.07, $t_{602} = 6.67$, $p < 0.0001$), and females do

TABLE 1 | Average home range size (ha, mean ± *SD*, sample size between brackets) of male and female Eurasian red squirrels and Mt. Graham red squirrels per season (breeding and seed) and independent of season (Total).

Period	Eurasian Red Squirrel		Mt. Graham Red Squirrel	
	Males	Females	Males	Females
Breeding	29.90 ± 31.14 (43)	14.46 ± 7.57 (28)	10.43 ± 15.41 (180)	5.49 ± 6.90 (208)
Seed	22.30 ± 14.79 (38)	12.32 ± 8.76 (30)	3.90 ± 5.81 (149)	5.70 ± 12.56 (183)
Total	26.33 ± 25.00 (81)	13.36 ± 8.21 (58)	7.48 ± 12.47 (329)	5.59 ± 9.94 (391)

TABLE 2 | Linear mixed model parameter estimates for important drivers of squirrel home range size (ln transformed).

Parameter	Estimates	*t*-test (df)	*P*
Intercept	2.98 ± 0.15	19.6 (69)	<0.0001
Species[a]	−1.12 ± 0.13	8.33 (508)	<0.0001
Sex[b]	−0.54 ± 0.16	3.45 (462)	0.0006
Season[c]	−0.38 ± 0.11	3.51 (614)	0.0005
Species * sex		1.64 (406)	0.10
Species * season		1.31 (608)	0.19
Sex * season		4.98 (570)	<0.0001

See section "Materials and Methods" for details. Adjusted df of t test with the Satterthwaite method. [a]ERS held as reference value. [b]Male held as reference value. [c]Breeding season held as reference value.

not (DLSM = 0.06 ± 0.07, $t_{581} = 0.94$, $p = 0.78$). Consequently, males use larger home ranges than females in the breeding season, but not in the (autumn) seed season (DLSM males vs. females breeding season = 0.40 ± 0.09, $t_{481} = 4.25$, $p = 0.0002$; seed season = 0.02 ± 0.10, $t_{503} = 0.19$, $p = 0.99$). Note that this effect is mainly due to MGRS whose males leave their territories for mating opportunities in the breeding season (**Table 1**).

The model with the species as independent variable showed a better fit than the model with body mass for the core are size (difference in LogLikelihood = 37.65, *df* = 1, $p < 0.0001$). Differences between the species, sexes, and seasons, and the above-described interactions persist when using 85% KDE_{adj} core area size (see **Supplementary Material 3**). Overall, core areas of male ERS were about four times larger than of male MGRS; those of female ERS were about two to three times larger than of female MGRS.

Core-Area Overlap

We used data from 1938 home range core overlaps from 408 different squirrels over a 16-year period. The type III ANOVA of the LMM model revealed that ERS had higher core-area overlap than MGRS [Species effect $F_{(1,\ 443)} = 39.2$, $p < 0.0001$; **Table 3**]. The sex of the individual by sex of overlapper effect was significant [male by males (MM), male by females (MF), female by males (FM), and female by females (FF)] [$F_{(3,\ 1286)} = 22.5$, $p < 0.0001$] although the season effect was not [$F_{(1,\ 1796)} = 0.42$, $p = 0.52$]. However, the interactions of species by sex of the individual by sex of overlapper [$F_{(3,\ 1326)} = 31.6$, $p < 0.0001$] and of season by sex of the individual by sex of overlapper [$F_{(3,\ 1619)} = 3.03$, $p = 0.029$] were statistically significant although the three-factor interaction was not [$F_{(3,\ 1619)} = 2.40$, $p = 0.066$]. Because of the species effect and significant interactions with species, we further analyzed the core-area overlap patterns for each species.

In ERS, patterns of core-area overlap differed by sex and with season (**Table 3** and **SM 4.1**). In the breeding season, female–female (FF) overlap was smaller than all other categories (DLSM, all *p*s < 0.001). In the seed season, FF overlap was smaller than female–male (FM) overlap ($p < 0.0001$), confirming intrasexual territoriality among adult female ERS. During the breeding season, ERS females were more strongly overlapped by males (FM) than males by males (MM) or males by females (MF) (DLSM, all *p*s < 0.001). During the seed season, there were no significant differences in proportion core-area overlap among the different combinations of the sexes (all *p*s > 0.05) except the abovementioned FF with FM.

In MGRS, percentage overlap of 85% KDE core areas was small and core-area overlap patterns between the sexes did not change with season (**Table 3** and **SM 4.2**). Males had a higher core-area overlap with other males than with females (DLSM both seasons, all *p*s < 0.0001). Male–female (MF) overlap was also slightly smaller than female–female overlap (DLSM both seasons, all *p*s < 0.05; **SM 4.2**).

DISCUSSION

In this study, we compared the space use of the Mt. Graham red squirrel and the Eurasian red squirrel, two species with similar body size and food resources but different foraging strategies and social organization. Because of the slight difference in body mass between the two species, we first tested the importance of body mass in explaining the variation in home range and core area size. The study by Tucker et al. (2014) predicted a much smaller difference than the threefold larger home ranges of ERS measured in this study. Note also that the home ranges used by both squirrel species were much larger than predicted values: on average, 21 ha or six times larger for ERS and 6.45 ha or three times larger for MGRS. The big discrepancy between the predicted and observed size of home ranges suggests how other factors, other than body mass and diet, could affect and define the space use of these species.

TABLE 3 | Average core-area overlap (%, mean ± *SD*, sample size between parentheses) of male and female Eurasian red squirrels (ERS) and Mt. Graham red squirrels (MGRS) by season (breeding and seed) and independent of season (total).

Sex	ERS			MGRS		
	Breeding	Seed	Total	Breeding	Seed	Total
FF	3.75 ± 11.23 (28)	4.73 ± 10.61 (28)	4.23 ± 10.84 (38)	4.80 ± 17.11 (131)	4.02 ± 15.61 (131)	4.41 ± 16.37 (170)
FM	11.39 ± 20.91 (28)	11.22 ± 24.24 (28)	11.29 ± 22.45 (38)	7.57 ± 22.35 (131)	6.01 ± 17.79 (131)	6.79 ± 20.21 (170)
MF	11.23 ± 22.75 (41)	14.03 ± 23.84 (34)	12.51 ± 23.15 (52)	3.16 ± 13.75 (125)	3.46 ± 14.64 (124)	3.30 ± 14.17 (154)
MM	11.90 ± 22.89 (41)	5.76 ± 12.27 (33)	9.13 ± 19.00 (52)	7.55 ± 19.87 (125)	7.78 ± 22.91 (124)	7.52 ± 21.39 (154)

Sex indicates the combination of sex of the animal and sex of the overlapping animals (FF, female by females; FM, female by males; MF, male by females; MM, male by males).

Moreover, because of analysis of the models' fit, body mass did not seem to specifically influence the variation in home range size, confirming a low importance of this trait in our study system and the comparable sizes of the two squirrels. We found, instead, that, although living in similar mixed-conifer ecosystems, the timing in food resource availability is very different between the western United States and the Italian Alps, and it has been a powerful selective force upon the social evolution and space use of the two squirrels. Masting, or the synchronous, episodic flood of resources, is a reproductive strategy in trees thought to have evolved as a co-evolutionary response to seed predation (Boutin et al., 2006; Steele, 2008). A slow process such as the physical environment and climate along with millennia in an active arms race with seed predator communities (e.g., Benkman, 2010) have driven divergent tree morphologies and life history strategies. In mixed-conifer forests of the western United States, such as on Mt. Graham, many conifers produce cones that ripen and release their seeds in autumn of the *same* year (Burns and Honkala, 1990). To capitalize on this highly ephemeral and pulsed resource (Boutin et al., 2006), squirrels in the genus *Tamiasciurus* clip the immature cones and cache them in middens before the cones open and release seeds. The moist microclimate of the midden preserves the closed cones, allowing access to a rich food supply throughout the winter and following spring (Smith and Mannan, 1994; Hurly and Lourie, 1997). In the Alps, cones from pine and spruce species do not open until mid or late April the *following* spring, permitting the ERS to feed on cones in the tree canopy during autumn and winter and feed on scatter-hoard cones (of pines) in late spring when no new cones are available in the trees (Wauters and Dhondt, 1987; Molinari et al., 2006). Hence, for ERS, food resources are more strongly spaced and cannot be defended against conspecifics, and this difference in food distribution and defensibility has ultimately led to the divergent space use patterns and social organization for the two squirrel species. The differences in resource timing, availability, and defensibility between the two forest ecosystems are reflected in species- and population-level patterns of home range size and home range overlap for the two squirrels, demonstrated by the fact that across sexes, MGRS had smaller, exclusive home ranges and core areas compared to ERS, supporting our first two predictions.

At the population and individual levels, resource availability, timing of breeding, and the sex of individuals can exert seasonal influences on home range size and exclusivity. Social interactions have an important role in shaping animal movements, especially during the breeding season, when one or both sexes might need to adapt their space use to encounter potential mates (Madison, 1980; Lazenby-Cohen and Cockburn, 1991; Van Beest et al., 2011). Thus, we hypothesized that home ranges would be smaller for both species in autumn when the new seed crop becomes available and larger for both species during the spring breeding season (prediction 4). During spring, we predicted that MGRS would range farther in search of mates, and ERS would range farther primarily in search of scatter-hoarded food. However, we found that only male MGRS increased their home range during the breeding season. In contrast, we did not find any seasonal variation in the home range sizes of either male or female ERS. The difference between the two species can be explained by their different social organization and mating behavior. Male ERS home ranges always overlap with several females to increase their chances of reproducing during the breeding season when three to five neighboring males engage in mating chases (Wauters et al., 1990). The dominant male tends to defend the estrus female from lower ranked competitors and obtains the majority of matings, but in some cases, females accept or even solicit copulations by subordinate males (promiscuous mating system with male-defense polygyny; Schwagmeyer, 1990; Wauters et al., 1990; Waterman, 2008). In contrast, male and female MGRS maintain nearly exclusive territories throughout the year with the exception of spring and summer breeding forays by males, wherein they temporarily leave their food-based territory to increase mating opportunities with neighboring estrus females (Koprowski et al., 2008; Lane et al., 2009). During the mating chase, many males can fight to obtain copulations with the estrous female: a scramble-competition mating system (Lane et al., 2009). This relationship between space use and mating system is demonstrated by male MGRS having larger home ranges than females during the breeding season (partially supporting predictions 3 and 5) although this pattern was not observed for ERSs.

Seasonality and sex of individuals in the population also influenced home range exclusivity as reflected in patterns of core-area overlap between sexes of both species. Analysis of core-area exclusivity also allowed us to test the degree to which species-specific social systems (territorial vs. non-territorial species) are upheld for the populations studied here. In general, core-area overlap among ERS was much higher than observed in MGRS except for ERS F-F overlap. Low intrasexual overlap among female ERS (F-F) supports marked female intra-sexual territoriality documented in previous studies (Wauters and Dhondt, 1992; Di Pierro et al., 2008; Romeo et al., 2010). Female ERS defend exclusive core areas against members of the same sex because their home range is strictly related to food resources and habitat quality. Defense of an exclusive core area is important for successful reproduction as only dominant females are capable of rearing offspring (Wauters and Dhondt, 1989, 1992). In MGRS, core-area overlap was consistently low across seasons. Although we found an increase of MGRS male home range size during the breeding period, the core-area size remained constant throughout the year for both sexes with little F-M overlap. The stable size of the core area can be explained by tree squirrel reproductive physiology and the scramble-competition mating system of MGRS. Female tree squirrels are in estrus for about 1 single day (often even less than 24 h) during a breeding season of 2–4 months. As a result, females are relatively asynchronous, and males appear to monitor reproductive status by olfaction (Gurnell, 1987; Arbetan, 1992; Steele and Koprowski, 2001). Dozens of males can be attracted to a female about 5 days before estrus, e.g., Thompson (1977), so the fast excursions of males into female territories may not affect core-area size and relative overlap because males keep their center of activity around their middens to defend the food resource from conspecifics

(Gerhardt, 2005; Donald and Boutin, 2011). Thus, population-level patterns of territoriality in MGRS and non-territoriality in Italian ERS are similar to species-level patterns (prediction 2). Although seasonality and sex of individuals do contribute to variation in home range size and exclusivity at the population level, a long co-evolutionary history in ecosystems with different timing in and defensibility of resources appears to be the dominant driver in the divergent foraging, social, and mating systems of the two species. The differences in foraging, social, and mating systems, in turn, drive species-level differences in home range size and exclusivity.

We capitalized on long-term data sets (Arregoitia et al., 2018) available for two ecologically similar species allowing us to test the importance of individual-, population-, and species-level ecological drivers (McLoughlin and Ferguson, 2000) of home range size and exclusivity. Our models took into account the heterogeneity of our data set with larger sample sizes over a longer time period for the MGRS to avoid any bias in our results. Moreover, because of the important role of climate and food resources in shaping animal movements over short and long periods of time (e.g., Lurz et al., 2000; Van Beest et al., 2011; Morellet et al., 2013), our models considered possible annual fluctuations. Although tree species are different, the two forest ecosystems produce similar food items in terms of range in cone and seed size and range in energy content, and yearly fluctuations in seed production are of the same order of magnitude in both the Alps and Mt. Graham (**Figure 2**; see also **Supplementary Material 1**). In future studies, we will test how annual fluctuations in conifer seed production affect home range size of both species over time and relate annual variation in home range sizes to temporal trends in climate, habitat disturbance, and habitat loss.

CONCLUSION

Tree-life history strategies dictate the availability and predictability of resources – strong selective forces that have contributed to divergent foraging and social systems among tree squirrels (e.g., territorial vs. non-territorial; larder hoarding vs. scatter-hoarding) (Smith, 1970; Steele, 2008). Such species-level constraints on spacing and foraging are ultimately reflected in species-specific patterns of home range size and exclusivity (McLoughlin and Ferguson, 2000) and contribute to observed deviations from the mean home range–body mass scaling relationship for ecologically similar species of slightly different body size.

Although faster processes, such as seasonality and annual fluctuations in the availability and quality of resources at multiple scales, are important drivers of individual- and population-level home range dynamics (Maher and Lott, 2000; McLoughlin et al., 2001; Van Beest et al., 2011), it is important to consider the co-evolutionary history of species within their respective ecosystems to understand higher-level constraints on home range size, foraging strategy, and social systems (Ofstad et al., 2016). Processes such as the co-evolution between animals and their resource base and competition for resources (Connell, 1980; Benkman, 2010) lead to niche differentiation and speciation and are likely the ultimate factors driving the variation in home range size for ecologically similar species of comparable mass.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the all field work on Mt. Graham red squirrels was conducted under the University of Arizona Institutional Animal Care and Use Committee protocol # 08-024, Arizona Game and Fish Department scientific collecting permit # SP654189, U.S. Fish and Wildlife Service permit # TE041875-0 and adhered to the American Society of Mammalogists guidelines for the use of wild mammals in research (Sikes et al., 2016). All field work on Eurasian red squirrels in Italy was conducted by the University of Insubria staff under the permits: decree 1488 of 14/07/2000, decree 7489 of 29/04/2002, decree 5663 of 05/04/2004, decree 2456 of 07/03/2006, and decree 1702 of 23/02/2009 of Direzione Generale Agricoltura of the Lombardy Region, Italy.

AUTHOR CONTRIBUTIONS

LW, AM, and JK collaborated to develop the framework and ideas in this manuscript, managed, and obtained funding for long-term ecological studies, and gave final approval for publication. FB, LW, MJM, and MVM compiled the long-term data sets. FB and MVM analyzed the data. MVM, MJM, and LW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00174/full#supplementary-material

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Ancient Divergence Driven by Geographic Isolation and Ecological Adaptation in Forest Dependent Sundaland Tree Squirrels

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A surprising amount of hidden phylogenetic diversity exists in the small to medium size, drab colored squirrels of the genus *Sundasciurus*. This genus is endemic to Sundaland and the Philippines, where it is widespread. An earlier revision of this genus found that the high elevation 'populations' of the widespread, lowland slender squirrel (*S. tenuis*) were different species. Previous phylogenies based on mitochondrial cytochrome *b* sequences also suggested that the widespread, lowland Low's squirrel (*S. lowii*) and the narrow endemic Fraternal squirrel (*S. fraterculus*) are not reciprocally monophyletic. Additionally, deep divergences have been identified between lineages within Low's squirrel that date to the early Pliocene. Here we focus on evaluating the relationships and differences within and between populations of these two nominal species using whole mitochondrial genome sequences, nuclear intron sequences, and morphology. We reassess the taxonomy of this group, revalidate the species status of Robinson's squirrel (*Sundasciurus robinsoni* Bonhote, 1903) support the species level recognition of the Natuna squirrel (*Sundasciurus natunensis* Thomas, 1895) and identify three other lineages that require further study. We estimate times of divergence and integrate geologic history to find that most of the divergences are pre-Pleistocene, and thus predate the Pleistocene flooding of Sundaland. Biogeographic, and ecological factors may have played a more important role than climatic factors in generating these patterns. While divergence in allopatry seems to be the main process driving speciation in lowland Sundaland squirrels (*Sundasciurus*), ecomorphological and behavioral adaptations in this clade suggest an important role of niche divergence.

Keywords: ***Sundasciurus*, mammal, Borneo, biogeography, speciation, systematics, rodent, Sciuridae**

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INTRODUCTION

The small brown tree squirrels of the genus *Sundasciurus* are endemic to Sundaland and the Philippines. This genus was described by Moore (1958) based on the presence of a Y-shaped trans-bullar bony septum and includes species previously assigned to *Sciurus* and *Tomeutes* (Thomas, 1915; Chasen, 1940). Most species were widespread across multiple landmasses in Sundaland. This genus has received more attention than other genera in the subfamily (Callosciurinae/Nannosciurinae). Deep genetic divergences associated with habitat (such as highland or lowland forest) or geography (such as land mass) were obscured by conservative morphology and have resulted in multiple revisions of the genus in the last few years, increasing the number of species from 11 to 17 (Heaney, 1979; Den Tex et al., 2010; Thorington et al., 2012; Hawkins et al., 2016a). These latest systematic revisions have largely focused on high elevation 'populations' of widespread taxa which in all cases turned out to be very divergent and merited specific status. The recognition of these species was supported by both genetic and morphological lines of evidence. The genus was divided into two subgenera by Moore (1958). These subgenera, *Aletesciurus* and *Sundasciurus,* were defined based on morphological criteria (presence/absence of a sagittal crest, an inconspicuous/inflated antero-mesial lobe, and skull size), but these were not found to be reciprocally monophyletic with molecular data (Den Tex et al., 2010; Hawkins et al., 2016a). At that time, taxonomic rearrangements were not performed because of limited sampling and low support of key nodes in the tree, suggesting that additional data were necessary. The subgenus *Aletesciurus* contains one widely distributed species (*Sundasciurus hippurus* of the Malay Peninsula, Sumatra, and Borneo), a narrowly distributed Bornean mountain endemic (*S. everetti*), and nine species distributed in the Philippines (*S. samarensis, S. philippinensis, S. mindanensis, S. rabori, S. steerii, S. moellendorffi, S. davensis, S. juvencus,* and *S. hoogstraali*) (Chasen, 1937; Den Tex et al., 2010; Hawkins et al., 2016a). The subgenus *Sundasciurus* includes seven species restricted to Sundaland in two clades: a mainly highland clade on a long branch (*S. altitudinis, S. brookei, S. jentinki, S. tahan,* and *S. tenuis*) and a widespread lowland clade sister to the other two (*S. fraterculus* and *S. lowii)* (Thorington and Hoffmann, 2005; Den Tex et al., 2010; Thorington et al., 2012; Hawkins et al., 2016a). Many of these widespread species contain multiple subspecies described from a limited number of specimens that were generally identified by differences in pelage coloration (Corbet and Hill, 1992; Thorington et al., 2012). In order to evaluate the diversity in the genus and uncover other unidentified lineages, we used populations as the unit of analysis.

Despite recent research on the phylogenetics of some of the members of the genus *Sundasciurus*, less attention has been paid to lowland Sunda squirrels. Widespread across Sundaland, lowland species of rodents have been shown to have more and stronger genetic structure associated with land masses, as compared to larger mammals but these divergences have not yet led to taxonomic revisions. Given the forest-dependent nature of these squirrels, an improved knowledge of their evolutionary history is key for our understanding of the dynamics of Sundaland rainforests (Den Tex et al., 2010). Deep divergences and speciation events may be the consequence of past isolation of landmasses or forest pockets while lack of genetic structure could shed light on recent episodes of land or forest connection. Conflicting patterns have been shown across different phylogeographic studies on mammals distributed across Sundaland (Mason et al., 2011; Leonard et al., 2015). Some species of murids, carnivores, suids, greater mouse deer and pangolins have weak or no genetic structure across Sundaland and/or within Borneo (Gorog et al., 2004; Achmadi et al., 2013; Patel et al., 2017; Mason et al., 2019; Veron et al., 2020) while others, such as Sunda rats, viverrids, colugos or the lesser mouse deer, show much deeper divergences some of which predate to the Pleistocene (Den Tex et al., 2010; Mason et al., 2011; Camacho-Sánchez, 2017; Veron et al., 2019). Recently, Husson et al. (2019) provided substantial evidence for a new paleogeographic scenario in which prior to 400 thousand years ago (ka) the Sunda shelf would have been continuously exposed. According to this study, dispersal across this exposed shelf would have been possible through this period for terrestrial species. Nevertheless, geological connectivity does not necessarily imply population connectivity, different dates of divergences between populations on different land masses might reflect different dispersal capabilities across non-forested ecological barriers such as the possible "savanna corridor" in interior Sundaland (Heaney, 1991; Sheldon et al., 2015).

Within the genus *Sundasciurus*, previous phylogenies based on mitochondrial cytochrome *b* sequences have identified deep divergences between lineages within Low's squirrel (*S. lowii*), and a lack of reciprocal monophyly between Low's squirrel and the Fraternal squirrel (*S. fraterculus*) from the Mentawai islands (Den Tex et al., 2010; Hawkins et al., 2016a). Low's squirrel is a good example of a widespread, lowland forest dwelling tree squirrel. It is currently listed as a Species of Least Concern by the IUCN, largely based on its wide historic distribution (Meijaard, 2016). However, the lowland tropical forest is rapidly disappearing in this region due to human induced habitat modifications and its current status and distribution need to be re-evaluated. Furthermore, it is possible that the widely distributed Low's squirrel is actually a complex of multiple species each with a more restricted range. Low's squirrel contains seven nominal subspecies distributed across Borneo (*S. l. lowii*), northern Borneo's offshore islets Banguey and Balambangan (*S. l. bangueyae*), the Malay Peninsula (*S. l. robinsoni*), southern Malay Peninsula Riau offshore islands (*S. l. seimundi*), Sumatra (*S. l. humilis*), west Sumatra Batu offshore islands (*S. l. balae*), and the Natuna islands (*S. l. natunensis*) (Thorington and Hoffmann, 2005; Thorington et al., 2012) all based on morphology (**Figure 1**).

In this study, we use genetic data including complete mitochondrial genomes and twelve nuclear loci from across the *Sundasciurus lowii* and *S. fraterculus* distribution to construct a well-resolved phylogeny of the group, and to date divergences. These results are placed in the context of the morphological variation and distribution of the different taxa in order to re-evaluate and resolve the number of species in this group. Additionally, a comprehensive taxonomic evaluation of this species complex will have important conservation implications that could lead to the re-assessment of the IUCN Red

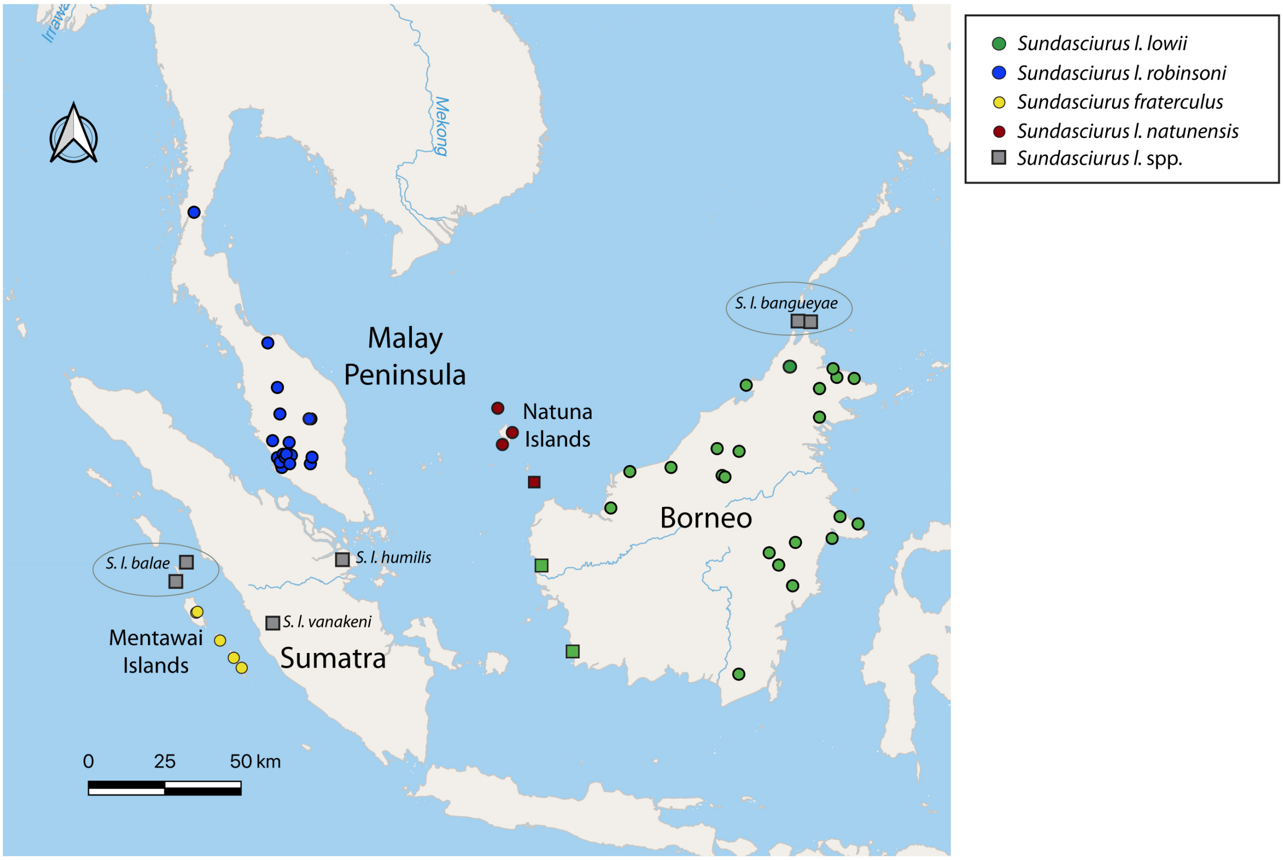

FIGURE 1 | Map of Sundaland showing the distribution of the different subspecies of the Low-Fraternal squirrel species complex, with dots indicating sample collection locations of specimens included in the morphometric analyses. Samples are colored according to the four highly supported major clades recovered in the mitogenome-based phylogenies of **Figures 2**, **3**. Populations indicated by a red circle have been considered *S. l. natunensis* but the morphological data in this study suggests they are more similar to *S. l. lowii*. Populations indicated with a green square have been considered *S. l. lowii* but the morphological data in this study suggests these they are more similar to *S. l. natunensis*. Molecular evidence from these populations is needed to clarify their taxonomic status. Populations included in the morphological analyses but not the genetic analyses are indicated in gray.

List category for some of these squirrels based on their updated distributional ranges. It will also provide a better understanding of the ecological and evolutionary processes that led to the high diversification rates of these forest dependent squirrels in this region.

MATERIALS AND METHODS

Materials

A total of 11 samples of *Sundasciurus lowii* and *S. fraterculus* from the major landmasses and isolated archipelago populations (Malay Peninsula, Borneo, Mentawai Islands and Natuna Islands) and 29 outgroups were included in the molecular part of the study (**Supplementary Table S1**). Populations from Sumatra and satellite islands (Batu and Riau) were not sampled, because only type specimens are available (*S. l. humilis, S. l. balae, S. l. seimundi*). In any case, different studies have suggested recent connections among Sumatra and Peninsular Malaysia (Ruedi and Fumagalli, 1996; Mason et al., 2011; Camacho-Sánchez et al., 2017) and among these satellite islands and the nearest landmasses (Mason et al., 2019) so our geographic coverage could potentially represent most of the genetic structure of this complex. Twelve samples were derived from tissue collected from historical specimens housed in three museums, 10 samples were modern tissue coming from museum loans and 14 sample sequences were downloaded from Genbank. Four samples were obtained from specimens collected during a previous expedition to Borneo (Camacho-Sánchez et al., 2019) and these were all taken according to the guidelines of the American Society of Mammalogists (Sikes, 2016) and as approved by Institutional Animal Care and Use Committees (Smithsonian Institution, National Museum of Natural History, proposal number 2012-04 and Estación Biológica de Doñana proposal number CGL2010-21424). Field research was approved by Sabah Parks [TS/PTD/5/4 Jld. 45 (33) and TS/PTD/5/4 Jld. 47 (25)], the Economic Planning Unit (100-24/1/299) and the Sabah Biodiversity Council (Ref:TK/PP:8/8Jld.2). All recognized species and subspecies of this complex were included in the morphological analyses (**Figure 1** and **Supplementary Table S2**).

DNA Extraction, Amplification, Enrichment and Sequencing

DNA was extracted using phenol-chloroform with ethanol precipitation. Amicon Ultra 0.5 mL filters were used for the final clean instead of ethanol precipitation for historic samples. All museum samples were processed in an isolated ancient DNA laboratory following strict protocols to control for contamination.

Twelve nuclear loci (introns) previously found to be present in single copies and informative in closely related mammalian species (Igea et al., 2010) were targeted. Primer sets for four of those introns developed for *Tamiasciurus* squirrels by Chavez et al. (2014) worked in our taxa as a multiplex. For the other eight loci, we designed primers following the steps of Forcina et al. (2020). We developed 15 primer sets to amplify smaller fragments (ranging from 88-233 base pairs) of these same loci for the low quality specimens (**Supplementary Table S1**). PCR conditions for the two modern DNA and four historic DNA multiplexes are in **Supplementary Table S3**. PCR products were purified using streptavidin magnetic 'SPRI' beads (Rohland and Reich, 2012) and visualized on agarose gels. Given the degraded nature of historic DNA, three replicate PCRs were run per sample. Amplicon libraries were cleaned and checked on a gel with reference standards for quantification (Image Lab 5.2.1 software), and were pooled at equimolar concentrations. Illumina TruSeq adapters with an individual index were added to the purified PCR products in a second PCR as performed in Camacho-Sánchez et al. (2017).

We used target capture to recover complete mitochondrial genomes. We used an Illumina modification to the protocol based on Maricic et al. (2010) to enrich complete mitogenomes from historic samples. Enrichment conditions followed kit protocols as described in Hawkins et al. (2016b). Briefly, samples were denatured, incubated for ∼24 h, recovered the baits, and amplified from the p5/p7 priming site on the Illumina adapters for a limited number of PCR cycles to generate concentrations of mitochondrial DNA which could be sequenced. PCR products were purified as above.

Dual-indexed shotgun libraries were sequenced on an Illumina MiSeq using 2 × 300 (600 cycle) PE at the Center for Conservation Genomics, Smithsonian Conservation Biology Institute.

Preprocessing and Quality Scanning of Sequencing Data

Adaptor removal and quality trimming was performed with Trimmomatic (Bolger et al., 2014) with the sliding window parameter set to 5:20, read minimum length parameter to 50 bp and leading and trailing to 5. Single quality scans were run on the raw fastq files before and after trimming with FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016) was run to create a single report visualizing the output from all the samples of each run, enabling global trends and biases to be quickly identified. Mitochondrial genome reads were preprocessed following the protocol described in Hawkins et al. (2016b).

Assembly and Alignment of Mitochondrial Genomes

Quality trimmed reads were mapped to a reference mitochondrial genome with BWA-MEM algorithm or with Geneious Mapper iterative algorithm (up to 10 iterations and medium-sensitivity) for some divergent samples. The output of BWA was converted to BAM files with SAMtools (Li et al., 2009). These were later sorted, merged and PCR duplicates removed. Finally, BAM files were imported to Geneious where consensus sequences were called (minimum 5x and 75% threshold). Samples mapped in Geneious were inspected for duplicate removal with Dedupe plugin of the BBTools package v. 35.82 (Bushnell, 2015). We aligned our sequences with Callosciurinae whole mitochondrial sequences available in Genbank (**Supplementary Table S1**) with MAFFT v7.450 Geneious plugin automatic algorithm (Katoh and Standley, 2013) under default parameters (scoring matrix:200PAM/K = 2, gap open penalty = 1.53, offset value = 0.123). The control region was removed from the mitogenome assemblies because it was poorly assembled in many historic samples and has been shown to provide low phylogenetic resolution and overestimation of divergence times (Duchêne et al., 2011). ND6 is present in the light strand and was thereby reverse complemented. Protein-coding regions were translated and inspected for frameshift mutations and for the presence of unexpected stop codons to prevent inclusion of NUMTs. We computed summary statistics of the alignment with AMAS (Borowiec, 2016).

Genotyping and Alignment of Nuclear Data

For nuclear sequences, we imported the trimmed reads to Geneious and mapped these to the closest homologous sequences found in Genbank (*Marmota marmota* (NW_015351277.1, NW_015351233, XM_027934759, NW_015351257, NW_015351271, NW_01535121) and *Tamasciurus hudsonicus* (KF883544, KF883925, KF885067) with Geneious Mapper (low-medium sensitivity and up to 5 iterations). We called consensus sequences in Geneious with a minimum 5× coverage and 75% consensus threshold. Each locus was aligned independently with MAFFT v7.450 Geneious plugin automatic algorithm (Katoh and Standley, 2013) under default parameters (scoring matrix:200PAM/K = 2, gap open penalty = 1.53, offset value = 0.123). We translated the alignments to amino acids and inspected them for insertions, deletions and premature stop codons to prevent inclusion of paralogous sequences. Amplicon sizes exceeded the length generated by the Illumina sequencing for two loci (GDAP1, 795 bp and P4HA2, 758 bp). This generated missing data in the center of those alignments (203 and 295 bp), which were trimmed.

All nuclear sequences were resolved into statistically probable haplotypes using PHASE 2.1.1 (Stephens, 2005) with an acceptance threshold of 0.90. The online application SeqPHASE (Flot, 2010) was used to convert FASTA files to PHASE input files, as well as to convert PHASE output back to FASTA format. Due to the different size of modern and historic sample sequences, the initial gene alignments contained sections of missing data for the shorter historic samples. Since PHASE can not deal with missing data in heterozygous sites, two alignments (historical and modern samples) were generated for each gene to remove most missing data and overcome this problem. Genotypes that were still unresolved following PHASE were phased manually based on the original BAM alignment (Camacho-Sánchez, 2017).

Phylogenetic Analyses and Haplotype Networks

We performed a likelihood ratio test (LRT) with MEGA 7 to test the clock-like behavior of the mitochondrial sequences. A strict molecular clock was rejected. The optimal partitioning scheme and substitution model was selected by PartitionFinder 2.1.1.2 (Lanfear et al., 2016). We followed Mackiewicz et al. (2019) considering 3 codon positions for each individual protein coding gene and separate partitions for each of the RNA genes, but retained contiguous t-RNAs because individual t-RNA alignments would be too short to be informative (Nicolas et al., 2019). This reduced the number of initial partitions from 67 to 53. We used the "greedy" algorithm with branch length estimated as "unlinked", AICc criteria and "–raxml" command line option to search for the best-fit partitioning scheme (11 partitions) and substitution model (GTR + GAMMA). We tested for saturation plotting transitions and transversions of each alignment partition against each other and against raw/uncorrected pairwise genetic distances with the APE R package and "dist.dna" command. We also performed Xia substitution saturation tests in Dambe 7 (Xia, 2018) for the 3rd codon of some fast-evolving genes (e.g., Cytochrome *b*) as a complementary line of evidence. We did not find saturation so all partitions were kept in downstream analyses. We performed phylogenetic inference through a maximum likelihood framework with RAxML (Stamatakis, 2014) on the mitochondrial genome dataset. We specified the output of PartitionFinder2 as input for RAxML (Stamatakis, 2014). We selected the rapid bootstrapping algorithm, GTR GAMMA substitution model and followed the extended majority rule stopping criterion (-autoMRE). The maximum likelihood phylogeny was used for the BEAST analyses.

Divergence times were estimated with our mitochondrial dataset in BEAST2 (Bouckaert et al., 2014). The mitochondrial genome alignment was split into the best partition schemes as identified by Partitionfinder2 with AMAS. We performed site modeling through a Bayesian approach with bmodeltest, selecting mutation rate estimation and empirical frequencies priors (Bouckaert and Drummond, 2017). Following MEGA7 tests, a lognormal (uncorrelated) relaxed clock was used under a Yule speciation tree, with linked partitions and default operators. Only one sequence per putative species was kept to comply with Yule speciation prior assumptions. Three independent runs of Markov chains (MCMC) for Monte Carlo simulations were run for 50,000,000 generations, with parameters and trees sampled every 5000 generations. Convergence was checked using Tracer 1.7.1 (Rambaut et al., 2018). For each run, the first 10% of sampled trees were discarded as burn-in. Two fossil constraints were selected to calibrate the phylogeny. A fossil of *Callosciurus sp.* (specimen number: YGSP 21682; left upper third molar that conforms to the proportions of living *Callosciurus*) from locality Y589 (Chinji formation, Siwaliks, Pakistan) dated by paleomagnetic correlation to 14 Ma (Flynn, 2003) for the Callosciurus and Sundasciurus clade; and a stem form of *Tamiops* (specimen numbers: Z2438, Z2439, Z2440, Z2441) from Z122 (Vihowa Formation) dated by paleomagnetic correlation to 18.7 Ma (L. Flynn, pers. comm.) as the oldest known fossil for the *Callosciurus, Sundasciurus*, *Tamiops*, and *Dremomys* clade. All constraints were set using hard minimum bounds and soft upper bounds using a lognormal prior, as suggested by Parham et al. (2012).

We performed TCS haplotype networks with popART (Leigh and Bryant, 2015) separately, for the modern and historic alignments of the mitochondrial genomes and nuclear intron sequences. We computed uncorrected pairwise genetic distances on the Cytochrome *b* gene with the APE R package and dist.dna command (Paradis et al., 2004). Results were visualized with ade4:table.paint. Finally, a multidimensional scaling analysis was performed for both datasets with stats:cmdscale and ggplot2. We defined the *a priori* groups for the species tree analysis within our ingroup following four criteria: Cytochrome *b* uncorrected genetic distances > 5% (Baker and Bradley, 2006) reciprocal monophyly in mitochondrial genome tree, clusters in MDS, presence of private alleles in the intron haplotype networks and phenotype differentiation. Based on these criteria we considered six groups within our ingroup as input for the *BEAST species tree: *Sundasciurus fraterculus, S. lowii robinsoni, S. l. natunensis, S. l. lowii* from Sabah, *S. l. lowii* from Sarawak, and *S. l. lowii* from East Kalimantan.

We included all intron phased alleles available per group in the *BEAST analysis, and unlinked partitions, selected an uncorrected lognormal clock, bmodeltest with empirical frequencies and mutation rate estimation, analytical population size integration model and Yule speciation prior. Three independent runs were conducted for 50000000 generations Markov chains (MCMC) for Monte Carlo simulations, with parameters and trees sampled every 5000 generations.

Species delimitation was conducted using the program BPP X1.2.2 (Yang, 2015) details regarding this analysis are specified in the **Supplementary Material File 1**.

Morphological Data Collection

The specimens examined here are housed in the following natural history repositories: American Museum of Natural History, New York, United States (AMNH); Natural History Museum, London, United Kingdom (BMNH); Estación Biológica de Doñana, Seville, Spain (EBD); Field Museum of Natural History, Chicago, Illinois (FMNH); Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts (MCZ); and National Museum of Natural History, Smithsonian Institution, Washington, DC, United States (USNM) **Supplementary Table S2**. Skins were carefully examined to avoid any misidentifications and a geographically balanced subset was photographed for further evaluation. During our initial surveys of the squirrel collections, we found that a high proportion of the skulls were broken and therefore decided to undertake traditional morphometric analyses instead of geometric morphometrics. Dental eruption and wear patterns were checked to age each specimen. Fusion of the presphenoid-basisphenoid and basisphenoid-basioccipital sutures were not informative to age individuals in this taxon since some large-sized adults with very worn molars had non-fused sutures. Only adults were included in the morphometric analyses. We combined data from males and females in the morphometric analyses because sexual dimorphism is uncommon in tree squirrels (Moore and Tate, 1965; Tenzin et al., 2013; C̀anády et al., 2015). However, if certain characters were sexually dimorphic, results would be interpreted in the context of phenotypic variation among putative species inclusive of potential patterns of sexual dimorphism, thereby providing a more conservative assessment of divergence (Meik et al., 2018). Seventeen cranial measurements were taken with a Fowler High Precision electronic digital caliper to the nearest 0.01 mm by AH (**Supplementary Table S2**). These were occipitonasal length (ONL), zygomatic breadth (ZB), interorbital breadth (IB), length of nasals (LN), anterior nasal breadth (ANB), length of rostrum (LR), height of rostrum (HR), mastoid breadth (MB), height of braincase (HBC), length of orbit (LO), length of diastema (LD), length of bony palate (LBP), breadth of bony palate at fourth premolar (BBP), length of auditory bulla (LB), crown length of maxillary cheek teeth (CLPMM), median palatal length (MPL) and least breadth of caudal point of zygomatic process of frontal bone (LBC). All measurements are as defined in Musser et al. (2010) except median palatal length (MPL) and least breadth of caudal point of zygomatic process of frontal bone (LBC), which are defined in Hayashida et al. (2007). In addition, given the curved rostrum of our target taxa, the height of rostrum (HR) was modified after conducting preliminary error measuring tests. We define it as the distance from dorsal surface of the nasals to ventral surface of the premaxillary at the premaxillary-maxillary suture and taken perpendicularly to the molar row axis. External measurements, head-body length (HB), tail (T), hindfoot (HF), ear (E) and weight (W), were taken directly from specimen labels (**Supplementary Table S2**). We examined, measured and photographed all of the types of our target taxa.

Morphometric Statistical Analyses

We performed standard univariate descriptive statistics (mean, standard deviation and observed range) for each of the subspecies (Thorington and Hoffmann, 2005) and also for each of the *S. lowii* Borneo populations in a separate analysis (**Supplementary Table S4**). We log-transformed each measurement to its standard deviation prior to computing the principal component analyses (PCA) so that the data was analyzed on the basis of correlations instead of covariances. PCA was implemented by the prcomp command in R studio (Allaire, 2012; R Core Team, 2018). Results were extracted and visualized with the following functions of the factoextra package (Kassambara and Mundt, 2017): fviz_pca_ind, plots PCA results; fviz_pca_biplot, biplot of individuals and variables; get_eigenvalue, extracts eigenvalues, variance percentage and cumulative variance percentage; get_pca_var, outputs each variable's contribution to variance.

We conducted a discriminant analysis of the principal components (DAPC) with the dapc function of adegenet (Jombart and Ahmed, 2011). Identification of the clusters was made with find.clusters. This function first transforms the data using PCA, asking the user to specify the number of retained principal components (PCs) interactively. Then, it runs k-means algorithm with increasing values of k and computes associated summary statistics (by default, BIC). We performed a cross-validation with the xvalDapc() function with default parameters to decide the number of PCs to retain. This function runs 30 replicates of cross-validation for a number of PCs less than the total number of variables. After having an approximate idea of the PCA axes range to be retained, we re-ran the cross-validation with 1000 replicates. Different number of clusters had similar BIC values so a DAPC was run for each scenario (3-10 clusters). Different DAPCs (retaining 13-3 PCs and 6-3 discriminant functions) with *a priori* grouping for each of the subspecies (Wiley, 1978; Thorington and Hoffmann, 2005) and for each of the *S. lowii* Borneo populations (in a separate analysis) were also performed. We interpreted group memberships with the functions summary, compoplot and assignplot to test how clear-cut clusters were. These functions indicate the proportions of successful reassignment (based on the discriminant functions) of individuals to their original clusters. To increase the number of samples (from 55 to 152) and include the types, we made bivariate plots of craniodental variables based on the output of the DAPC and standard external measurements.

Registration of Nomenclature

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser at urn:lsid:zoobank.org:pub:727A0DA1-2B92-4400-A3C2-CE816BE00B1F

RESULTS

Whole mitochondrial genomes were constructed from 24 individuals, and their alignment (without control region) was 15735 bp, with 5557 parsimony informative sites and 8.97% missing data. Missing data was mainly in the sequences from the eleven historic samples (15–47%). Mitochondrial genomes of all ingroup taxa were successfully assembled, although only 53% of one *Sundasciurus lowii natunensis* from two attempted samples could be reconstructed. Similarly, *Sundasciurus lowii natunensis* was the only taxon from which we failed to amplify any introns. Three intron loci were not informative or did not amplify correctly (GAD2, SLC17A9, Ulk1b) and were excluded from downstream analyses.

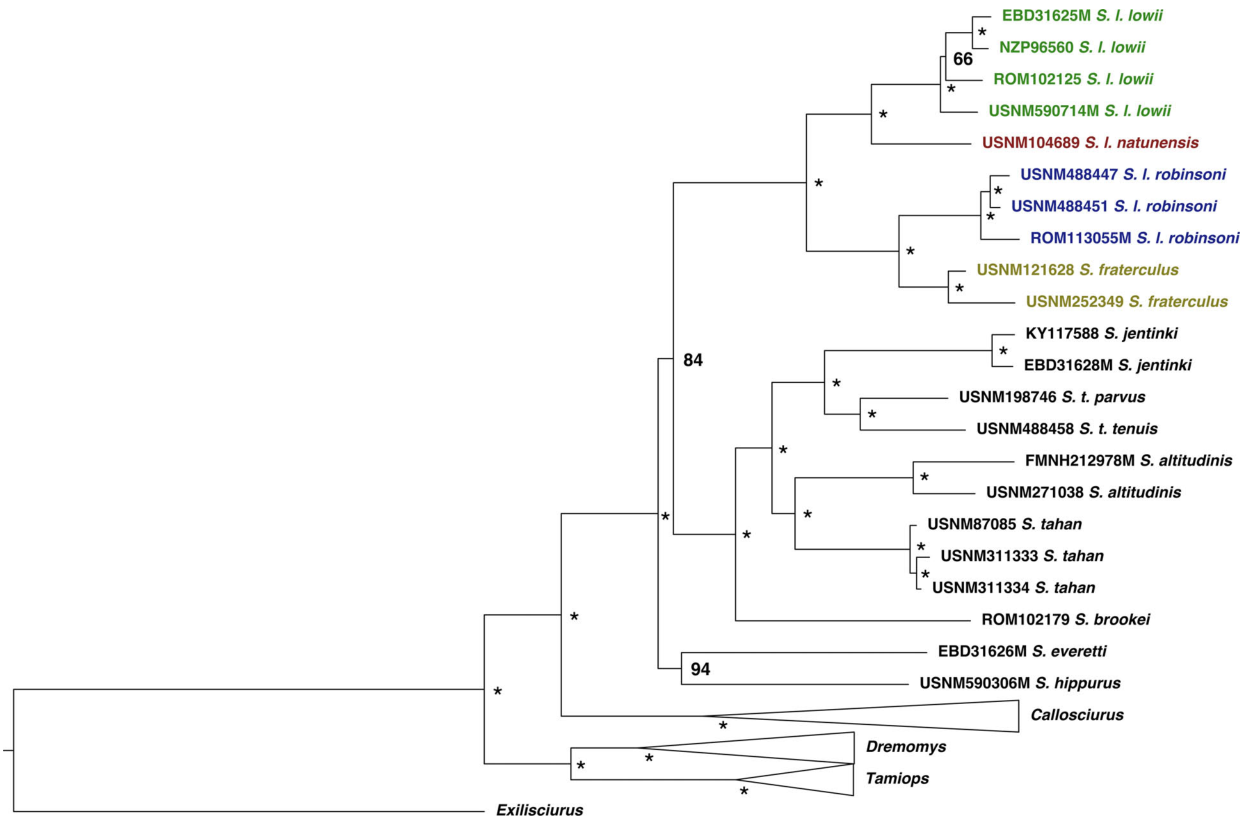

FIGURE 2 | Mitogenome-based maximum likelihood phylogeny for Sunda squirrels. Highly supported nodes (PP > 0.95) are shown with an asterisk. Posterior probabilities are specified in less supported nodes. Ingroup samples of the four main clades are colored to match map in **Figure 1**. Outgroup samples are shown in black. Outgroup genera clades are collapsed for clarity.

Mitochondrial Genome Phylogenetic Relationships and Divergence Dating

All nodes were strongly supported (Bootstrap support (BP) > 84/ Posterior probability (PP) > 0.98) in the mitochondrial genome maximum likelihood tree (ML) and Bayesian maximum clade credibility tree (BMCC) and the topology was consistent between approaches (**Figures 2**, **3**). Only the relationships among *S. l. lowii* had weak support in the ML tree (BP = 0.66) but strong support in the BMCC (PP = 0.99). These tree topologies were consistent with previous studies (Den Tex et al., 2010; Hawkins et al., 2016a). The Low's squirrel complex contained two divergent clades from east and west Sundaland. The former included *S. l. lowii* from Borneo and *S. l. natunensis* from the Natunas, while the latter had *S. l. robinsoni* from peninsular Malaysia and *S. fraterculus* from Mentawai Islands. The sister relationship of *S. fraterculus* with *S. l. robinsoni* makes *S. lowii sensu lato* paraphyletic as in Den Tex et al. (2010). Within *S. l. lowii*, the population from Sarawak was sister to the clade containing the ones from Sabah and East Kalimantan. All genera (*Tamiops, Dremomys, Callosciurus* and *Sundasciurus*) were recovered as monophyletic. Previously suggested subgenera within *Sundasciurus* (*Aletesciurus and Sundasciurus*; Moore, 1958) were not found to be reciprocally monophyletic. Previous studies either could not resolve the lower nodes or found them not to be monophyletic (Den Tex et al., 2010; Hawkins et al., 2016a) and thus had suggested suspending that nomenclature (Hawkins et al., 2016a).

The time to the most recent common ancestor (TMRCA) of Callosciurinae and *Sundasciurus* was estimated at 26.86 (95% HPD: 20.00–33.85) and 12.25 (95% HPD: 10.67–13.67) million years ago (MYA). These dates are older than those previously estimated by Mercer and Roth (2003) who associated the diversification of Sundaland genera with the lowest pre-Pleistocene sea stand in the Cenozoic about 11 MYA. TRMCA for the subgenus *Sundasciurus* and *Sundasciurus lowii* complex were estimated at 11.65 (95% HPD: 10.09–13.11) and 7.01 (95% HPD: 5.74–8.33) MYA. At 4.41 (95% HPD: 3.39–5.53) MYA one of *Sundasciurus lowii* complex lineages split into the ancestors of *S. l. lowii* clade and *S. l. natunensis*. At 3.20 (95% HPD: 2.30–4.12) MYA the other *Sundasciurus lowii* complex lineage split into the ancestors of *S. fraterculus* and *S. robinsoni.* The lowland *Sundasciurus tenuis* and highland *S. altitudinis* exhibited remarkable deep levels of intraspecific divergence dated at 3.96 (95% HPD: 2.82–5.05) and 2.85 (95% HPD: 2.04–3.79) MYA. These dates are older to what was previously estimated from Cytochrome *b* by Den Tex et al. (2010).

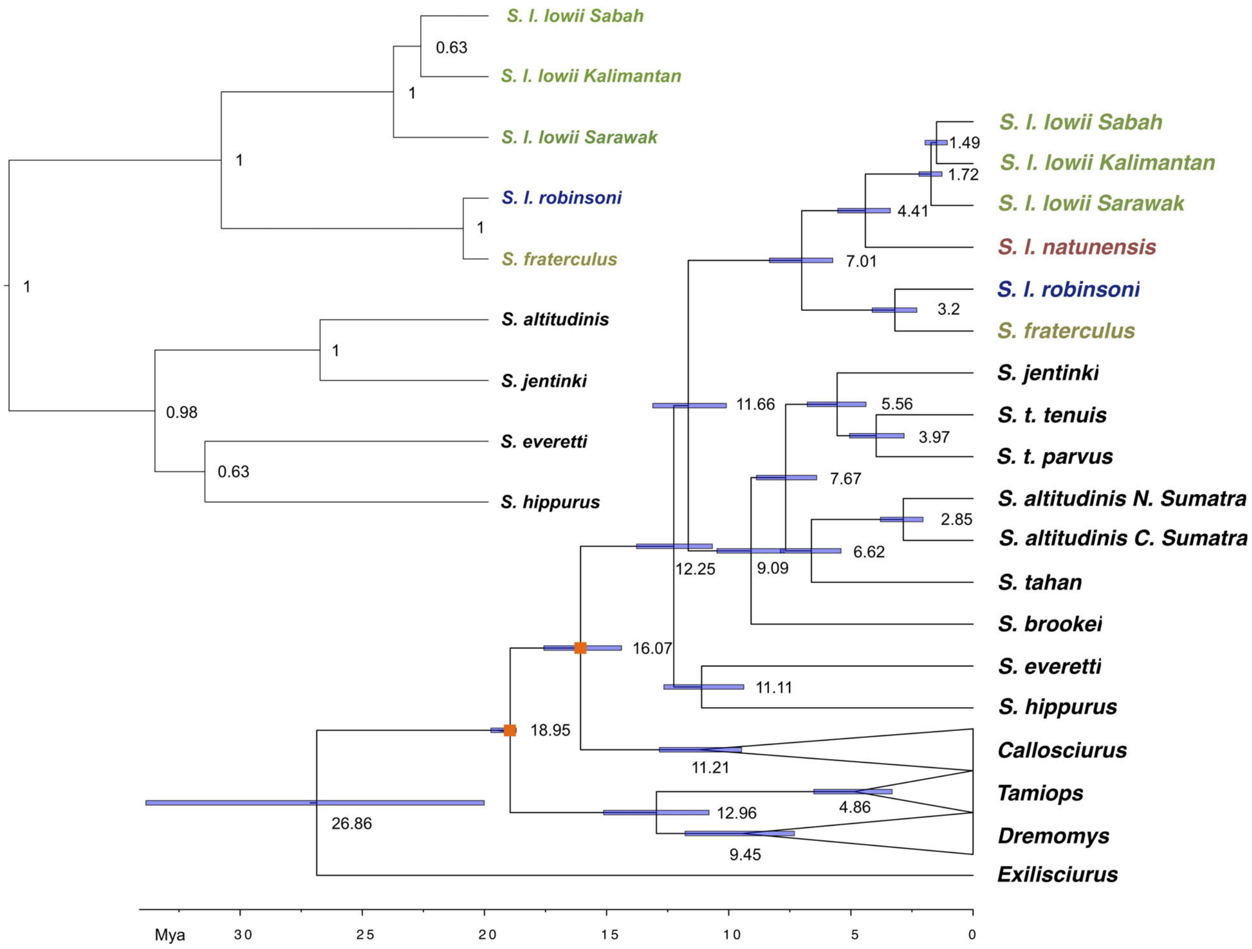

FIGURE 3 | Mitochondrial genome Bayesian maximum clade credibility tree including all *Sundasciurus* species from Sundaland. Dates are indicated on the bar at the bottom of the figure in millions of years before present (Mya) and on nodes. Nodes used to calibrate the dating are marked with an orange box. All nodes are fully supported (PP = 1). Inset shows a species tree of the Low's and Fraternal squirrel species complex, as estimated from nine nuclear loci using *BEAST. Ingroup samples of the four main clades are colored to match **Figure 1** map. Outgroup samples are shown in black. Outgroup genera clades are collapsed for clarity.

Divergence and Private Alleles

Many subspecies had private alleles (**Figure 4** and **Supplementary Figure S1**). The taxa *S. l. robinsoni* with *S. fraterculus* and *S. l. lowii* had private alleles for all introns. Within Borneo *S. l. lowii* of Sabah, *S. l. lowii* of Kalimantan and *S. l. lowii* of Sarawak had private alleles for seven out of nine introns. The narrow endemic *S. fraterculus* had private alleles for three of nine introns.

Cytochrome *b* uncorrected genetic distances among some of the taxa (*S. l. robinsoni*, *S. l. natunensis*, *S. l. lowii* of Sabah and *S. l. lowii* of Sarawak) were above 5% (**Supplementary Figure S2**), a scale of divergence which may suggest a revision of the status of those taxa could be of interest (Baker and Bradley, 2006). Genetic distances among these taxa were higher than that observed among other recognized species (**Supplementary Figure S2**). These results further highlight that while Cytochrome *b* distances might indicate potential unrecognized genetic species, it should never be used alone to define species. We note that high percent sequence divergence levels were also observed between *S. t. tenuis* and *S. t. parvus,* and among populations within *S. hippurus*, *S. mindanensis,* and *S. altitudinis* (**Supplementary Figure S2**), suggesting that additional work on these groups in the future could shed more light on the evolution of this genus.

Multilocus Species Tree and Species Delimitation

Phylogenetic relationships inferred by the intron species tree constructed in *BEAST were concordant with the mitochondrial genome tree topology. Only paraphyly of the subgenus *Sundasciurus* in the nuclear species tree was inconsistent with the mitochondrial topology, but concordant with Hawkins et al. (2016a) **Figure 3**. However, deep nodes such as the MRCA of *S. everetti* and *S. hippurus* or shallow ones such as the relationships among Bornean *S. lowii* were weakly supported (PP = 0.62-0.63) while the other nodes were strongly supported (PP > 0.99).

Our analyses of nuclear data were consistent across the independent BPP runs and among the five prior combinations.

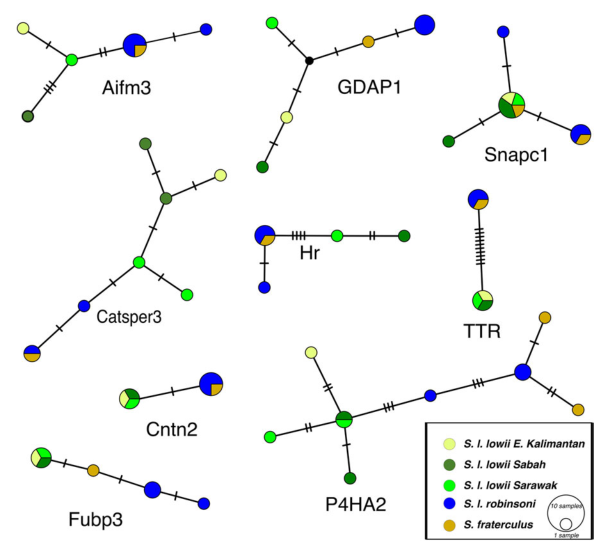

FIGURE 4 | TCS haplotype networks for each of the nine polymorphic introns studied. The size of the pie charts corresponds to the frequency of the haplotype; transversal lines represent single base pair differences; black dots represent missing haplotypes.

We inferred a posterior probability of 1.0 for all species postulated by the *BEAST guide tree except *S. fraterculus* and *S. l. lowii* from E. Kalimantan. The former was not supported (PP = 0.07–0.26) and the latter went from weakly to moderately supported (PP = 0.51–0.95). The most likely scenario in every run supported all species except *S. fraterculus* (PP = 0.41–0.88).

Morphometric Results

A high degree of morphometric differentiation among the different populations was observed (**Figures 5, 6**) with a large part (82%) of the variance explained by the first principal component. PC1 exhibited different loadings for most variables, with crown length of maxillary cheek teeth (17%), length of nasals (14%) and breadth of bony palate (10%) driving most of the variation. PC1 discriminated the larger-sized subspecies *S. l. lowii* and *S. l. bangueyae* from the smaller-sized subspecies *S. l. robinsoni, S. l. humilis, S. l. balae* and *S. fraterculus*. PC2 explained 5.7% of the variance and was mainly correlated with shape robustness/broadness, bulla and molar row length. The variables with a higher contribution were crown length of maxillary cheek teeth (26%), interorbital breadth (21%), anterior nasal breadth (20%), length of bulla (11%), and least breadth of caudal point of zygomatic process of frontal bone (7%). It discriminated the relatively gracile and large molar *S. l. robinsoni*, southern Natuna *S. l. natunensis,* east and west Kalimantan *S. l. lowii* and *S. l. bangueyae,* from the relatively robust/broad shaped and small molar *S. fraterculus, S. l. balae, S. humilis,* Sarawak and most Sabah samples of *S. l. lowii,* and northern Natuna *S. l. natunensis*. PC3 explained 3.3% of the variance and was correlated with anterior nasal breadth (35%), crown length of maxillary cheek teeth (14%), length of bulla (10%) and mastoid breadth (9%). It discriminated the narrow-nasal shaped *S. l. robinsoni, S. l. balae, S. humilis,* northern Natuna *S. l. natunensis* and east and west Kalimantan *S. l. lowii* from broad-nasal shaped *S. fraterculus, S. l. bangueyae,* southern Natuna *S. l. natunensis* and Sarawak and east-central Kalimantan *S. l. lowii.* Sabah *S. l. lowii* fell in between.

The DAPC discriminated most subspecies (**Supplementary Figure S3**). Some overlap was observed between *S. l. balae* and *S. l. robinsoni,* and between *S. l. lowii* and *S. l. bangueyae*. The Mount Kerinci sample of *S. l. humilis* (belonging to the junior synonym *S. l. vanakeni*) had an intermediate position between *S. fraterculus* and *S. l. robinsoni,* while the only west Bornean sample of *S. l. lowii* was clustered between other Borneo *S. l. lowii* and *S. l. natunensis.* Only samples from East Kalimantan could be discriminated from the remaining Bornean populations. This analysis identified crown length of maxillary cheek teeth, bony palate breadth, orbit length, rostrum length, interorbital breadth, and bulla length, as the measurements most useful for distinguishing among all taxa (**Supplementary File S1**).

Taxonomic Revision

Sundasciurus robinsoni **(Bonhote, 1903) – Robinson's squirrel**

Sciurus robinsoni Bonhote (1903):24.—Type locality. Bukit Besar, Nawngchik, Patani, Peninsular Thailand, 2500 ft.
Holotype. BMNH 3.2.6.55, skull, skin, adult female, collected 30 August 1901 by C. H. Robinson and N. Annandale

Sciurus balae Miller, 1903:14.—Type locality. Tana Bala, Batu Islands, Sumatra
Sciurus piniensis Miller, 1903:14.—Type locality. Pulo Pinie, Batu Islands, Sumatra
Sciurus seimundi Thomas and Wroughton, 1909:440.—Type locality. Kundur Island, Riau Islands
Sciurus humilis Miller, 1913:24.—Type locality. Kateman river, East Sumatra
Sciurus vanakeni Robinson and Kloss, 1916:270.—Type locality. Barong Bharu, Korinchi, Sumatra, 4000 ft.

Subspecific Taxonomy

Sundasciurus robinsoni (Bonhote, 1903)
Sciurus robinsoni alacris Thomas, 1908e: 306.—Type locality. Selangor-Pahang border, Malaya, 3000ft.
Sciurus seimundi Thomas and Wroughton, 1909:440.—Type locality. Kundur Island, Riau Islands

Sundasciurus robinsoni balae (Miller, 1903)
Sciurus balae Miller, 1903:14.—Type locality. Tana Bala, Batu Islands, Sumatra
Sciurus piniensis Miller, 1903:14.—Type locality. Pulo Pinie, Batu Islands, Sumatra
Sciurus humilis Miller, 1913:24.—Type locality. Kateman river, East Sumatra

Sundasciurus robinsoni vanakeni (Robinson and Kloss, 1916)
Sciurus vanakeni Robinson and Kloss, 1916:270.—Type locality. Barong Bharu, Korinchi, Sumatra, 4000 ft.

Emended Comparison With Other *Sundasciurus* Species

The species *Sundasciurus robinsoni* has a dorsum that ranges from medium brown with orange agouti to dark brown (*S. r. vanakeni*), and its venter ranges from white to pale yellow/buff white, with a reduction in the extent of this pale coloration and a lack of distinct margins in the case of *S. r. vanakeni*. Some populations (*S. r. balae* and *S. r. vanakeni*) have a grayish ventral coloration in limbs while others do not (*S. r. robinsoni*). It can be easily distinguished from other medium-sized western Sundaland *Sundasciurus* based on its ventral coloration and tail. All populations of *S. fraterculus* except Siberut, *S. tahan*, and *S. altitudinis* have a venter fur coloration homogeneously admixed with gray. The only other medium sized squirrel found in syntopy, *S. tenuis*, is also usually ventrally darker (admixed with gray) and dorsally lighter, with reddish-brown coloration on the shoulders and hips, white/pallid yellow hair tips present on tail, and a relatively thinner and longer tail (85–95 % of head-body length; Corbet and Hill, 1992) than *S. robinsoni* (56–84% of head-body length). Males of *S. fraterculus, S. tahan* and *S. tenuis* have a darker orange wash in the scrotal area than *S. robinsoni,* which is peach colored. Bonhote (1903) pointed out *S. robinsoni* has a "paler tinge" than *S. lowii* and *S. natunensis*, who have a more reddish brown agouti fur. The taxa also differ in variation of fur length on the tail, and body size and proportions. Moderate variation of hair length along the tail is observed in *S. robinsoni*, *S. fraterculus*, *S. natunensis* (and *S. lowii* Northwest Borneo populations) and *S. tenuis*. Extensive variation with short hair close to the base and longer hair at the tips giving a tapered appearance is observed in *S. lowii* and *S. tahan*. A tapered tail, but thickest at the base is found in *S. fraterculus* (**Supplementary Figure S4**). *S. robinsoni* is smaller (head-body range: 112–140 mm; body mass range: 49–77 g) than *S. lowii* (head-body range: 127–169 mm; body mass range: 60–125 g), somewhat smaller but overlapping in size with *S. natunensis* (head-body range: 133–152 mm) and slightly larger but quite overlapping in size with *S. fraterculus* (head-body range: 109–125 mm). Although the absolute tail length of *S. robinsoni* is about the same as *S. lowii, S. fraterculus,* and *S. natunensis,* it is relatively longer (56–85% of head-body length as opposed to 53–76%, 62 – 72%, and 57 – 67%, respectively). Within *S. robinsoni*, *S. r. balae* has a relatively shorter tail than the other two subspecies. *S. robinsoni* also has a relatively larger ear length (9–15% of head-body length) than *S. lowii* (7–13% of head-body length) and absolutely and relatively larger ear length than *S. fraterculus* (9% of head-body length) and *S. natunensis* (8% of head-body length) (**Supplementary Table S4**).

As with overall body size, the skull of *S. robinsoni* is smaller (33.60–36.43 mm) than *S. lowii* (36.93–41.03 mm); somewhat smaller but overlapping in size with *S. natunensis* (35.35–36.55 mm), and somewhat larger but overlapping in size with *S. fraterculus* (32.46–34.51 mm) (**Supplementary Table S4**). The skull of all of the small/medium-sized *Sundasciurus* are quite similar and conserved in proportion. Not many discrete characters have been found to distinguish the different species. Bonhote's statement "the bony palate extends well back the last molar which is not the case with *S. lowii*" can be considered accurate for *S. robinsoni*, and be extended to *S. fraterculus* and *S. natunensis* as well. However, the bony palate does not always end in line with the last molars in *S. lowii*, and does sometimes end after, as in the other species, but quite closer. Another feature described by Bonhote, "the bullae are more flattened and rounded in *S. robinsoni* than *S. lowii* and do not project so far downwards" does not apply when additional specimens are examined. Incisor size and projection, however, do seem to be quite diagnostic. Bonhote (1903) pointed out "the molar series of *S. robinsoni* is much shorter and smaller than *S. lowii*, but incisors are about the same size." In line with this statement, the height of the molars of *S. robinsoni* is clearly shorter than that of *S. lowii* and *S. natunensis.* While the incisors of *S. robinsoni* are less orthodont than *S. tenuis,* they are more orthodont than *S. lowii, S. fraterculus, S. natunensis* and *S. tahan*, which are conspicuously proodont. Finally, *S. robinsoni* has chisel-shaped incisors with sharp-angled beveled edges as in *S. fraterculus, S. natunensis*, *S. lowii lingungensis* and some specimens from west Borneo, but in contrast to *S. lowii lowii* from the remainder of Borneo

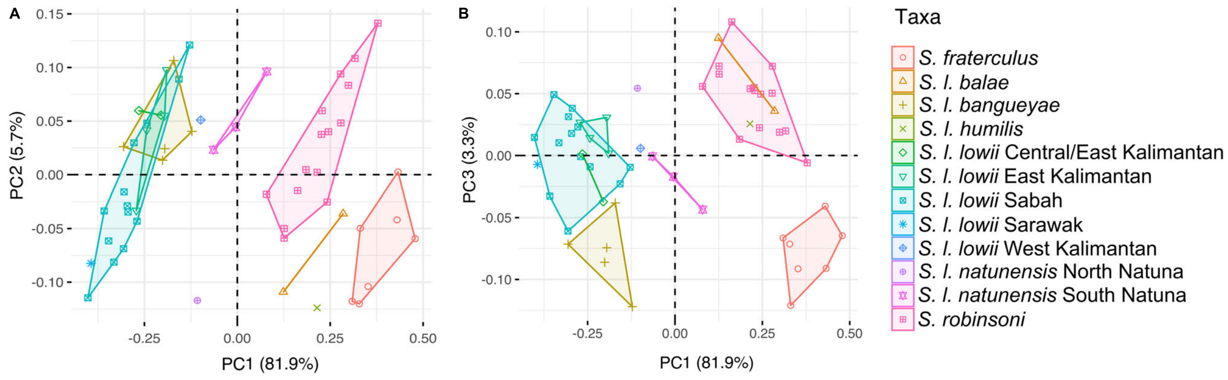

FIGURE 5 | Inter- and intraspecific morphometric variation among species of the Low's and Fraternal squirrel complex. **(A)** shows first and second principal components from an analysis of 17 cranial dimensions, while **(B)** shows first and third principal components of that same analysis. Samples are labeled and colored according to the different subspecies but subspecies with high genetic/phenotypic variation (*S. l. lowii* and *S. l. natunensis*) have been split into geography-based categories.

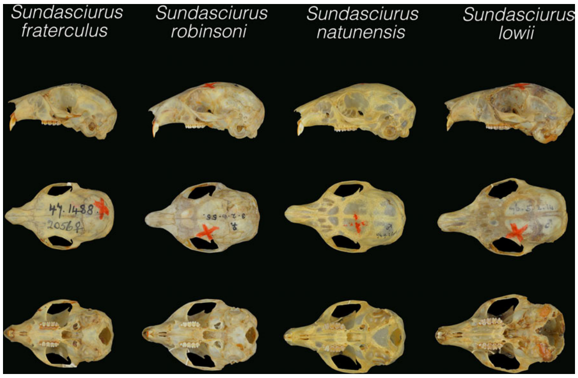

FIGURE 6 | Dorsal, palatal, and lateral views of the holotype skulls of *Sundasciurus lowii* (NHM76.5.2.14), *Sundasciurus natunensis* (NHM94.9.28.40) and *Sundasciurus robinsoni* (NHM3.2.6.55). Given the highly damaged state of the holotype skull of *Sundasciurus fraterculus* (NHM95.1.9.12), the type of *Sundasciurus fraterculus siberu* (NHM47.1488) is shown instead for the sake of comparison. All pictures are at the same scale.

(**Figure 6**). *S. robinsoni* was characterized by the PCA analysis as having the most gracile skull, with a relatively short interorbital breadth, anterior nasal breadth and least frontal bone breadth (**Figure 5**). It also has a shorter molar row (5.57–6.16 mm), palatal length (16.03–17.83 mm) and breadth (6.51–7.5 mm) than *S. lowii* (6.63–7.81 mm, 18.15–20.65 mm, 7.99–8.89 mm), but a relatively larger braincase.

Sundasciurus robinsoni has a shorter muzzle than *S. natunensis*, so it can be differentiated based on a shorter rostrum length (13.13–14.86/14.84–15.36 mm), palatal length (16.03–17.83/17.48–18.44 mm), and relative and absolute narrower bony palate (6.51–7.5/7.81–8.07 mm). *S. robinsoni* has a relative and absolute shorter molar row (5.57–6.16 mm) and smaller bullae (6.17–7.34 mm) than *S. natunensis* (6.72–7.15 mm; 7.26–7.65 mm) but a relatively larger proportion of bony palate of the total palatal length, relatively larger height of rostrum and relatively larger braincase (**Supplementary Figure S5**). Postorbital processes are relatively shorter and less pointed in *S. robinsoni* than *S. natunensis.*

There are major distinctions between almost all populations of *S. robinsoni* and *S. fraterculus* analyzed here. However, the intermediate position of the *incertae sedis* Kerinci population, that we have tentatively considered a subspecies (*Sundasciurus robinsoni vanakeni*) until molecular lines of evidence are available, generates an overlap among *S. robinsoni* and *S. fraterculus* for most diagnostic characters. The nominal *S. r. robinsoni* and *S. r. balae* have larger tympanic bullae (6.46–7.34 mm) than *S. fraterculus* (5.88–6.43 mm) but there is overlap with the inclusion of *S. r. vanakeni* (6.17–7.34 mm) in the comparison. The larger maxillary cheek teeth of *S. robinsoni* (5.83–6.16 mm) than *S. fraterculus* (5.32–5.79 mm) becomes another invalid character once *S. r. vanakeni* (5.6–6.16 mm) is included in the comparison. Finally, the shorter anterior nasal breadth (4.27–4.88 mm) of *S. robinsoni* as compared to *S. fraterculus* (4.79–5.31 mm) also loses its diagnostic power with the inclusion of *S. r. vanakeni* (4.27–5.06 mm).

Although there are clear distinctions among these two species there are no discrete craniodental measurements to distinguish them. However, the first and third components of the PCA effectively separated these, especially the latter. PC3 loadings confirm that despite the previously mentioned overlapping measurements (anterior nasal breadth, maxillary cheek teeth and tympanic bullae length), their combination can still efficiently differentiate these taxa. Mastoid breadth, diastema length and interorbital breadth also seem to be diagnostic to a lesser degree with moderate contribution in the PC3 loadings (**Supplementary File S1**). *S. robinsoni* seems to have a somewhat broader mastoid breadth and narrower interorbital breadth than *S. fraterculus. S. robinsoni* also exhibits a relatively narrower least frontal bone breadth than *S. fraterculus*. Finally, *S. robinsoni* has a relatively shorter muzzle than *S. fraterculus,* which is reflected in a relatively shorter length and height of rostrum (**Supplementary Figure S5**).

Distribution: Malay Peninsula, Sumatra, and Batu and Rhio Archipelagos. The subspecies *S. r. robinsoni* and *S. r. balae* have been recorded from 0–610 m, on the Malay Peninsula and Rhio and Sumatra and Batu, respectively, while *S. r. vanakeni* has been recorded between 900–1372 m on Mount Kerinci.

Sundasciurus natunensis (Thomas, 1895)- Natuna squirrel.
Sciurus lowii natunensis Thomas, 1895:26.—Type locality. "Sirhassen Island" (Serasan), Natuna Islands.
Holotype. BMNH 94.9.28.40, skull, skin, adult male, collected 23 September 1893 by A. H. Everett.

Emended comparison with other *Sundasciurus* species.

Comparisons of *S. natunensis* with *S. robinsoni* are detailed in the previous section.

The slight brown pinkish flush described by Thomas (1895) was only found to be present in the type series and is likely an effect of the preservation of these specimens. As Thomas reasoned, these were "skinned out of spirit" and certain yellows turned into red in other specimens of that same expedition. Therefore, this species exhibits the same coloration as *S. lowii,* and can be distinguished from *S. fraterculus* based on paler ventral coloration. A major external distinction is that *S. natunensis* (together with west Borneo *S. l. lowii*) lack that tapered appearance in its tail (described in the previous section) that characterizes *S. lowii* (from remaining Borneo and northern Natunas), with a thinner tail base, and *S. fraterculus,* with a thicker tail base (**Supplementary Figure 4**). Thomas (1895) described *S. natunensis,* in relation to *S. lowii*, as having "smaller size, shorter feet and longer ears, which in the typical variety is a mere low rim, while the Natuna subspecies has a distinct upstanding conch". The data presented here show smaller but largely overlapping head-body size (133.3–152. mm) and shorter but overlapping feet (33–35 mm with claw) than *S. lowii* (127–169 mm; 32–40 mm with claw). However, the only ear measurement available for *S. natunensis* (provided in the original description) is smaller (12 mm) than *S. lowii* (12–17 mm), not larger. Nevertheless, in relation to this, perhaps the most distinctive characteristic of *S. natunensis* is its relatively larger bullae than *S. lowii*. Besides this, the skulls of *S. natunensis* and *S. lowii* are quite similar and conserved in proportion, and only differ on the relatively thinner and less deep rostrum and narrower palate of *S. natunensis* (**Figure 6** and **Supplementary Figure S5**). The skull of *S. natunensis* (33.60–36.43 mm) is smaller than that of *S. lowii* (36.93–41.03 mm), as Thomas (1895) pointed out. The other non-overlapping characters are braincase height (12.34–13.01 mm) and orbit length (8.33–9.05 mm), which are also larger in *S. lowii (*13.07–14.30 mm; 9.03 – 10.06 mm). Our expanded dataset also supports Thomas' statement that *S. natunensis* "postorbital processes are longer and slenderer" than those of *S. lowii* (except West Borneo populations), but also than *S. fraterculus* and *S. robinsoni*. The presence of chisel-shaped incisors with sharp-angled beveled edges are an additional diagnostic feature to differentiate *S. natunensis* from *S. lowii*.

The species *S. natunensis* has a larger skull (35.35–36.55 mm), rostrum (14.84–15.36 mm), mastoid breadth (15.66 – 16.56 mm), diastema length (8.91–9.68 mm), bony palate breadth (7.81–8.03 mm), upper molar row length (6.72–7.15 mm) and bulla length (7.26–7.65) than *S. fraterculus (*32.46–34.51 mm; 13.550 – 14.83 mm; 14.48–15.43 mm; 7.84–8.67 mm; 6.40–6.99 mm; 5.32–5.79 mm; 5.88–6.43 mm). In addition, these taxa also differ in skull proportions, with *S. natunensis* having a relatively more gracile skull than *S. fraterculus,* which exhibits a broader skull (IB and LBC) and thicker rostrum (HR) relative to skull length.

Distribution: Southern Natuna islands (only recorded in Sirhassen Island), and possibly in west Borneo. Despite discrete characters such as postorbital processes and tail shape of the latter populations closely resemble *S. natunensis*, we currently consider the taxonomic status of west Borneo populations as *incertae sedis* given the intermediate phenotypic position of these among *S. natunensis* and *S. lowii* shown in the PCA and DAPC and the lack of genetic evidence. Genetic data from these, southern Sumatra and northern Natuna populations is needed to clarify the taxonomy and distribution of this group.

***Sundasciurus lowii* (Thomas, 1895) -Low's squirrel.**
Sciurus lowii Thomas, 1895: 253.—Type locality. "Lumbidan, on the mainland opposite Labuan", Sarawak, Borneo. Holotype. BMNH 76. 5. 2. 14., skull, skin, adult male, collected by H. Low (unknown date).

Sciurus lingungensis Miller, 1901:123.—Type locality. Lingung Island, Near Bunguran Island, Natuna Islands.

Subspecific Taxonomy

Sundasciurus lowii lowii (Thomas, 1895)

Sundasciurus lowii bangueyae (Thomas, 1908)
Sciurus lowii bangueyae Thomas, 1908:387.— Type locality. "Banguey Island" (Banggi)

Sundasciurus lowii lingungensis (Miller, 1901)
Sciurus lingungensis Miller, 1901:123.—Type locality. Lingung Island, Near Bunguran Island, Natuna Islands

Distribution: *S. l. lowii* is present in Borneo, *S. l. bangueyae* in Banggi and Balambangan islands, (and possibly Malawali island as well) and *S. l. lingungensis* in the Northern Natunas (recorded in Bunguran, Lingung and Laut islands).

Taxonomic Notes

Corbet and Hill (1992) pointed out that geographical variation in *Sundasciurus lowii sensu lato* is slight. Many of the currently recognized subspecies are not morphologically distinct from others. We consider *Sundasciurus robinsoni balae* and *Sundasciurus robinsoni vanakeni* valid subspecies based on their ventral coloration and craniodental differentiation (**Figure 5** and **Supplementary Figure S3**). We synonymize former *Sundasciurus lowii humilis* with *Sundasciurus robinsoni balae* due to external resemblance (ventral grayish coloration in limbs). We also synonymize based on external resemblance (lack of ventral grayish coloration in limbs) *Sundasciurus lowii seimundi* with *Sundasciurus robinsoni robinsoni*. Finally, we consider *Sundasciurus lowii lingungensis* and *Sundasciurus lowii bangueyae* valid subspecies given their craniodental differentiation (**Figure 5** and **Supplementary Figure S3**).

Molecular species delimitation analyses suggest the presence of three species in Borneo within *S. lowii* (in Sabah, Sarawak and East Kalimantan). However, phenotypic divergence seems to be slight and the fine scale distribution of the morphs is unknown. The only Sarawak sample included in the PCA was clustered separately but close to Sabah + east Kalimantan, that largely overlapped. Regarding the DAPC, only east Kalimantan samples were differentiated from the remaining overlapping populations. Relative size of interorbital breadth seems to differentiate a small number of Sarawak specimens from the remaining *S. lowii*, but these samples are clustered very close to the other *S. lowii*. Sarawak specimens also seem to have darker fur than Sabah and East Kalimantan *S. lowii* and a lack of blond tips on the tail, which are only observed in Sabah populations. We consider these populations unconfirmed candidate species (Padial et al., 2010) until a better molecular and morphological sampling is performed in terms of geographic coverage and number of specimens included.

As pointed out by Hawkins et al. (2016a) the two subgenera, *Aletesciurus* and *Sundasciurus,* proposed by Moore (1958) are not supported as reciprocally monophyletic groups. The discrete characters (presence of sagittal crest, skull size, and shape of anterior-mesial lobe of auditory bullae) described as distinguishing subgenera were not valid for all species. For instance, the Palawan mountain squirrel (*Sundasciurus rabori*) belongs to *Aletesciurus* but lacks a sagittal crest and has an intermediate antero-mesial lobe and skull size (40.9-43.6 mm) among both subgenera (Heaney, 1979; Hawkins et al., 2016a). The northern Palawan tree squirrel (*Sundasciurus juvencus*) shows an antero-mesial lobe that resembles that of the subgenus *Sundasciurus* despite being assigned to *Aletesciurus*. Finally, different species of the subgenus *Sundasciurus* such as *S. lowii*, *S. tahan* or *S. altitudinis* have skulls that reach sizes (41–43 mm) that overlap with those of *S. rabori* (authors unpublished data). Although these discrete characters do not support the subgenera *Aletesciurus* and *Sundasciurus*, Heaney (1979) found support for them in his morphometric analyses. Molecular phylogenies suggest that there are 5–6 major phylogenetic lineages within *Sundasciurus*, which correspond to monophyly in the subgenus *Aletesciurus* (which contains 3 divergent clades), and paraphyly in the subgenus *Sundasciurus* (which also contains 2–3 divergent clades) (Den Tex et al., 2010; Hawkins et al., 2016a). The lack of universally valid diagnostic features and the phylogenetic evidence from this and previous studies demonstrate that current *Sundasciurus* subgeneric classification is invalid, so we synonymize *Aletesciurus* with *Sundasciurus*.

DISCUSSION

Deep genetic and moderate phenotypic divergences between the lowland Sunda tree squirrels formerly recognized under the species *Sundasciurus lowii* were confirmed. The consistency between these two lines of evidence along with geography required the revalidation of *Sundasciurus robinsoni* and species level recognition of *S. natunensi*s.

The patterns of divergence suggest ancient diversification events between these taxa that predate the Pleistocene. This does not support the Pleistocene speciation-pump hypothesis as the main driver behind the high levels of diversity found in Sundaland (Brown et al., 2013; Husson et al., 2019). The ancient patterns of divergence that we found in Sunda squirrels are consistent with those found in other small vertebrates and invertebrates (Klaus et al., 2013; Grismer et al., 2017; Karin et al., 2017; Manawatthana et al., 2017). This is particularly interesting in light of recent geological work suggesting that the Sunda shelf was continuously exposed until 400 ka (Husson et al., 2019). This suggests that dispersal should have been fluid across the entire shelf until this point. The much older times of divergence suggest that there was substantial structure, and likely speciation prior to the inundation of the Sunda shelf in the Middle Pleistocene.

The dated phylogeny also suggests that Sunda squirrels experienced a rapid radiation, given the almost simultaneous diversification event during the middle-late Miocene transition among major lineages of *Sundasciurus*. The mito-nuclear discordant relationship among these major lineages supports this hypothesis. There are two potential processes that might have driven this gene discordance, incomplete lineage sorting and hybridization, both of which have been viewed as byproducts of rapid radiations (Degnan and Rosenberg, 2006; Wiens et al., 2006; McLean et al., 2016). The latter has proved to be a widespread process across Sciuridae (Garroway et al., 2010; Chang et al., 2011; Chavez et al., 2014; McLean et al., 2016; Hawkins et al., 2016b), and may be the result of speciation outpacing the evolution of reproductive incompatibilities (Wiens et al., 2006). Interestingly, similar diversification patterns have also been shown during this narrow timeframe in stream toads, halfbeaks and colugos (De Bruyn et al., 2013; Grismer et al., 2017; Mason et al., 2019).

Land bridges that were formed after the inundation of the Sunda shelf may have allowed disparate dispersal rates between the different land masses. Whether or not a species successfully dispersed during these periods was likely due to a number of factors including the available habitat on the exposed shelf, distance between land masses, and presence or absence of close relatives/competitors for the same resources. Previous studies have suggested that during this time, despite shelf exposure, many rainforest taxa remained isolated (Den Tex et al., 2010; Leonard et al., 2015; Mason et al., 2019).

The Mentawai Islands are geographically very close to Sumatra, but were not connected by land bridges to Sumatra when other land masses were connected due to a deep-sea trench between them (Voris, 2000; Roberts et al., 2011). The endemic fraternal squirrel became isolated from Robinson's squirrel, which was distributed across Sumatra and the Malay Peninsula in the Pliocene. This level of deep divergence between taxa on Mentawai and sister taxa elsewhere in Sundaland has also been identified in other taxa such as gibbons, civets and skinks (Chan et al., 2010; Patou et al., 2010; Karin et al., 2017) and even deeper divergence was found in macaques (Evans et al., 2017). This sister relationship between Mentawai and Sumatra-Malay Peninsula populations (Meijaard and Groves, 2004; Chan et al., 2010; Patou et al., 2010; Arifin et al., 2018) however, is not the most common in mammals (Wilting et al., 2012). For example, the Mentawai treeshrew (*Tupaia chrysogaster*) is sister to the endemic Bornean Long-footed treeshrew (*T. longipes*, Roberts et al., 2011) and the Mentawai endemic Koopman's pencil-tailed tree mouse (*Chiropodomys karlkoopmani*) is sister to the Bornean endemic greater pencil-tailed tree mouse (*C. major/C. calamianensis)* (Musser and Carleton, 2005).

Although bathymetry suggests that the Mentawai islands have not been directly connected to Sumatra, it is possible that they have been connected to the Batu Islands which have been connected to Sumatra, creating a peninsula parallel to the latter (Voris, 2000; Roberts et al., 2011). Potential episodes of gene flow across this past connection in both directions could explain the lack of differentiation in nuclear introns but high mitochondrial divergence and important phenotypic differentiation among populations. The intermediate phenotype of the Siberut population, the closest Mentawai island to the Batu archipelago, which externally resembles Robinson's squirrel but is clustered within the craniodental variation of the Fraternal squirrel would also support this. In accordance with this peninsular dispersal corridor, Karin et al. (2017) suggest a divergence of ∼2.60 Mya among skink populations of Nias (Batu Islands) and Siberut (Mentawai Islands) which would be slightly after to the divergence among Mentawai and Malay Peninsula squirrels. Given that western Sumatra and Mentawai have been suggested to be a forest refuge (Morley, 2000; Lim et al., 2010) it is not straight-forward to assess the direction of the potential dispersal events. Intron haplotype networks suggest that *S. fraterculus* is within the diversity of *S. robinsoni*, which could suggest a Malay Peninsula to Sumatra to Batu to Mentawai colonization. However, within Mentawai, just South Pagi was sampled, so data from other populations from Mentawai, Sumatra and Batu islands are needed to confirm this.

There is a deep, Late Miocene divergence between the western Robinson's squirrel and Fraternal squirrel and the eastern Low's squirrel and Natuna squirrel. At about the same time the western high elevation Sunda squirrels (*S. tahan* and *S. altitudinis*) diverged from the western slender and Jentink's squirrels. This period was also characterized by diversification across Sundaland in semiarboreal rough-skinned skinks, bulbuls, barbets and Sundaic freshwater crabs (Den Tex et al., 2010; Klaus et al., 2013; Karin et al., 2017; Manawatthana et al., 2017). These common patterns could be due to dispersal barriers following the development of the Bornean highlands and river systems into their more modern representation by ∼8–10 Mya (Hall, 2013; Mason et al., 2011; Yang and Rannala, 2010). Alternatively, vicariance during fragmentation of the Sunda shelf /oceanic dispersal between islands during periods of high sea-levels by ∼5–6 Mya (Karin et al., 2017) if there were early inundations, could also explain this pattern.

The Natuna Islands are located in the South China Sea between Borneo and mainland Asia, a key location for understanding the biogeography of the region. Few studies to date (Den Tex et al., 2010; Mason et al., 2011, 2019) have been able to gather any information from historic specimen samples from this archipelago possibly due to poor specimen and DNA preservation. A deep divergence between populations of colugos from the northern and southern Natuna islands has been identified, with the former populations closely related to northeastern Borneo populations and the latter populations to the rest of Borneo (Mason et al., 2019). Here we show that the Natuna squirrels from the southern islands are deeply divergent from Low's squirrels of central and east Borneo (**Figure 3**). These squirrels from the southern Natuna islands are also morphologically distinct from the northern Natuna squirrels, which are more similar to central and east Borneo specimens (**Figure 5**). Finally, populations from west Borneo have an intermediate phenotype (**Supplementary Figure S3**). These squirrels might have a similar pattern to the colugos, with one species on the southern Natuna islands and possibly extreme west Borneo (the Natuna tree squirrels, *S. natunensis*) and the other on the northern islands and rest of Borneo (*S. lowii*). However, additional genetic and morphologic data from the Natuna islands and Borneo is needed in order to confirm this hypothesis and the taxonomic status of west Borneo and north Natuna populations.

While divergence in allopatry seems to be the main process driving the speciation of Low's squirrels, ecomorphological and behavioral adaptations in this clade suggest an important role of post-vicariance/colonization niche divergence, and a lack of niche conservatism. Previous studies have shown a significant correlation between locomotion modes and morphological traits in squirrels and other rodents. Tail length is thought to be associated with arboreality in squirrels (Hayssen, 2008) and other small mammals such as deer mice (Horner, 1954; Kingsley et al., 2017) and treeshrews (Martin, 1968). Nations et al. (2019) found a positive relationship of tail length with climbing in Philippine murines, but also a large variance and low predictive power for this trait across all locomotor categories. Thorington and Heaney (1981) provided a comparative table with different ratios that also shows this trend (e.g., the most terrestrial *Dremomys rufigenis* and *S. lowii* exhibit the relative shortest tail among Callosciurinae). Arboreal Sigmondontines and tree squirrels are known to have a more rounded and inflated vault and shorter nasals than terrestrial species (Lu et al., 2014; Camargo et al., 2019*)*. Arboreal squirrels and Procyonids also seem to have shorter nasals or slightly shorter olfactory bulbs compared to terrestrial ones (Lu et al., 2014; Ahrens, 2014; Bertrand et al., 2019). A longer rostrum could imply a better sense of smell, which might be useful when living on the ground (Lu et al., 2014) while brain enlargement is an adaptation in arboreal species that use the space in three dimensions and need to integrate information of complex environments (Camargo et al., 2019*)*. The relatively long tail, larger braincase and shorter rostrum of *S. robinsoni* as compared to the terrestrial *S. lowii* and *S. natunensis* might suggest arboreality. Ecological field studies (Kemper and Bell, 1985; Sharma, 1992; Abdullah et al., 2001; Yasuda et al., 2003; Saiful and Nordin, 2004; Jayaraj et al., 2013) and diet data finding bark, fruit and insects (Harrison, 1962; Abdullah et al., 2001) are also consistent with an arboreal life style for *S. robinsoni*. On the other hand, *S. lowii* on Borneo seems to be much more terrestrial (Davis, 1962; Wells et al., 2004; Camacho-Sánchez et al., 2019) with a diet including small arthropods, fruits and fungus (Davis, 1962). The Fraternal squirrel seems to have intermediate habits, since it uses the ground extensively, but also climbs showing a preference for heights up to five meters, but avoids the upper canopy (Whitten, 1981). It has only been observed eating bark, moss and lichen, but stomach content analyses also found annelid remains, in accordance with more terrestrial behavior.

The relatively longer rostrum of *S. lowii*, *S. fraterculus* and *S. natunensis* with respect to *S. robinsoni* might be due to its terrestrial foraging behavior, reflecting a more developed olfactory bulb as an adaptation to a more insectivorous diet. In addition, the more robust skull of *S. fraterculus* with regard to *S. robinsoni* might reflect a possible adaptation to the harder dietary elements such as an increased consumption of chitin from insects, lichens and bark, and an absence of soft food items such as fruit (Whitten, 1981). These findings suggest that not only has there been a high degree of genetic and phenotypic divergence, but also that morphological changes have possibly been the result of natural selection driven by adaptation to different niches, and are not only due to drift caused by geographic isolation (Mayr, 1963). It is difficult to interpret if the different degrees of scansoriality found among these species are due to different levels of interspecific competition, predator avoidance, food limitation or a combination of some of these. In any case, differences in the squirrel and treeshrew communities among islands are notable. On the Mentawai islands, *S. fraterculus* has to share its habitat with just one diurnal treeshrew (*Tupaia chrysogaster*), one terrestrial squirrel (*Lariscus obscurus)* and one arboreal diurnal squirrel (*Callosciurus melanogaster*), allowing it a greater range of vertical habitat than its sister species (Whitten, 1981). On Borneo, *S. lowii* has to share its habitat with up to six diurnal treeshrews and seven species of diurnal squirrels. Two of the treeshrews (*Tupaia minor* and *Tupaia gracilis*) and most of the squirrels are arboreal, and three of the latter are bark gouging specialists (*Sundasciurus tenuis*, *Nannosciurus melanotis* and *Exilisciurus exilis*) (Emmons, 2000; Zelditch et al., 2017). However, the only two terrestrial squirrels (*Lariscus insignis* or *L. hosei* and *Rhinosciurus laticaudatus)* are in very low densities or possibly absent where Low's squirrel is present (Wells et al., 2007; Yasuda and Tsuyuki, 2012; Fitzmaurice, 2014; Chapman et al., 2018; Camacho-Sánchez et al., 2019; authors unpublished data) and are specialized on fruits and insects. On Sumatra and the Malay peninsula *S. robinsoni* is co-distributed with diurnal arboreal and terrestrial treeshrews (*Tupaia minor* and *T. glis/T. ferruginea)*, and up to ten diurnal squirrels, three of which are terrestrial (*Lariscus insignis, Menetes berdmorei* and *Rhinosciurus laticaudatus)* (Lekagul and McNeely, 1977). These terrestrial squirrels seem to be more common in mainland field-studies than in Borneo (Kemper and Bell, 1985; Yasuda et al., 2000; Syakirah et al., 2001; Yasuda et al., 2003; Jayaraj et al., 2013). On the other hand, the bark gouging niche seems to be less exploited in Peninsular Malaysia, with just *S. tenuis* (Zelditch et al., 2017). This could have potentially added evolutionary selective pressures on the ancestor of *S. robinsoni* to alter its diet to avoid competition with other species. The relatively larger and more orthodont incisors of this species with regard to its closest relatives are also in line with this hypothesis. Convergence towards a bark gouging diet seems to have arisen at least seven times in Sciuridae and it exhibits higher rates of convergence relative to the number of living species (10 species) within that dietary niche (Zelditch et al., 2017).

Within Borneo, deep genetic divergences were detected across *Sundasciurus lowii*. Bornean populations exhibit a high level of genetic and some morphological diversity, but the fine scale distribution of this diversity is not yet characterized. Molecular species delimitation analyses suggest candidate species found in Sabah, Central Sarawak and East Kalimantan. However, much more sampling will be required to clarify their taxonomy. As in previous studies on Sunda squirrels, our results also point towards a possible underestimation of highland species diversity given the high genetic divergence observed among populations of *S. altitudinis* and high phenotypic divergence found between Mount Kerinci mid-elevation (900–1400 m) populations (*S. r. vanakeni*) and the remaining Sumatra and Malay Peninsula lowland populations (*S. r. robinsoni* and *S. r. balae*). The lack of molecular evidence for this pelage and craniodentally distinct population suggests to consider *S. r. vanakeni* as an unconfirmed candidate species (Padial et al., 2010). Given that a previous attempt failed to amplify DNA from the only known series of specimens of this taxon (Den Tex et al., 2010) future collection of new specimens along an elevational gradient in Kerinci will be needed to confirm the specific status of this population.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Smithsonian Institution, National Museum of Natural History, proposal number 2012-04 and Estación Biológica de Doñana proposal number CGL2010-21424.

AUTHOR CONTRIBUTIONS

AH, MH, JM, and JL designed the study. AH, MH, and AA performed field and museum data collection. AH and MH performed laboratory work and statistical analyses. AH and JL wrote the manuscript with input from MH, AA, and JM. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00208/full#supplementary-material

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Fitness Estimation for Ecological Studies: An Evaluation in Columbian Ground Squirrels

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The topics of evolution by natural selection and ecological interactions are closely intertwined. Thus, measurements of evolutionary fitness are ubiquitous in the ecological literature. As an empirical problem, the components of fitness, reproduction and survival, may be analyzed to produce fitness estimates in several ways. These can be divided into annual estimates that are most appropriate for short-term (e.g., annual) and experimental studies, and lifetime fitness estimates that are most appropriate for evaluating functional organismal traits. The latter are appropriate for comparative studies of natural selection in species with lifetimes that extend over several years. These estimates may also be particularly useful for estimating the direct and indirect components of inclusive fitness, an important topic for the evolution of cooperation. We reviewed examples of some of these alternatives from our research on Columbian ground squirrels (*Urocitellus columbianus*). Using empirical data, we also test the degree of correspondence of annual fitness, lifetime reproductive success, and individual fitness measures that are based on matrix methods. We conclude that correspondence of different methods is not strong, though each method appears most appropriate for different types of traits and research questions about fitness differences among trait forms.

Keywords: annual fitness, Columbian ground squirrels, individual fitness, lifetime reproductive success, matrix algebra, natural selection, population dynamics

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INTRODUCTION

In his seminal book on evolutionary ecology, G. Evelyn Hutchinson described the interaction between ecology and evolution as "the ecological theater and the evolutionary play" (Hutchinson, 1965). The idea is that ecological conditions provide the selective influences on phenotypic traits that evolve through the process of natural selection (Darwin, 1859). In a population, phenotypic traits may change in frequency over time, and this change can be inferred from fitness differences, patterns of reproduction and survival that are associated with different trait forms. This evolutionary principle, that trait adaptation by natural section occurs in ecological environments, has been applied to many empirical studies, and is assumed by many more. Studies of natural selection on traits commonly measure changes in trait frequencies that occur over time, either from year to year or from generation to generation [e.g., studies reviewed by Endler (1986) and Charmantier et al. (2014)]. Such changes are produced by variations in reproduction and survival, and are used as surrogate measures of evolutionary fitness for the trait forms that individuals carry.

Empirical studies often group individuals that express alternative forms of a trait into "trait groups" or use individuals to represent a continuum of trait values (termed "trait forms" for continuous traits). Although trait fitness is typically estimated through the reproduction and survival of individuals that carry specific trait forms, It is the change in frequency of traits – not individuals – that is the result of natural selection. In this sense, trait groups can be considered alternative adaptations that respond to environmental conditions. Good examples are studies of the influence of global changes in climate on the timing of reproductive events or other elements of a species' lifecycle (e.g., Visser and Both, 2005; Chamaillé-Jammes et al., 2006; Parmesan, 2006; Lane et al., 2012; Tafani et al., 2013; Dobson et al., 2016; Radchuk et al., 2019). An important caveat is that individuals have many traits, some of which may be genetically correlated through linkage disequilibrium or pleiotropy (e.g., Duckworth and Kruuk, 2009; Bize et al., 2017; Mullon et al., 2018). Thus, single-trait studies may not identify the traits that are the targets of selection, including important evolutionary tradeoffs, and the actual interactions with agents of selection in the environment (Falconer, 1952; Lande and Arnold, 1983; Roff and Fairbairn, 2012). In addition, the fitness of a trait form is relative to the success of alternative trait forms, as populations dynamically change. Hence, the fitness of traits is relative to what other individuals are doing at a given moment in time for a population that may be increasing or decreasing. Measuring fitness for alternative trait forms is thus complicated by the need to account for population demographics (Metcalf and Pavard, 2007).

Nonetheless, studies of single or a few traits provide a good starting place for more in-depth examinations of suites of traits and suites of environmental factors. Early studies examined the influence of environmental events on morphological traits and differential survival (e.g., Bumpus, 1899; Kettlewell, 1961; Lande and Arnold, 1983), but later studies examined both reproduction and survival (e.g., Pemberton et al., 1999). Measures that combine reproduction and survival (e.g., annual fitness; Qvarnström et al., 2006) have been applied to examine traits expressed on an annual basis such as reproductive phenology (e.g., Lane et al., 2012; Dobson et al., 2017). Other measures examine traits expressed for a lifetime, like foraging patterns (using lifetime reproductive success, LRS; Clutton-Brock, 1988; Altmann, 1991) or age at first breeding (via individual fitness from matrix methods; McGraw and Caswell, 1996). Lifetime reproductive success is not sensitive to the timing of successful reproductive events (that might occur at any point in a lifecycle), but individual fitness measures are sensitive to when such events occur (Brommer et al., 2002). The impact of this difference on studies of natural selection is currently unknown (Brommer et al., 2004; Reid et al., 2019).

Fitness is a population parameter, though it is often estimated for individuals. A variety of fitness estimates have been developed for individuals, using demographic data from populations (e.g., Clutton-Brock, 1988; McGraw and Caswell, 1996; Qvarnström et al., 2006; Viblanc et al., 2010; Rubach et al., in press). However, natural selection operates on trait groups in populations, or rather on suites of correlated traits. Thus, individuals are divided into groups, effectively sub-populations, that exhibit alternative expressions of the trait(s) of interest. When we compare these alternative trait groups (or forms, for continuous traits), a rough idea of the association of the trait form and fitness (reproduction or survival, but best is a combination of the two) can be obtained. This is a rough estimate of selection on the trait forms. One problem is that the trait of interest may be genetically correlated with other traits that constrain or magnify the change in trait forms from one generation to the next (e.g., Lande and Arnold, 1983; Price and Langen, 1992). Thus, identifying which trait forms are the actual "targets" of selection can be difficult. Another problem for inference about natural selection is that we may have little idea about the heritability of trait forms. However, given caution, it is instructive to understand which trait forms appear associated with fitness differences and which do not, remembering that selection and response to selection are two different things.

Our purpose is to compare and contrast different methods of measuring fitness, applied during the course of our long-term research program on Columbian ground squirrels (*Urocitellus columbianus*). We provide examples from past studies using different fitness measures, all based on empirical studies of reproduction and survival during 28 years of research on ground squirrels. In these examples, we used information about reproduction, survival, and traits of individuals to evaluate fitness of alternative trait groups or trait forms. We used annual fitness estimates in some studies, and lifetime fitness in others. In some cases, experimental treatments provided the alternative trait groups, but we also applied continuous variables to estimate trait forms. In one study, we examined direct and indirect components of inclusive fitness of cooperative behavior from helpful close kin. Herein, our goals were: first, to review our long-term studies of reproduction and survival in a comparison of annual and lifetime estimates of fitness, and lifetime reproductive success and individual fitness estimates from matrix models. We expected that annual and lifetime estimates of fitness might not correspond closely, since environmental conditions may vary among years (Lane et al., 2012; Dobson et al., 2016). Second, to test this expectation, by comparing annual and alternative lifetime fitness measures for correspondence. Different methods should correspond closely, if they give an accurate estimate of the fitness of individuals and their traits.

MATERIALS AND METHODS

Field Site and Ground Squirrels

Columbian ground squirrels (*Urocitellus columbianus*) were studied from 1992 to 2019 in the Sheep River Provincial Park in Alberta, Canada [50°38′N, 114°39′W; elevation 1,550 m; see population #3 of Figure 1 in Dobson (1994)]. The study population was about 1.8 ha of meadow surrounded by pine-spruce forest on 3 sides and the gorge of the Sheep River on the final side. The meadowland contained grasses and forbs that provided forage for the ground squirrels, and was honeycombed with burrows that were dug out each year by these semi-fossorial rodents. Natural predators included coyotes (*Canis latrans*), red foxes (*Vulpes vulpes*), long-tailed weasels (*Mustela franata*), golden eagles (*Aquila chrysaetos*), and northern goshawks

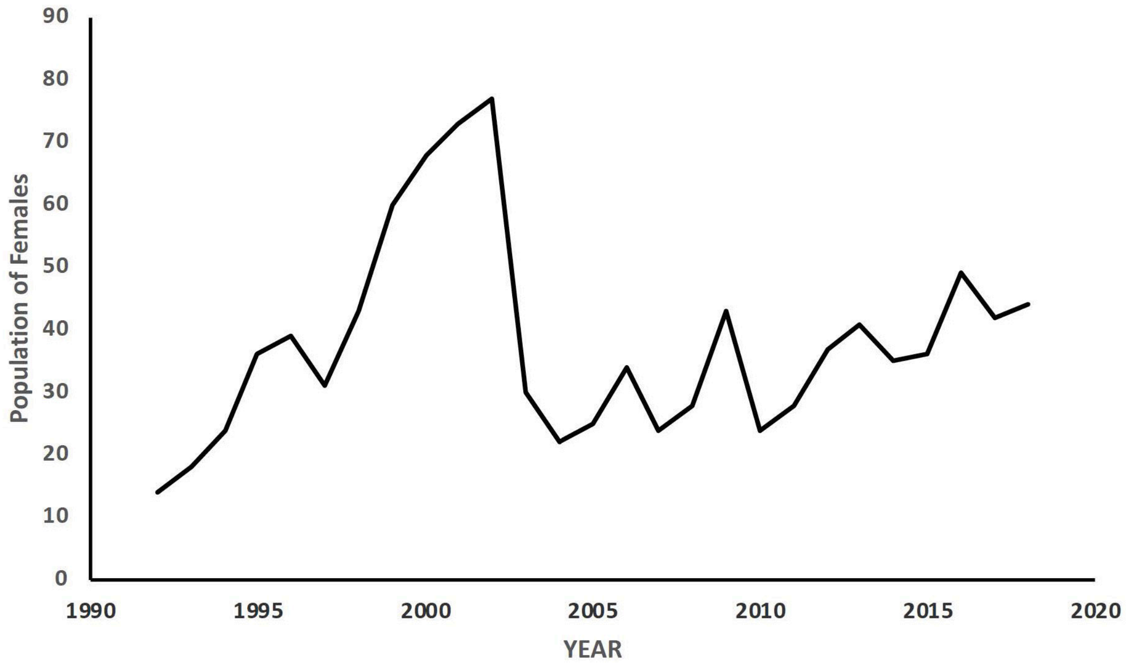

FIGURE 1 | Spring population size of females in a colony of female Columbian ground squirrels (≥1 year old) in Sheep River Provincial Park, Alberta Canada from 1992 to 2019 [used with permission, from Rubach et al. (in press)].

(*Accipiter gentilis*). Columbian ground squirrels hibernate for 8–9 months each year and have a single breeding season during their short period of annual activity (Dobson and Murie, 1987; Dobson et al., 1992). Our studies have focused primarily on female ground squirrels, because they are matrilocal and many were monitored over their complete lifespans (only 3 of 338 adult females were immigrants, over 28 years). Female ground squirrels can live up to 9–10 years (an exceptional female lived to 14 years), and usually begin reproduction at 2–3 years of age (Broussard et al., 2008), though yearling females occasionally reproduce in our population (Rubach et al., in press).

Each spring, we trapped all ground squirrels within 3 days of emergence from hibernation in live traps (13 × 13 × 40 cm; Tomahawk Live Trap, Hazelhurst, WIS, United States) baited with a small amount of peanut butter. Each individual was induced to enter a cloth bag for initial handling. We weighed each ground squirrel to the nearest 5 g in the handling bag with a Pesola spring-slide balance (1000 g; Schindellegi, Switzerland), fitted untagged individuals with a pair of numbered metal ear tags (Monel #1005-1; National Band and Tag, Newport, Kentucky), and measured their head width (zygomatic arch breadth) to the closest 0.1 mm with a pair of dial calipers. Each ground squirrel was examined for sexual condition (males abdominal or scrotal testes; females for appearance of the vulva, closed, open, open and swollen, and with or without a copulatory plug), presence and abundance of fleas, and wounds. We gave each ground squirrel a unique black mark on the pelage with black dye (human hair dye; Lady Clairol Hydrience, Procter & Gamble Co., Cincinnati, OH, United States) for later visual identification. Because unmarked individuals could be quickly identified and trapped, we were able to capture, mark, and examine every ground squirrel in the population that emerged from hibernation in every year of the study.

In the spring, female ground squirrels were watched daily for characteristic reproductive behavior (and regularly trapped to check the appearance of their vulva), to determine their single annual day of estrus, during which they are typically receptive to mating males for a period of 5–6 h. Subsequently, females typically went through 24 days of gestation and gave birth underground in single-entrance "natal burrows" where the young are nursed and protected by territorial mothers (Murie and Harris, 1988) during about 27 days of lactation (Murie and Harris, 1982). Juvenile ground squirrels first emerge above ground from their natal burrows near the time of weaning. Because mothers kept juveniles in single-entrance natal burrows, we were able to both identify the mother and capture emerging juveniles, usually on their first day above ground. Juveniles were trapped (Tomahawk or other cage traps), ear-tagged, examined to identify sex, examined for presence and number of fleas, and dye-marked with unique symbols. Males typically dispersed to other populations as yearlings, or remained residents on the study site but resided away from the area of their natal nest [and thus only occasionally interacted with female kin; dispersal pattern reviewed in Neuhaus (2006)]. In contrast, females were highly philopatric and interactive, very rarely dispersing to other populations (Arnaud et al., 2012). Thus, we were able to build lifetime records of reproduction and survival for females that could be used to estimate a variety of fitness measures.

Fitness Estimates

Annual Fitness

Our fitness estimates were applied to females, because females were matrilocal (Arnaud et al., 2012) and paternity was not measured in all years of our study (Raveh et al., 2010; Balmer et al., 2019). Using the long-term data, we calculated annual fitness as the mean number of gene copies that each female had represented in the following year (Qvarnström et al., 2006). A female had one copy if she survived to the next year, plus a half copy for each of her new offspring that also survived to the following year. For the estimate, these values were added together.

Lifetime Reproductive Success

Lifetime reproductive success (LRS) was measured by simply adding up the number of offspring that each female produced over her lifetime. We did this for offspring produced near the time of weaning, when young first emerged from natal burrows. We also constructed a relative index of lifetime reproductive success of each mother compared to their peers, by regressing lifetime reproductive success for each female onto the lifetime reproductive success of her cohort. The residuals of this regression were used as a relative index (LRS_{rel}).

Individual (Lifetime) Fitness

We calculated individual fitness estimates (λ_{ind}) for each female of the population that had lived-out her entire lifespan (thus, excluding those alive at the end of the study), following the matrix approach of McGraw and Caswell (1996). For each female, we constructed a matrix that had half her reproductive output (at either weaning of offspring, or offspring that survived their first hibernation season) specified on the top row of the matrix. This represented her annual contribution to reproductive production, the other half being from the male. The matrix had ones on the off diagonal, and zeros in all other unfilled elements (for an example, see Viblanc et al., 2010). The dominant right eigenvalue of this matrix (of value λ_{ind}) was a growth parameter for the matrix, and was taken as the lifetime individual fitness of the female and thus also her phenotype. The method was explained in detail by McGraw and Caswell (1996).

The individual fitness measure of McGraw and Caswell (1996) required additional attention. Any estimate of fitness of an individual is relative to others in the population. Over a lifetime that may extend several years, rodent populations are well known to fluctuate (e.g., Boonstra and Krebs, 2012; Fauteux et al., 2016; Brommer et al., 2017; Bonnet and Postma, 2018). For example, our population initially grew for about 10 years, then declined by close to 50% over 2 years, and then had a relative stable and gently increasing period for about 15 years (**Figure 1**). During times of population increase, a female with an estimated λ_{ind} of 1.0 does poorly compared to others in the population, since population growth (and the estimated fitness of an average female in the population) would have a λ value greater than 1.0. A female would be doing quite well, however, if the population was decreasing (population $\lambda < 1.0$). Thus, it may be necessary to adjust λ_{ind} for changes in population size or population growth, if one wishes to compare the traits among females that experience different populations changes over their lifetimes.

We adjusted our lifetime individual fitness measure for changes in the population in two ways. First, we regressed individual fitness (λ_{ind}) on a measure of population change in which comparative population values were produced by specifying proportional year-to-year population changes into a matrix in lieu of reproductive fertility values, but still with values of one in the off diagonal (to create $\lambda_{\Delta N}$, after Viblanc et al., 2010; Dobson et al., 2012). These matrices were constructed for the same years as each female's lifetime, and gave an indication of how the population, on average and over all females, was changing over time. Then λ_{ind} values were regressed on $\lambda_{\Delta N}$ values. The residual values of this analysis adjust for changes in population size during a female's lifetime, and we added 1.0 to these to make interpretation easier (producing $\lambda_{rel\Delta N}$). Second, we calculated actual population growth using a Leslie matrix (Leslie, 1945) for the cohort of each female, during that female's lifetime, and then similarly regressed λ_{ind} on λ_{Leslie} and added 1.0 to the residuals of the regression (producing λ_{relL}; after Rubach et al., in press). $\lambda_{rel\Delta N}$ and λ_{relL} were used as estimates of a female's fitness relative to her competitors, compared to the population at large and compared to her cohort, during her lifetime.

Direct and Indirect Components of Inclusive Fitness:

We estimated the direct and indirect components of inclusive fitness in two related ways. Inclusive fitness was relative to a particular trait, the presence of close kin ("genial neighbors") that demonstrably improve reproductive success and λ_{ind} relative to changes in population size ($\lambda_{\Delta N}$), to produce $\lambda_{rel\Delta}$ (after Viblanc et al., 2010; Dobson et al., 2012). First, we computed $\lambda_{rel\Delta N}$ for mothers with and without co-breeding close kin and compared these values. Next, we estimated inclusive fitness from a direct component (the mean relative individual fitness of mothers without kin present) and an indirect component (averaged: relative individual fitness for relatives present minus the mean fitness of mothers without kin present, times the degree of relatedness). These estimates were calculated based on the number of weaned offspring and included as kin only those relatives that appear to be recognized as close kin in the field (King, 1989). "Uterine kin" (viz., mothers, daughters, and littermate sisters) recognize one another via social familiarization in the natal nest-burrow (Hare and Murie, 1996). Other females were classed as distant and non-kin, and used for comparison to close kin. Further details of inclusive fitness calculations can be found in Dobson et al. (2012).

RESULTS AND DISCUSSION

Fitness Estimates in Columbian Ground Squirrels: Review From a Long-Term Study

Annual Fitness Estimates

Raveh et al. (2015) conducted an experiment on reproductive Columbian ground squirrel females that involved the removal of fleas (*Oropsylla* spp.) using a spot-on pet insecticide. The

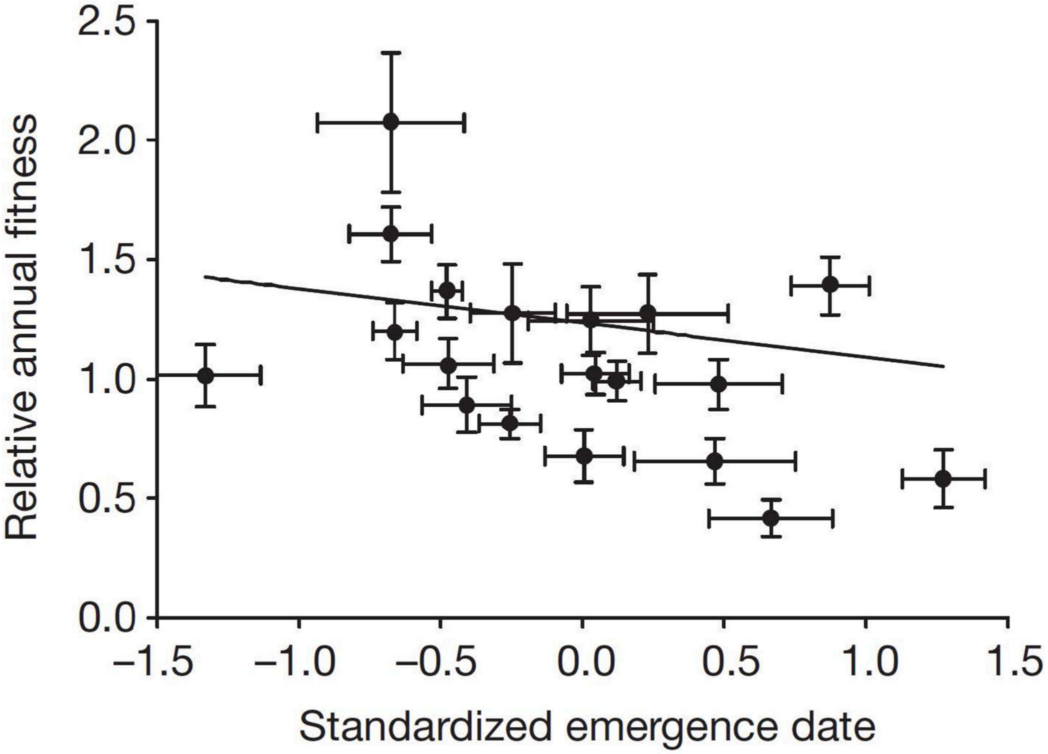

FIGURE 2 | Influence of mean emergence date from hibernation on mean annual fitness of adult female Columbian ground squirrels over a period of 20 years. The influence of standardized emergence date (standardized to a mean of zero and unit variance) on mean relative annual fitness (individual values divided by the population mean). From a mean-centered selection analysis, the between-individual selection gradient = –0.14 (highest posterior density interval = –0.27 to –0.03) [used with permission, from Lane et al. (2012)].

experiment artificially created the trait groups "with natural flea loads" and "with no fleas," and thus tested for a fitness cost to parasitism. The results indicated no significant difference between treated and untreated mothers using the Qvarnström et al. (2006) annual fitness measure (respectively; 1.13 ± 0.13, $n = 28$; and 1.06 ± 0.14, $n = 26$; mixed model, $x^2 = 0.06$, df = 1, $P = 0.80$). In addition, they found no significant difference in the annual fitness measure for mothers and young that occupied nests that were infested with fleas during lactation from nests that were not infested (respectively; 1.25 ± 0.16, $n = 20$; and 1.08 ± 0.14, $n = 26$; mixed model, x2 = 1.29, df = 1, $P = 0.26$). The conclusion of the study was that the removal of ectoparasites was not a significant influence on annual fitness, nor on several other measures related to maternal fitness. The Qvarnström et al. (2006) method was an appropriate measure for the experimental contrast and natural comparison of the ectoparasite "treatments," and two trait groups (viz., with and without parasites) were used in both comparisons. This method tracks the number of gene copies that were passed on in the population from 1 year to the next: one complete copy if the mother survives, and a half copy of her genes passes on through each of her male and female offspring.

Viblanc et al. (2016) applied annual fitness to a comparative network study of aggression by adult female Columbian ground squirrels. There were no significant differences in fitness among reproductive females according to the aggression that they received from other mothers. But aggression directed toward non-close kin was 2.3 times greater than aggression directed toward reproductive close kin [randomized network analysis, $P < 0.001$; Figure 3 in Viblanc et al. (2016)]. Mothers that most commonly committed aggression toward other females had significantly greater annual fitness [randomized network analysis, $P = 0.004$; see Figure 4 in Viblanc et al. (2016)]. The trait forms were different levels of committed aggression (chases and fights). Here, the Qvarnström et al. (2006) method was an appropriate measure of network analyses from a single year of behavioral data, but in this case the trait form was a continuous axis.

Annual fitness can also be applied to longer-term studies, such as those examining influences of changing climate. In Columbian ground squirrels, variations in spring and summer climates have significant influences on annual fitness (Lane et al., 2012; Dobson et al., 2016). Over a 20-year period, when adult females emerged from hibernation earlier, their annual fitness was greater (**Figure 2**). For this study, the trait forms were different dates of emergence from hibernation, a variable that was repeated for most individuals among years. Emergence from hibernation was influenced by snowmelt, and lower fitness ensued when spring melt-off of snowpack was later (**Figure 3**). Additionally, dry hot conditions during summer also had a strong negative influence on fitness (**Figure 4**). These seasonal climatic influences on the fitness of adult females produced strong influences on population

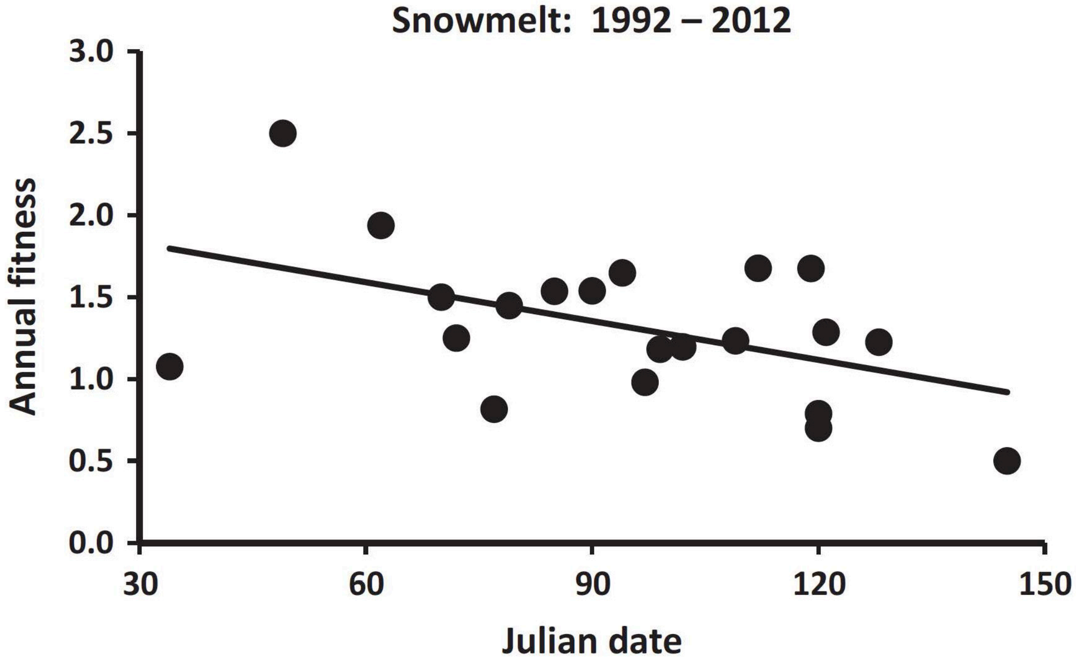

FIGURE 3 | Regression of annual fitness on Julian date of snowmelt. Points represent annual averages, between 1992 and 2012. The trendline reflects the significant negative regression slope ($\beta = 0.008 \pm 0.003$ SE, $R^2 = 0.23$, $F = 5.8$, d.f. = 1,19, $P = 0.03$) [used with permission, from Dobson et al. (2016)].

size as well, so that a year of especially late spring melt-off of snow and hot and dry conditions in summer were associated with a nearly 50% decline in the population (Dobson et al., 2016). In these studies, annual fitness was an appropriate index for comparisons, in part because annual events were studied. The Qvarnström et al. (2006) method has been used in a similar manner in several comparative and experimental field studies (e.g., Arnaud et al., 2013; Lane et al., 2015; Hoogland and Brown, 2016; Lane et al., 2019).

Lifetime Reproductive Success and Individual Fitness Estimates

Interest in the individuals that carry traits often leads to comparisons of the lifetime reproductive success of individuals with different phenotypic values of traits. But natural selection applies to traits and combinations of traits, rather than individuals per se. The frequencies of trait groups change over time when natural selection occurs. Thus, rather than the number of offspring or even grand-offspring for measuring changes due to natural selection (Brommer et al., 2004; Reid et al., 2019), the most useful measure is the growth rate of trait forms or associations of trait forms among generations. The most commonly used measure of increase in trait forms is lifetime reproductive success (e.g., Clutton-Brock, 1988; Grafen, 1988; Merilä and Sheldon, 2000; Jensen et al., 2004; Descamps et al., 2006; McLoughlin et al., 2007).

The growth of trait groups can also be estimated by calculation of individual fitness over the lifetime of individuals in the population (McGraw and Caswell, 1996). This method uses matrix algebra (classically used for estimating population growth) to estimate the increase in trait forms from changes in the frequency of phenotypic traits. The logic of doing this follows a long history of measuring fitness from the intrinsic growth rate of individuals that carry different trait forms (Stearns, 1992; Roff, 2002). A major advantage of the individual fitness approach is that it is sensitive to the timing of reproduction, so that offspring produced early in a mother's life contribute more to fitness than those produced later (Brommer et al., 2002).

Age at first reproduction

Demographic theory suggests that offspring produced early in life, if they carry a particular trait form, may themselves begin to reproduce earlier, thus contributing to further spread of the trait form over time (Stearns, 1992). Individual fitness was devised for an examination of age at reproductive maturity in Sparrowhawks (*Accipiter nisus*) and blue tits (*Parus caeruleus*) (McGraw and Caswell, 1996). For Columbian ground squirrels, a similar exploration of individual fitness revealed greater individual fitness values for individuals that begin to reproduce successfully at an earlier age (Rubach et al., in press). This was done with a calculation similar to that of McGraw and Caswell (1996). The number of weaned offspring was used to estimate reproductive success. Regression of λ_{ind} [as in McGraw and Caswell (1996)] on λ_{Leslie} (cohort growth) showed that an adjustment for changes in population growth was needed ($R^2 = 0.52$, $F = 52.6$, $P < 0.0001$, $n = 148$). Thus, Rubach et al. (in press) used the residuals of this regression (λ_{relL}) to estimate relative individual fitness. λ_{relL} differed significantly among females that first reproduced at ages 1, 2, and 3–5 years old (**Figure 5**). Lifetime reproductive success, however, showed no significant difference among the trait groups

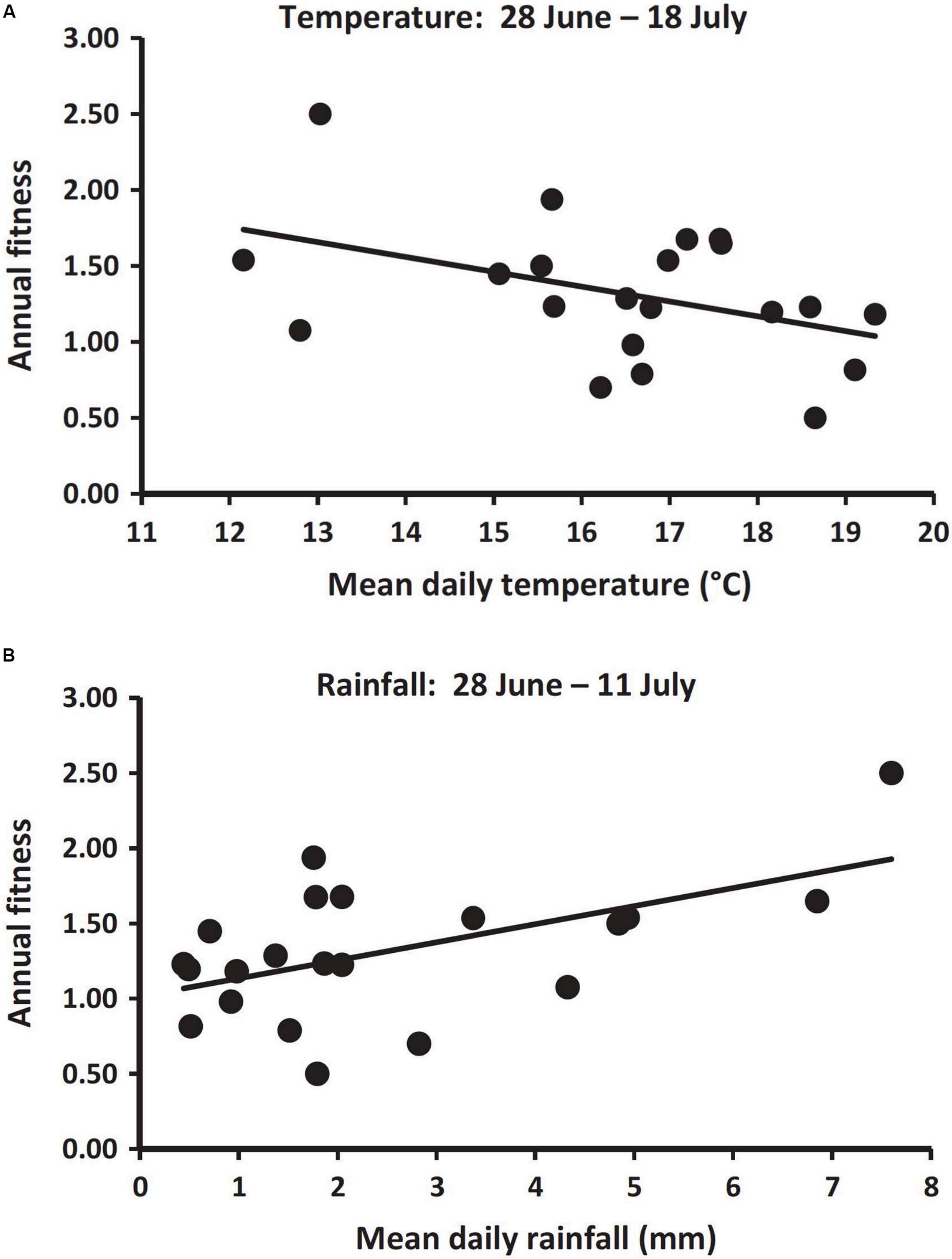

FIGURE 4 | Regression of annual fitness on **(A)** early summer temperature and **(B)** early summer rainfall after the young of the year were weaned (late June and July), when all ground squirrels were fattening for subsequent emergence into hibernation. Points represent daily period averages for 21 different years (1992–2012). Trendlines reflect significant negative and positive slopes, respectively, (β = 0.12 ± 0.04 SE, $R^2 = 0.18$, d.f. = 1,19, $F = 4.1$, $P = 0.05$; and β = 0.12 ± 0.04 SE, $R^2 = 0.30$, d.f. = 1,19, $F = 8.1$, $P = 0.01$) [used with permission, from Dobson et al. (2016)].

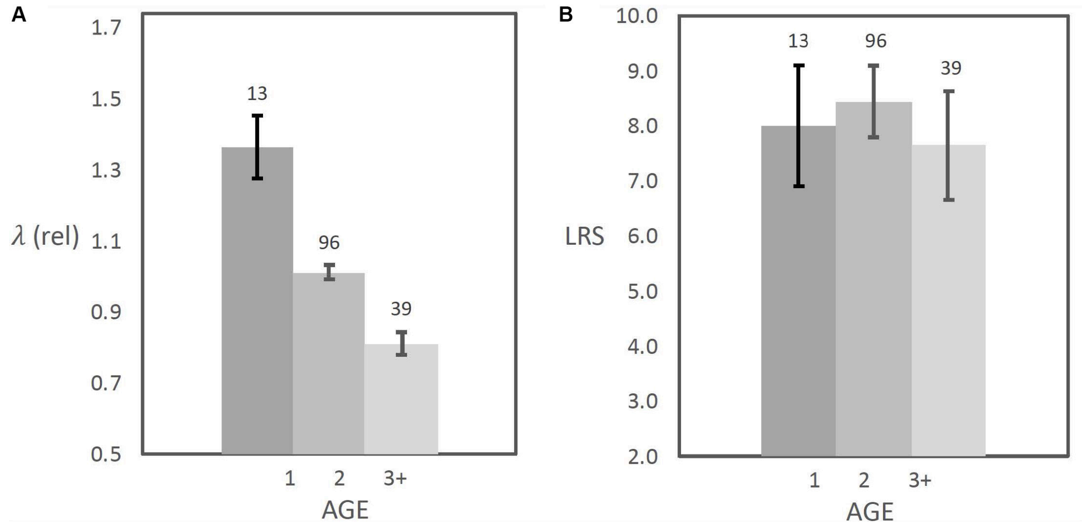

FIGURE 5 | Fitness estimate for females that first reproduced at different ages, from **(A)** means adjusted lambda (measured as λ_{relL}) and from **(B)** means lifetime reproductive success, both with standard errors, and reproductive success estimated from number of weaned offspring. Differences among ages were significant for λ_{relL}, but not for lifetime reproductive success (respectively; $R^2 = 0.309$, d.f. = 1,146, $F = 65.3$, $P < 0.0001$; $R^2 = 0.001$, d.f. = 1,146, $F = 0.20$, $P = 0.65$). Samples from 28 years (1992–2019) were included [used with permission, from Rubach et al. (in press)].

(i.e., females that first reproduce at ages of 1, 2, and 3 and above). Statistically comparing lifetime reproductive success for mothers to that of their peers (i.e., their cohort lifetime reproductive success, to produce $\mathrm{LRS}_{\mathrm{rel}}$) produced little difference among females that first reproduced at different ages. Additionally, an earlier study that used lifetime reproductive success as a fitness measure found no significant difference between females that first reproduced at ages 2 and 3 (Neuhaus et al., 2004).

Examples that examine age at maturity have presented the strongest case for using individual fitness estimates, since the age at which reproduction begins has a strong influence on population growth (Cole, 1954; Oli and Dobson, 2003). Examples of fitness differences that appear to favor early breeding include blue tits and sparrowhawks (McGraw and Caswell, 1996), Ural owls (Brommer et al., 1998), wood ducks (*Aix sponsa*) (Oli et al., 2002), and yellow-bellied marmots (*Marmota flaviventris*) (Oli and Armitage, 2003). Oli and Armitage (2008), found that female marmots that delayed breeding suffered a loss of inclusive fitness, even when direct fitness was augmented by indirect fitness benefits from reproduction of close kin. The attempt of Rubach et al. (in press) to compare the lifetime reproductive success of individual females to that of their cohorts ($\mathrm{LRS}_{\mathrm{rel}}$) yielded no beneficial insights to simple use of lifetime reproductive success. Thus, based on present evidence, no measure of fitness, whether annual or over the lifespan, is certain to provide an accurate description of natural selection or evolutionary response to natural selection. For long-term studies that examine complete lifespans, a cautious approach might be to apply a relative measure based on individual fitness and either on changes in population size or on population growth rate.

Relative individual fitness (e.g., λ_{relL} or $\lambda_{\mathrm{rel}\Delta\mathrm{N}}$) takes changes in population size into account, and gives an estimate of the growth rate of the different trait groups. By asking how fitness differed among the trait groups, Rubach et al. (in press) assumed that females that reproduce at different ages have a trait that can be passed on to future generations: this is the assumption of genetic variation and heritability. While this idea might be challenged, the whole point of looking at a fitness measure is the search for an evolutionary advantage. If a trait undergoes selection, but exhibits no response to selection (viz., due to limited heritability, antagonistic pleiotropy, or genetic correlations with other traits) the results are perhaps less interesting. Additionally, when the number of offspring that survived until their first possible reproductive season was used to estimate reproductive success, the advantage for earlier reproduction by mothers was not quite significant, though it still had a small to medium effect size. Production of the next generation is meaningful for natural selection in terms of offspring that themselves survive to reproduce in the next generation (e.g., Boyce and Perrins, 1987). Naturally, the different trait groups might vary due to environmental factors or random variations in resource acquisition over time, so it is important to remember that offspring may not express the trait forms of their parents, particularly for phenotypically plastic traits, where expression might be influenced by the environment.

Kin Selection and Inclusive Fitness

Individual fitness measures are most appropriate for traits that are expressed once during an individual's lifetime, as is the case with many developmental traits. Age at maturity is one such, but many traits of temperate species are expressed on an annual basis (e.g., litter size, phenology of reproduction, seasonal cycles in activity or body mass, etc.). Nonetheless, some evolutionary characteristics may vary during an individual's lifetime, but the primary interest is in the cumulative effects of the social or ecological environment on fitness. Inclusive fitness is an example

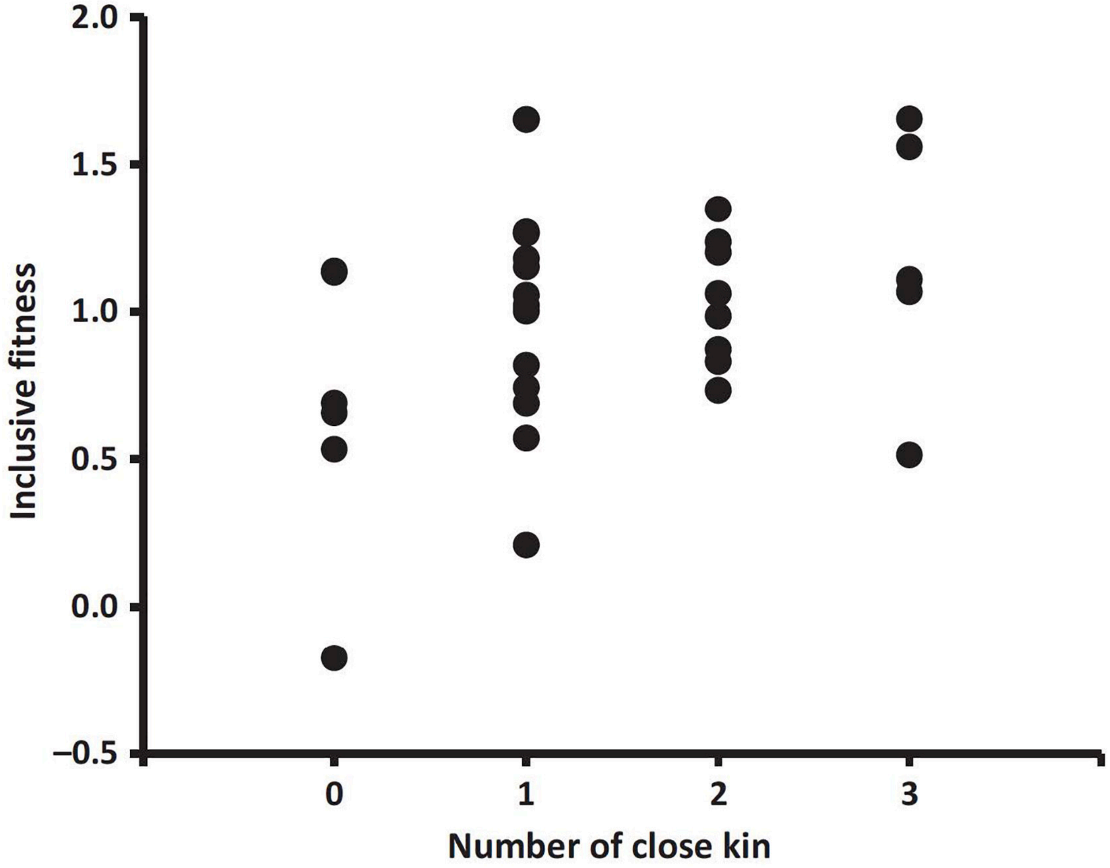

FIGURE 6 | Estimated inclusive fitness and number of co-surviving close kin (mother-daughter and littermate sister dyads) that were both of reproductive age (2 years old and older) and actively reproducing at the same time ($r = 0.425$, $n = 35$, $P = 0.005$; data from 1992 to 2008) [used with permission, from Dobson et al. (2012)].

of an advantageous phenomenon (the presence of cooperative and reproductive close kin that augment maternal fitness) that can accumulate over a lifespan. Here, the trait is usually some sort of behavioral cooperation with close genetic relatives, so that kin selection is a possible influence on the behaviors (Hamilton, 1964). In this case, inclusive fitness (an individual's total fitness) has two components: fitness accrued by an individual in the absence of help from genetic relatives (the direct component) and fitness accrued from the help that the individual gives to genetic relatives (the indirect component).

Our examination of possible kinship advantages in fitness terms began with an examination of whether there was a difference in the direct fitness component between female Columbian ground squirrels that reproduced in the presence of close kin and those that had no co-reproductive close kin with which to cooperate (Viblanc et al., 2010). The form that cooperation took was lowered aggression (viz. greater tolerance) during co-reproduction of adult females and their mother, littermate sisters, and daughters (King, 1989; Viblanc et al., 2016). The numbers of co-breeding close kin females per year (during the reproductive lifespan) were the trait forms. We found a significant association of the number of co-breeding close female kin and number of offspring at weaning, though at a medium effect size (estimated from path analysis of $\lambda_{\text{rel}\Delta\text{N}}$; $\rho = 0.29$, $P = 0.01$, $n = 70$). In turn, litter size was highly significantly associated with relative individual fitness ($\rho = 0.79$, $P < 0.0001$, $n = 70$), resulting in a small-to-medium indirect effect of number of close kin on fitness (indirect path coefficient, $\rho = 0.23$, significant when both direct coefficients are significant, Cohen, 1988). Viblanc et al.'s (2010) use of relative individual fitness ($\lambda_{\text{rel}\Delta\text{N}}$) was necessary because individual fitness (λ_{ind}) was significantly associated with changes in population density (estimated from $\lambda_{\Delta\text{N}}$; $R^2 = 0.33$, $F = 33.387$, $n = 70$, $P = 0.001$, $n = 70$).

To extend our study of kin effects, we estimated inclusive fitness, to include the indirect component in addition to the direct component previously calculated (Dobson et al., 2012). Our goal was to evaluate the possible importance of an indirect component to inclusive fitness in Columbian ground squirrels. Mothers were classified as having co-breeding close kin versus not having close kin during their reproductive lifespans, so the alternative trait groups were the presence or absence of potentially helpful close kin. The number of weaned offspring was used to estimate female reproductive success, and the analyses used an adjustment for changes in the population during a female's lifetime (in this case, λ_{ind} was regressed on $\lambda_{\Delta\text{N}}$ and 1.0 was added to the

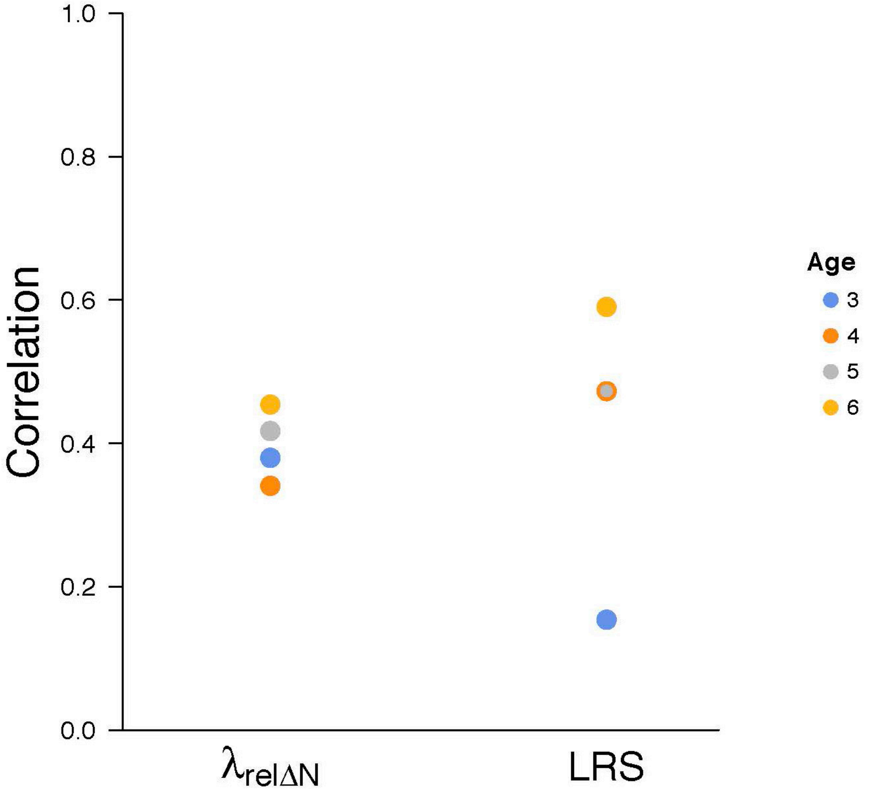

FIGURE 7 | Comparison of annual fitness values for each reproducing female, and her lifetime adjusted individual fitness (measured as $\lambda_{\text{rel}\Delta}$) and lifetime reproductive success (both lifetime estimates calculated from number of offspring at weaning). For annual fitness, all mothers 3-years old (*n* = 101), 4-years old (*n* = 80), 5-years old (*n* = 59), and 6-years old (*n* = 32) were included.

residuals, to produce $\lambda_{\text{rel}\Delta\text{N}}$ for each female). Relative indirect fitness accounted for over 40% of a mother's inclusive fitness, a substantial and significant amount (0.43 ± 0.08 SE, $t = 5.71$, d.f. = 28, $P < 0.0001$). In addition, as the number of close co-breeding close relatives increased, a mother's inclusive fitness increased significantly (**Figure 6**). The analyses were greatly facilitated by use of the relative individual fitness approach.

Empirical Comparison of Annual Fitness, Lifetime Reproductive Success, and Individual Fitness

The use of annual and lifetime fitness estimates made us question how closely these estimates correspond. To compare annual and lifetime measures, we used samples of females when they were of different prime breeding ages, namely 3, 4, 5, and 6 years old (respectively; 101, 80, 59, 32 mothers). Of course, these values for individual females were not independent and were from different years over the 28-year study period. The number of offspring at weaning was used to estimate reproductive success for these subsequent analyses. We compared the annual fitness values for the females in each age group separately to the relative lifetime individual fitness estimates ($\lambda_{\text{rel}\Delta\text{N}}$) and to the lifetime reproductive success of these same females with correlations (**Figure 7**; e.g., a single datum would be an annual value for a 3-year-old female and her lifetime fitness, the latter estimated by relative individual fitness $\lambda_{\text{rel}\Delta\text{N}}$ or estimated by lifetime reproductive success). The values of the correlations were used as indications of effect size (Cohen, 1988) for the similarities of annual and lifetime values, with small ($r = 0.10$), medium ($r = 0.30$), and large effects ($r = 0.50$). Similarity of estimates of the association of annual and individual fitness (unadjusted) were consistently between medium and large, averaging around 40% ($r = 0.380$). Similarity of annual fitness and lifetime reproductive success also averaged around 40% ($r = 0.423$), but were much more variable. The annual estimates of fitness were meant to reveal the influence of an experiment or annual comparisons. Since year-to-year variations in the environment occurred and these variations might well average out during an extended lifetime (9–12 years for 6 of the females in our sample of 101 mothers), a modest effect size might have been expected.

When relative individual fitness ($\lambda_{\text{rel}\Delta\text{N}}$) was compared to lifetime reproductive success, the correlation was significant but fairly moderate (**Figure 8**; $r = 0.345$, $n = 132$ mothers, $t = 4.186$, $P < 0.0001$). When the cohort Leslie matrix was used to estimate relative individual fitness (λ_{relL}), however, the correlation with lifetime reproductive success was very low and insignificant ($r = 0.016$, $t = 0.184$, $P = 0.85$). The two estimates of relative individual fitness ($\lambda_{\text{rel}\Delta\text{N}}$ and λ_{relL}) were strongly associated ($r = 0.817$, $t = 16.15$, $P < 0.0001$).

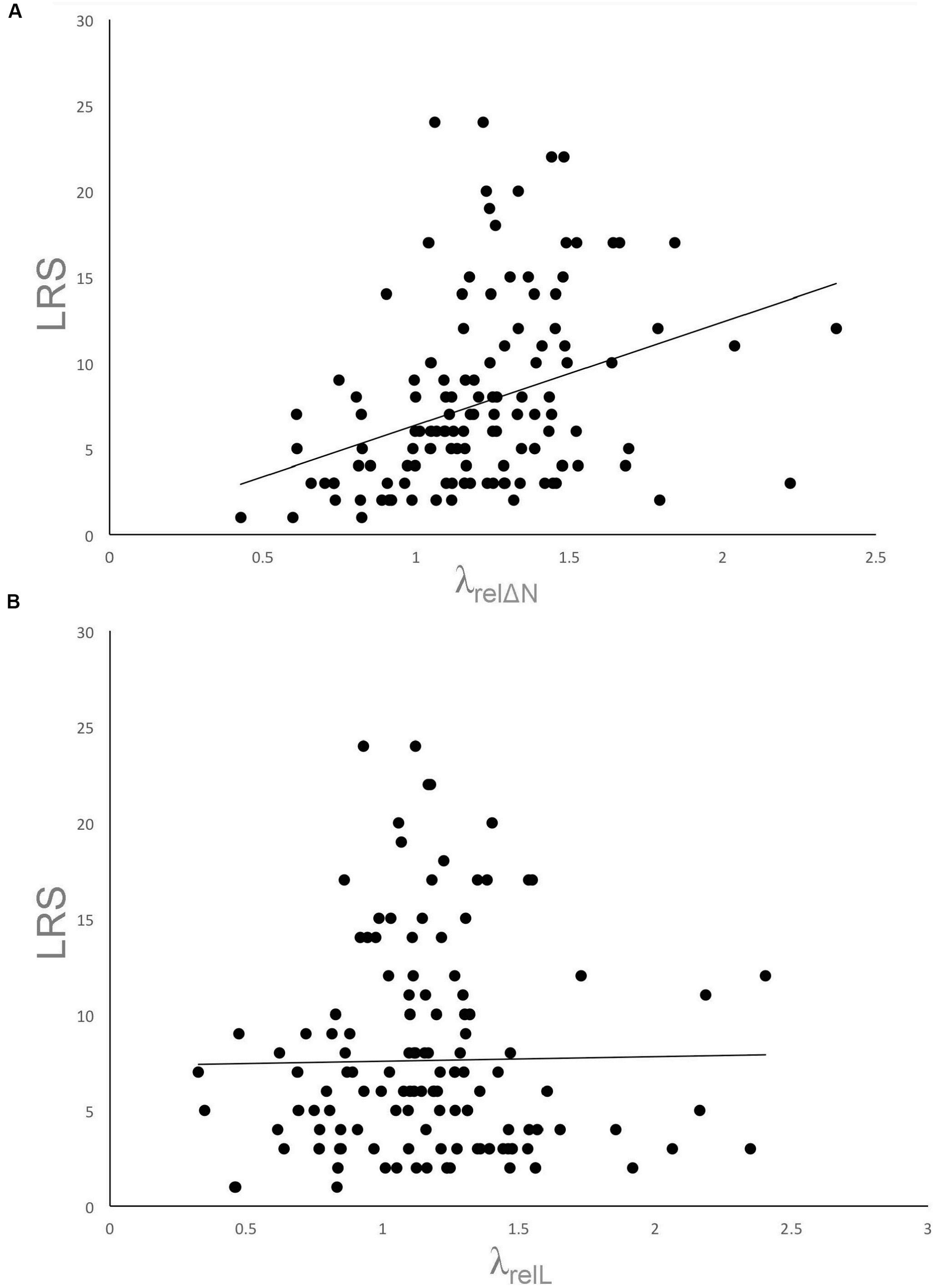

FIGURE 8 | Associations of lifetime reproductive success (LRS) and adjusted individual fitness **(A)** based on $\lambda_{rel\Delta N}$ **(B)** based on λ_{relL}. The former **(A)** exhibits significant association, but the latter **(B)** does not (see text). Trendlines are presented for visual inspection.

The lack of strong association of the relative indices of individual fitness and lifetime reproductive success indicate that the widespread use of lifetime reproductive success as a fitness measure might have to be reconsidered. On the other hand, both individual fitness and lifetime reproductive success were equally moderate predictors of the number of maternal gene copies passed on to future generations in collared flycatchers and Ural owls (respectively, *Ficedula albicollis*, *Strix uralensis*; Brommer et al., 2004), and in a study that included both male and female gene copies in song sparrows

TABLE 1 | Summary of methods for estimating fitness for empirical studies.

Method	Author	Formula	Situational use
Reproductive or survival or growth, or other phenotypes		Various and largely inferential, due to incomplete information	When there is no better method available for estimating fitness
Annual (gene copies)	Qvarnström et al., 2006	female survival (1/0) $+\frac{1}{2}$*(#offspring surviving)	Ecological experiments, annual events
Lifetime reproductive success	Grafen, 1988	# offspring in a lifetime	When survival/longevity is not known and temporal patterns are unimportant
Individual fitness	McGraw and Caswell, 1996	Individual population-growth matrix	Population number does not fluctuate, temporal patterns are important
Individual fitness adjusted for population size	Viblanc et al., 2010	Residual of individual fitness regressed on ΔN matrix +1.0	Population number fluctuates, temporal patterns are important
Individual fitness adjusted for cohort growth	Rubach et al., in press	Residual of individual fitness regressed on Leslie matrix for cohort +1.0	Population number fluctuates, temporal patterns are important

(*Melospiza melodia*; Reid et al., 2019). These studies were conducted, however, without obvious evidence of selection favoring particular traits.

CONCLUSION

In conclusion, when conducting experiments or looking at annual events, Qvarnström et al.'s (2006) annual fitness method seems to be a good tool that takes both reproduction and survival into account. Annual events were illustrated by the experimental ectoparasite (flea) removal treatment study (Raveh et al., 2015) and response to annual climatic conditions (Lane et al., 2012; Dobson et al., 2016). However, this method may be less appropriate for traits that occur as part of the ontogenetic sequence of events during the lifespan, or for judging longer-term success for conditions that have a cumulative influence on fitness. These latter cases were illustrated by the study of age at maturity (Rubach et al., in press), and the studies of kin selection (Viblanc et al., 2010) and inclusive fitness (Dobson et al., 2012). We summarize when we think these different estimates might be most appropriate in **Table 1**. Between the two ways that individual fitness (λ_{ind}) might be adjusted for changes in population dynamics ($\lambda_{\text{rel}\Delta\text{N}}$ and λ_{relL}), we prefer $\lambda_{\text{rel}\Delta\text{N}}$. This measure is based on changes in population size over time, and includes all competitors (in the present case, all females) in the population. By contrast, λ_{relL} compares a reproductive female only to others in her cohort, a subset of the population. Yet over their lifetime, individual in numerous species are exposed not only to individuals from the same cohort, but to overlapping generations of multiple cohorts. Thus, a fair comparison should contrast a particular individual against all individuals of the population over her lifetime. In any case, the choice of empirical fitness measures should be carefully considered, the one most appropriate to the research question, and amenable for the species under study (e.g., long vs. short-lived).

Other methods for estimating fitness are not directly applicable to the problem of comparing trait forms in empirical research, but may hold promise for future improvements. Perhaps the most attractive is the use of offspring from a pedigree, perhaps even including both male and female relatives (Brommer et al., 2004; Reid et al., 2019). An alternative to our use of changes in population size to adjust for environmental variation might involve fitness measures that take demographic and environmental stochasticity into account (e.g., Benton and Grant, 2000; Engen et al., 2009; Sæther and Engen, 2015). These measures have not yet been applied to alternative trait forms. Finally, methods that examine the comparative sensitivity of population growth to reproduction and survival (e.g., Dobson and Oli, 2001; Oli and Dobson, 2003; Coulson et al., 2006) might be used to answer similar questions about individual fitness.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, Auburn University.

AUTHOR CONTRIBUTIONS

FD and VV designed the study. FD wrote the manuscript. FD, VV, and JM collected the data and revised drafts of the manuscript. All authors contributed to the article and approved the submitted version.

IMPACT STATEMENT

Evolutionary biology studies how natural selection operates on traits and combinations of traits by comparing differences in fitness for individuals that exhibit different trait forms. Thus, how fitness is measured is a key issue for every evolutionary study. We used examples from our past research on Columbian ground squirrels to compare and contrast methods of measuring fitness. These include short-term, usually annual measurements, and alternative measures of lifetime reproduction. In particular, lifetime reproductive success and "individual fitness" measures (based on matrix methods) have provided conflicting results in past studies. In direct comparisons using our long-term, 28-year data set, alternative methods exhibited moderate but disappointing associations with one another. Methods to estimate fitness must be carefully chosen and considered with caution.

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Comparative Assessment of Ecological Plasticity of the Steppe Marmot Between Ukrainian and Kazakhstan Populations: Challenges of the Man-Induced Environmental Changes

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The steppe marmot inhabits a wide range of open dry grasslands in Eurasia. Throughout this vast area, marmot habitats have undergone major changes due to human activities. Long-term ecological monitoring was conducted in the European steppe marmot settlements (*Marmota bobak bobak*) in Northeastern Ukraine in 2001–2019. The data obtained were compared with the observations made in *M. b. schaganensis* settlements in Kazakhstan during the expedition in 2017. The goals of our investigation were (1) to estimate *M. bobak* ecological plasticity based on general vegetation parameters of its habitats and settlement structure, (2) to relate the population density of the European subspecies to the food base of its habitats, (3) to evaluate the population response of *M. b. bobak* to the abandonment of cattle grazing, (4) to ascertain new ecological adaptations (if any) to the habitat changes, and (5) to reveal the steppe marmot's status in the plant–herbivore interaction system in the grasslands of Northeastern Ukraine and Northern Kazakhstan. We have found differences in ecological features of *M. b. bobak* and *M. b. schaganensis*. The European subspecies was and continues to be a secondary pasture user. The Kazakhstan subspecies can be both secondary and primary users of the Asian dry steppes. Our studies have shown that the habitats of the European steppe marmot worsened dramatically (due to increased herbage height and cover of uneaten plant species together with litter) in comparison with those of the Kazakhstan subspecies. Presence in diverse habitats with a range of vegetation parameters as well as the differences between the settlement structures of *M. b. bobak* and *M. b. schaganensis* demonstrate the high ecological plasticity of the steppe marmot at the species level. At the same time, we have not found any new ecological adaptations that would ensure the survival of *M. b. bobak* settlements in modern conditions of the total cessation of cattle grazing.

Keywords: ***Marmota bobak*, food base, ecological plasticity, ecological adaptations, cattle grazing, abandonment**

INTRODUCTION

In the rodent family Sciuridae, marmots (*Marmota*), ground squirrels (*Spermophilus*), and prairie dogs (*Cynomys*) have a pronounced adaptation to life in burrows and to feeding on live green herbs. Species of the genus *Marmota* are highly variable in social behavior, covering the whole range from the solitary woodchuck (*M. monax*) to the highly socialized Olympic (*M. olympus*) and hoary (*M. caligata*) marmots (Bibikov, 1989; Armitage, 1998, 2014; Arnold, 1990). Marmots are hibernating animals and selective foragers (Bibikov, 1989; Frase and Armitage, 1989; Bassano et al., 1996; Massemin et al., 1996; Stallman and Holmes, 2002; Armitage, 2003; Garin et al., 2008). All species prefer forbs over grasses (Armitage, 2014), many of them choose plants with succulent leaves (Sludsky et al., 1969).

Marmots have a wide altitudinal range of their distribution, covering zonal steppes, mountain and alpine tundra, and mountain deserts. A unique behavioral adaptation of the genus *Marmota* is plugging the entrance to their winter burrow with a compact multimeter plug (soil mixed with feces) that virtually excludes heat exchange between the internal burrow environment and the external environment (Nikol'skii, 2009). The author defines "temperature niche" as "the entire diversity of temperature conditions of marmots, taking into account the depth of the burrows, season of the year and the geographical situation of local populations." According to Bibikov (1989), marmots are primary mountain animals. On the contrary, Nikol'skii and Rumiantsev (2012) suppose that the ancestral forms of marmots occupied a huge range thanks to the flat terrain of their primary habitats. The *bobak* group, which consists of *M. bobak*, *M. baibacina*, and *M. kastschenkoi*, is considered the youngest progressing branch of the Palearctic marmots' evolutionary tree (Brandler and Lyapunova, 2009; Brandler et al., 2010). Based on studies of mitochondrial DNA, Brandler (2009) proposes that *M. bobak* evolved as a plain-steppe form in the east part of the common historical range of *M. bobak* and *M. baibacina*.

The steppe marmot inhabits a wide range of open dry grasslands in Eurasia. Throughout the vast area, marmot habitats have undergone major changes due to human activities (Kirikov, 1959). Duration of the anthropogenic effect on marmot populations varied considerably depending on the population location. In the first half of the 20th century, the European subspecies of the steppe marmot (*M. b. bobak*) was under threat of extinction (Bibikov et al., 1990). The major threatening factors were the plowing of most native habitats and overhunting (Zimina and Isakov, 1980; Bibikov, 1989). At the same time, the number of the Kazakhstan subspecies (*M. b. schaganensis*) was estimated in millions of individuals (Sludsky et al., 1969). It inhabited the boundless virgin steppes of Kazakhstan, where the main anthropogenic effects were nomadic pastoralism and hunting (Sludsky et al., 1969; Zimina and Isakov, 1980).

The number of *M. b. bobak* in 1940–1950 did not exceed 5,000 individuals (Bibikov et al., 1990). Then, in the early 60s, there was a rapid "rebound" of the European subspecies. The marmots colonized the vast area of gullies and river valleys transformed into intensively used cattle pastures. The main reason for that is thought to be changes in the vegetation caused by cattle grazing (Seredneva and Nesgovorov, 1977; Seredneva, 1985). In the same period of time, the Kazakhstan subspecies experienced a large-scale anthropogenic impact of "the virgin lands campaign." In 1954–1960, 25 million hectares of virgin steppes were plowed in Kazakhstan (Brezhnev, 1978; Zimina and Isakov, 1980; Rachkovskaya and Bragina, 2012). Arable fields became the main marmot habitats, and only a part of the Kazakhstan population remained on the steppe pastures (Zimina and Isakov, 1980; Rumyantsev, 1991).

In the mid-1990s, the European subspecies accounted for about 350,000 individuals while the number of the Kazakhstan subspecies exceeded two million (Mashkin, 1997). There was a general impression that the steppe marmot had wide ecological plasticity and was capable of acquiring ecological adaptations in case of sharp changes in its natural environment (Zimina and Isakov, 1980; Bibikov et al., 1990; Rumyantsev, 1991; Mashkin, 1997). However, Rumyantsev (1991) notes that the marmots' settlements were located mainly along the margins of arable fields at the borders with the patches of virgin steppe. In the 21st century, the habitats of both subspecies have undergone new drastic changes. In the late 1990s, huge areas of arable fields in Kazakhstan were abandoned, and the grasslands began recovering due to spontaneous succession (Brinkert et al., 2016). Some fields were subsequently reclaimed (Kamp et al., 2011; Dara et al., 2018, 2019), and the use of herbicides in arable fields increased (Kamp et al., 2011). In Ukraine, the pastures were abandoned (Ronkin et al., 2009; Ronkin and Savchenko, 2016). In Kazakhstan, areas of pasture were also decreased dramatically (Mashkin et al., 2010), but the process was highly uneven (Kamp et al., 2012). The former nomadic grazing was replaced by sedentary grazing. As a result, now the surroundings of the villages are overgrazed while the areas located further from settlements are not grazed at all (Kamp et al., 2012; Deák et al., 2018).

Long-term investigations of species-specific responses to anthropogenic and/or natural impacts contribute to the understanding of the possible trajectories and outcomes of the ecosystem evolution as a whole. Steppe ecosystems have evolved as the ecosystems of the grazing type of functioning (Abaturov, 2006), where interactions between food plants and herbivorous mammals (plant–herbivore interaction) determine animal number, distribution, and population structure (Abaturov, 2005). The loss of large wild grazers (in our studies livestock) is supposed to change ecosystem functioning. We hypothesized that large-scale and rapid environmental changes may not only influence marmot population, but also encourage development of new adaptations, which will compensate for changes in their habitats. We proceeded from the fact that the development of ecological adaptations in response to previous habitat changes has been already observed in both subspecies (Bibikov et al., 1990; Rumyantsev, 1991; Mashkin, 1997). The goals of our investigation were as follows:

1. To estimate the *M. bobak* ecological plasticity based on general vegetation parameters of its habitats and settlement structure.

(2) To relate the population density of the European subspecies to the food base of its habitats.
(3) To evaluate the population response of *M. b. bobak* to the abandonment of cattle grazing.
(4) To ascertain new ecological adaptations (if any) to the habitat changes.
(5) To reveal the steppe marmot's status in the plant–herbivore interaction system in the grasslands of Northeastern Ukraine and Northern Kazakhstan.

MATERIALS AND METHODS

Study Areas

The research was carried out in Northeastern Ukraine (Kharkiv region) and Northern Kazakhstan (Kostanay and Karaganda regions) (**Figure 1**). The climate of Northeastern Ukraine is characterized as temperate continental with the hottest period in the second half of summer (end of June–August) and the coldest period in the second half of winter (January–February). The maximal recorded temperature during 2001–2019 was 39.4°C (July 2010, the authors' data) and the minimal 38.3°C (January 2006, the authors' data). An average annual rainfall ranges from 480 to 620 mm with a peak in June. The study area is desiccated with gullies and river valleys; the main soil type is chernozem. All flattened steppe plots had been transformed into arable fields by the end of the 19th century. The steppe marmot occurs in steppe gullies and floodplain meadows. Communities dominated by *Festuca valesiaca* agg., *Poa angustifolia*, and *Elytrigia repens* represent vegetation in the gullies while communities dominated by *Elytrigia repens*, *Poa angustifolia*, *Festuca rupicola*, and *Festuca pratensis* occupy the floodplain meadows (Ronkin and Savchenko, 2016). Chalk outcrops in the valleys of the Oskil, the Nyzhnia, and the Verkhnia Dvorichna rivers host specific plant communities of the class *Helianthemo-Thymetea* (Romashchenko et al., 1996). They alternate with the *Festucion valesiacae* communities forming the so-called chalk grasslands (Ronkin and Savchenko, 2016). In this chalky landscape, the marmot inhabits chalky steppe on the footslopes and neighboring floodplain meadows.

The climate of Northern Kazakhstan is sharply continental. The summer is usually dry and hot, but in some years, it can be cool and wet. The temperature averages 17–18°C in June and 20–21°C in July with a maximum of 40–44°C. The soil surface on certain hot days heats up to 50–60°C. In summer, there is a sharp difference in the day and night temperatures. The precipitation rate in various areas ranges from 250 to 400 mm; it decreases from north to south. Stable snow cover holds out for 5.0–5.5 months in the north and 4.0–4.5 months in the south. Approximately one third of the annual precipitation (70–100 mm) falls in winter. About 50% of the years are dry with no rains in June and/or in July. A combination of high daytime temperatures (30–35°C) and low relative humidity (30–40%) causes intense evaporation, which results in droughts. The chernozem soils are replaced in the south by the kastanozem ones.

Most of Northern Kazakhstan is located on flat sweeping landmass. Before the Soviet virgin lands campaign of the 1950s, it was covered with genuine steppes dominated by *Festuca valesiaca*, *Stipa zalesskii*, *Stipa lessingiana*, and *Peucedanum morisonii* (Rachkovskaya and Bragina, 2012; Demina and Bragina, 2014). The flat terrain determined total plowing and forming of large continuous fields in Northern Kazakhstan (2 × 2 km, the "standard" size of the Soviet virgin-land-campaign fields).

The material presented in this paper was collected in 2001–2019. A total of 20 settlements of *M. b. bobak* were investigated in Northeastern Ukraine (Kharkiv Region). At present, there are three types of habitats within the geographic range of the European subspecies: grazed grasslands in steppe gullies and river valleys, the same grasslands shortly after cessation of the cattle grazing, and long-abandoned pastures (Mashkin, 1997; Ronkin et al., 2009; Mashkin et al., 2010). In our study, we marked these types as "A," "B," and "C," respectively. Long-term ecological monitoring was conducted in three marmot settlements in the regional landscape park Velykoburlutskyi Steppe: (1) Nesterivka, (2) Zelenyi Hai, (3) Rohozianka, and two settlements in the national nature park Dvorichanskyi: (4) Kamianka and (5) Novomlynsk. In the Velykoburlutskyi Steppe, the marmots inhabit the gully steppe on chernozem soil, and in the Dvorichanskyi, they occur in the chalky steppe and neighboring floodplain meadows. In **Table 1**, each habitat type and settlement is provided with a code, years of monitoring, years of vegetation sampling, and the number of sample quadrates. The grazing cessation (and, accordingly, transformation of one habitat type into another) occurred in the study settlements in different years.

The data on vegetation and settlement structure of *M. b. bobak* were compared with those obtained in the expedition to Northern Kazakhstan in June 2017 aimed at studying settlements of *M. b. schaganensis*. The expedition route passed from Nur-Sultan through Akmola and Kostanay regions to the Karaganda region; then from the northwest of the Karaganda region through the Kostanay to the north of the Akmola region (**Figure 1**). The habitat spectrum of the Kazakhstan subspecies is much wider than that of the European one; it includes arable fields, roadsides, grazed and/or ungrazed steppes, and grazed and/or ungrazed former croplands (Koshkina et al., 2019). Moreover, these grasslands are divided into northern and southern variants depending on their location in the geographic range. In total, the data on nine settlements of the Kazakhstan subspecies are used in this work. We named the habitat types as follows: north locality: roadsides, north locality: arable fields, north locality: grazed steppe, north locality: grazed former croplands, north locality: ungrazed former croplands, south locality: grazed steppe, south locality: ungrazed steppe, south locality: grazed former croplands, and south locality: ungrazed former croplands.

Vegetation Data Collection

Vegetation data in *M. b. bobak* settlements were obtained from their foraging areas. No entire area of the marmot settlements is used for foraging activity. Despite the absence of visible reasons for ignoring (water bodies, wetlands, roads, and arable fields), there are no marmot's paths or burrows in such areas. We detected these areas only when the whole

FIGURE 1 | Location of the study areas (red circles) in Northeastern Ukraine (Kharkiv Region) and Northern Kazakhstan (Kostanay and Karaganda regions).

TABLE 1 | *M. b. bobak* habitats in Northeastern Ukraine and vegetation data collection.

Habitat type/Settlement	Code	Years of monitoring	Number of quadrates	Years of quadrate sampling
Grazed grasslands of Nesterivka	A1	2001–2014	23	2012–2013
Grazed grasslands of Zelenyi Hai	A2	2001–2017	7	2015
Grazed grasslands of Rohozianka	A3	2014–2017	9	2015
Grazed grasslands of Kamianka	A4	2013–2016	10	2015
Grasslands of Nesterivka shortly after abandonment	B1	2015–2017	6	2017
Grasslands of Kamianka shortly after abandonment	B4	2017–2018	5	2018
Grasslands of Novomlynsk shortly after abandonment	B5	2010–2012	7	2011
Long-abandoned grasslands of Nesterivka	C1	2018–2019	11	2019
Long-abandoned grasslands of Novomlynsk	C5	2013–2019	7	2019

vegetation within the settlement was short, which did not impede visual observation of foraging animals. As a rule of thumb, such vegetation state is accompanied by cattle grazing. Descriptions of typical plant communities of these unvisited areas were made to compare them with the foraging areas. Further in the text, these communities are referred to as ignored vegetation (or ignored areas).

General vegetation parameters were estimated in the quadrate 3.16 × 3.16 m (10 sq m). We estimated (1) total cover, (2) living cover of vascular plants, (3) cover of litter, (4) cover of forbs, (5) cover of grasses, and (6) herbage height. Then, we noted the cover of each vascular species if it was higher than 0.01%. To calculate parameters of the marmot food base in a given settlement (cover of food species and total cover of uneaten species together with litter) the covers of food/uneaten species were summarized. All these estimates were made in summer when all the grass layers had reached maximum growth. Herbage height was taken as the height of the main (most dense) herb layer; 11 measurements were made and then averaged.

In 2011–2019, 85 quadrates were sampled in five studied settlements of the European subspecies (**Table 1**). Of these, 70 quadrates were placed in the marmot foraging areas, 12 quadrates in the ignored vegetation of grazed grasslands of Nesterivka, and three quadrates in the ignored vegetation of grazed grasslands of Kamianka. After transition of one type of habitat into another (e.g., grazed grassland into the abandoned ones), new quadrates were sampled approximately at the same places. The proportion of the sampled quadrates (chernozem soil/chalky outcrops) roughly corresponds to the current ratio of the existing marmot settlements in these grasslands. In *M. b. schaganensis* settlements, we estimated the general vegetation parameters. In total, 62 estimates were made in nine settlements.

We performed testing on forb and grass species to compare with the marmot diet found earlier in 1989 (Ronkin and Savchenko, 2000). After grazing abandonment, the vegetation of marmot habitats changed dramatically. In 2010–2017, we continued to replenish and refine the list of food species in connection with the food base changes caused by the post-grazing succession. We recorded all plant species bitten by marmots in their natural settlements and agrocenoses. In addition, we monitored foraging preferences of free-living tame marmots to determine suitability of certain plant species as the main forage components. Two marmot families lived near our research center and were not afraid of people. During the growing season, freshly mowed plants were given to the animals twice a day in excess. We used plants defined as food species in our previous research. The fodder was heaped near a winter burrow. The plant species were considered suitable as the main forage component if the animals of all ages willingly ate a large amount of it every day. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Law of Ukraine "On the Animal World" (Article No. 9 "Basic Requirements and Principles for the Protection, Management and Reproduction of the Animal World"), the Committee on Bioethics of Kharkiv National University named after V. N. Karazin. The protocol was approved by the Committee on Bioethics of Kharkiv National University.

Data on the Settlement Structure

The winter burrow is considered the main attribute of the marmot home range and marmot family (Seredneva, 1986; Lenti Boero, 1996, 2001). In addition, marmots have summer and auxiliary burrows (Bibikov, 1989; Mashkin, 1997; Armitage, 2014). For the mapping of winter burrows, a handheld global positioning system device (Garmin Oregon, Magellan eXplorist) and the program QGIS v.2.18.8 were used. We mapped winter burrows of 132 families of *M. b. bobak* in five studied settlements and measured the distances ($n = 167$) between the nearest winter burrows of neighboring families. This distance is a key feature of both population density and environmental conditions. Smaller average distance indicates better food conditions in the marmot settlement (Seredneva, 1986).

To estimate *M. b. bobak* population response to the cattle grazing cessation and to ascertain new ecological adaptations (if any) to the habitat changes, we made censuses of the number of families in a settlement before and after the pasture abandonment. We also noted the total number of all types of burrows (summer and auxiliary) used by a family of marmots. If the number of families in the settlement changed, we took repeated measurements of the distances between inhabited winter burrows. To count the burrows used by marmots, we chose a typical plot of 25.5 ha in the central part of the Nesterivka settlement and divided it into 20 × 20 m quadrates. Within these quadrates, positions of all existing burrows were outlined. This work was completed in 2000–2001. In 2001–2002, we made a census of all burrows used by each marmot family in the study plot and adjacent areas (in total, 27 home ranges). In 2002–2019, we conducted annual monitoring of the site occupancy. In case of a decrease in the number of existing families, we repeated censuses of the number of burrows used by remaining families in the same home ranges.

During the study of *M. b. schaganensis*, we mapped inhabited winter burrows in its typical sustainable settlements, counted the total number of burrows used by a family of marmots, and measured the distances ($n = 91$) between the nearest winter burrows of neighboring families in all the studied settlements excluding roadsides (see above). We considered a given settlement sustainable if the families had offspring and the number of inhabited winter burrows was much higher than the number of uninhabited ones. Then, we compared the obtained parameters in the settlements of both European and Kazakhstan subspecies. The methods applied to solve certain research goals are shown in **Supplementary Figure S1**.

We consider population density in the marmot settlements as a number of present families per 1 ha of a given settlement. Based on previous research (Ronkin et al., 2009), we identified three population grades in marmot settlements: normal (predicted value 0.5–2 families/ha), decreased (0.3–0.5 families/ha), and collapsed (less than 0.3 families/ha). We determined the number of burrows used by a family by the fact of visiting and by indirect signs, such as freshly excavated soil and marmot feces near the burrow entrance. Before hibernation, the marmots plug the entrances of their winter burrows with small globules made of soil and their feces. These globules as well as old grass and hay (bedding) are additional proof of burrow use.

Statistical Analysis

Comparison of multiple independent samples was conducted using Kruskal–Wallis ANOVA by ranks. In most cases, the distribution in the data sets was different from normal ($P < 0.05$, Shapiro–Wilk and Kolmogorov–Smirnov normality tests). When comparing foraging areas within the settlements of both *M. b. bobak* and *M. b. schaganensis*, general vegetation parameters (living cover of vascular plants, cover of litter, cover of forbs, cover of grasses, and herbage height) were taken as dependent variables and the habitat type as a grouping variable. When comparing the food base in *M. b. bobak* settlements, the food base parameters (living cover of vascular plants, total cover of food species, total cover of uneaten species together with litter, cover of litter separately, and herbage height) were dependent variables, and the population grade (normal, decrease, and collapse) was a grouping variable. Comparison of *M. b. bobak* and *M. b. schaganensis* settlement structures was based on the distance between the nearest inhabited winter burrows as a dependent variable and the habitat type as a grouping variable. We used all the data obtained in the habitats of both *M. b. bobak* and *M. b. schaganensis* (see above) except the roadsides. Changes in the settlement structure of *M. b. bobak* were analyzed taking the distance between the nearest winter burrow as a dependent variable and the population grade as a grouping variable.

The *U*-test (Mann–Whitney) was used to determine the significance of differences in general vegetation parameters (living cover of vascular plants, cover of litter, cover of forbs, cover of grasses, and herbage height) in foraging areas. We used all the data obtained in the habitats of both *M. b. bobak* and *M. b. schaganensis* (see above) except the arable fields. Subspecies (*M. b. bobak*/*M. b. schaganensis*) was taken as a grouping variable. The *U*-test was also used to determine the significance of difference in distance between the nearest neighboring winter burrows in the settlements of *M. bobak*. Subspecies (*M. b. bobak*/*M. b. schaganensis*) was taken as a grouping variable.

The cluster analysis in the module of multivariate exploratory techniques was used for the habitat classification of *M. bobak*. Averaged values of the general vegetation parameters in each settlement were variables. We used all data obtained in the settlements of *M. b. bobak* (see above) except the ignored vegetation of chalky outcrops in Kamianka and the data on all studied settlements of *M. b. schaganensis*.

Statistical significance was set at $P < 0.05$. All the calculations were performed in the program StatSoft Statistica v8.0.

RESULTS

General Vegetation Parameters of the Habitats of *M. bobak* and the Structure of Its Settlement

The foraging areas of *M. b. bobak* were characterized by a wide range of general vegetation parameters (living cover of vascular plants, cover of litter, forbs, grasses, and herbage height) due to their location in grazed and/or abandoned grasslands. The herbage height ranged from 4 cm (grazed grasslands in total) to 69 cm (grasslands of Nesterivka shortly after abandonment, communities of the loose-bunch grasses *Poa angustifolia* and *Elytrigia repens*) (**Figure 2** and **Supplementary Figures S2A,B**). The cover of grasses had the largest range from 2% (grazed grasslands of Kamianka) to 77% (grazed grasslands of Rohozianka). The cover of litter changed in similar ranges from 1% (grazed grasslands in total) to 80% (long-abandoned grasslands of Novomlynsk, community of the loose-bunch grass *Elytrigia repens*). The lowest living cover of vascular plants was registered in the chalky steppe (40%, grazed grasslands of Kamianka) and that of the forbs in the communities of loose-bunch grasses (4%, grasslands of Nesterivka shortly after abandonment) (**Figures 2**, **3**).

The range of vegetation parameters in foraging areas of *M. b. schaganensis* was similar to that of *M. b. bobak* except the herbage height (**Figures 2**, **3**). The latter was 40 cm lower (4–27 cm), and its range was narrower than in the foraging areas of *M. b. bobak*. The cover of litter (0.1–75%) had the largest cover range. The lowest living cover of vascular plants was 25% (south locality: ungrazed steppe, communities dominated by *Festuca valesiaca* and *Artemisia* sp.), and the lowest cover of forbs was 2% (south locality: ungrazed former croplands, communities dominated by *Koeleria cristata* and *Stipa lessingiana*) (**Supplementary Figures S3A,B**). The living cover, the cover of litter, grasses, and herbage height in the foraging areas of the Kazakhstan subspecies, were lower than those in the foraging areas of the European subspecies (*U* test: $U = 1{,}059.5$, $n_1 = 58$, $n_2 = 70$, $P < 0.0001$; $U = 1{,}499.0$, $n_1 = 58$, $n_2 = 70$, $P < 0.05$; $U = 877.5$, $n_1 = 58$, $n_2 = 70$, $P < 0.0001$, and $U = 1{,}548.5$, $n_1 = 58$, $n_2 = 70$, $P < 0.05$, respectively).

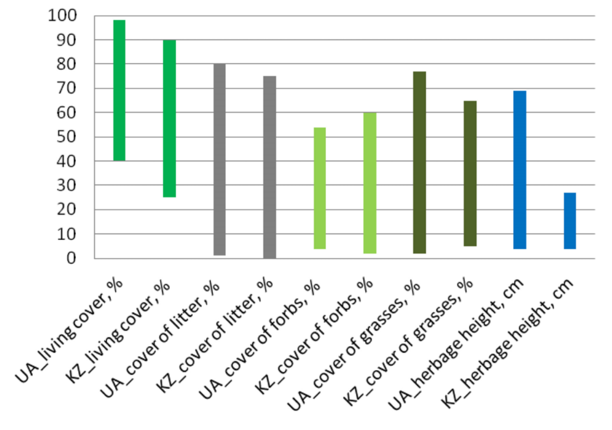

FIGURE 2 | Ranges of general vegetation parameters (living cover of vascular plants, cover of litter, forbs, grasses, and herbage height) in foraging areas of *M. b. bobak* and *M. b. schaganensis*.

The cluster analysis based on general vegetation parameters in the habitats of both marmot subspecies revealed three clusters (**Figure 4**). Although the arable fields were formally included in cluster 1, they stood apart from other habitats. The first and second clusters are characterized by low cover of litter and herbage height while the third cluster is distinguished by the high values of these parameters.

We did not find inhabited marmot settlements in vast areas of former croplands covered with dense vegetation dominated by *Stipa* cf. *zalesskii* (**Supplementary Figure S4A**). Dense stands of *Stipa pennata* in Northeastern Ukraine are also avoided by marmots (**Supplementary Figure S4B**).

The number of burrows per family in the studied settlements of *M. b. bobak* varied from 15 to 49. In the settlements of *M. b. schaganensis*, it was much lower than in the European subspecies, ranging from one (north locality: arable fields) to seven (north locality: grazed steppe). Mean distances between the nearest winter burrows of the neighboring families of *M. b. bobak* ranged from 76.7 ± 24.6 m ($n = 13$, grazed grasslands of Kamianka) to 231.5 ± 192.0 m ($n = 15$, long-abandoned grasslands of Novomlynsk). These distances in *M. b. schaganensis* settlements varied from 120.0 ± 41.2 m ($n = 6$, north locality: grazed former croplands) to 227.5 ± 43.7 m ($n = 6$, north locality: grazed steppe) (**Figure 5**). The comparative analysis of all investigated settlements showed that the mean distance between the winter burrows of *M. b. schaganensis* is higher (172.4 ± 63.4, $n = 91$) than that of *M. b. bobak* (111.1 ± 81.9, $n = 167$) ($U = 2{,}845.0$, $n_1 = 167$, $n_2 = 91$, $p < 0.001$).

Forage Preferences of Marmots

The list of plants that form the *M. b. bobak* food base in Northeastern Ukraine includes 41 species (**Supplementary Table S1**). These plants are common in various habitats of the European subspecies (*Poa angustifolia*, *Elytrigia repens*, *Achillea millefolium*, etc.); some of them are dominants and/or subdominants on the cattle pastures. For instance, in some grazing areas, the cover of *Poa angustifolia* could reach 75%. Mainly, the marmot's food species belong to the group of mesophytic forbs. Steppe bunch grasses are absent in its fodder. Moreover, six plant species were recognized as the main diet component when feeding tame marmots (*Lactuca serriola*, *Lactuca tatarica*, *Cirsium arvense*, *Sonchus arvensis*, *Medicago sativa*, and *Trifolium sativum*). They are not typical plants of *M. b. bobak* habitats, but they are able to form dense monospecies stands in agricultural lands.

The Population Density of the European Subspecies and the Food Base of Its Habitats

Food base parameters (living cover of vascular plants, total cover of food species, total cover of uneaten species together with litter, cover of litter separately, and herbage height) were related to the three population grades distinguished in the present

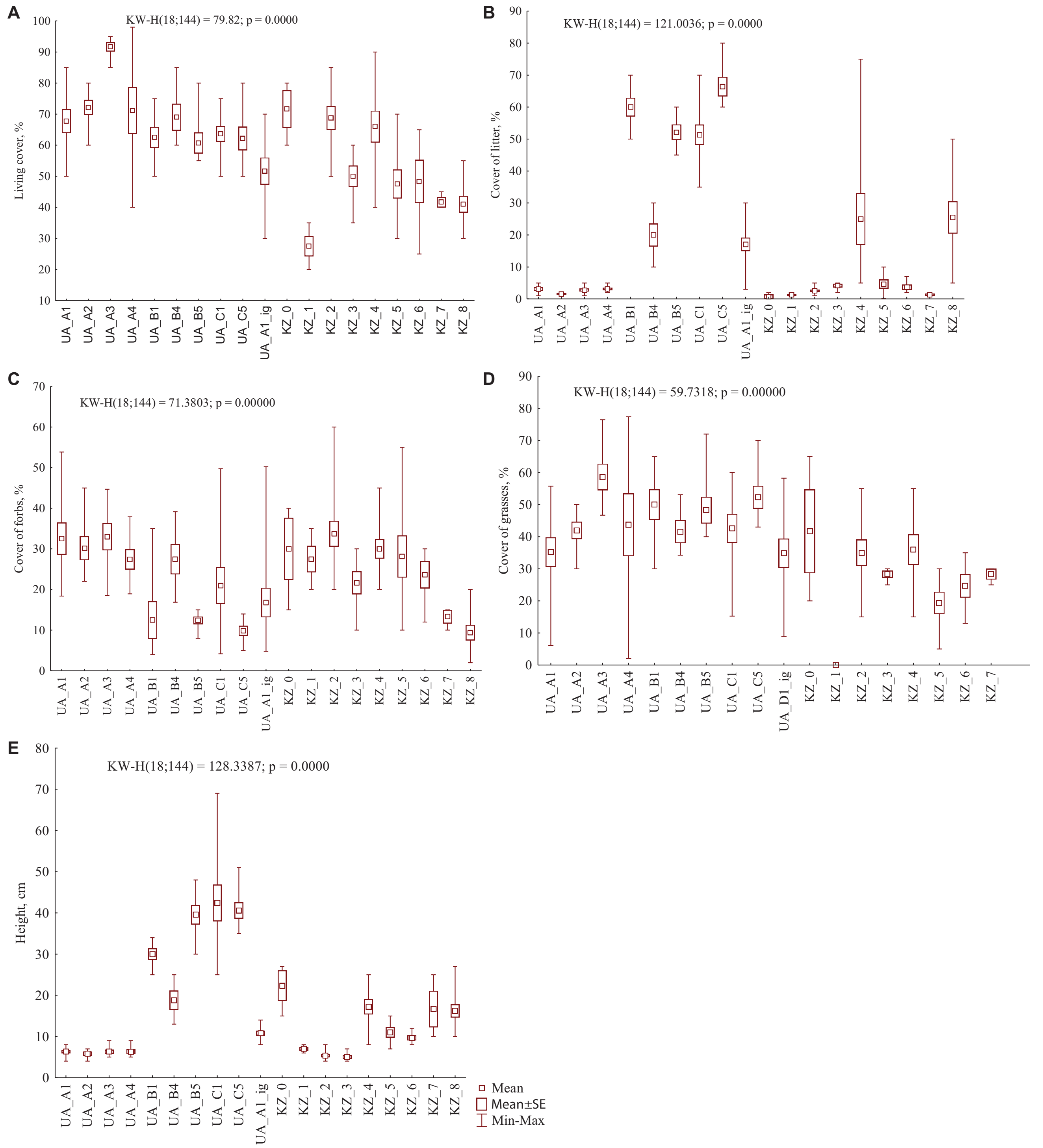

FIGURE 3 | General vegetation parameters (**A** – living cover, **B** – litter, **C** – forbs, **D** – grasses, and **E** – herbage height) in foraging areas of the studied settlements of *M. b. bobak* (UA) and *M. b. schaganensis* (KZ). UA_A1, grazed grasslands of Nesterivka; UA_A2, grazed grasslands of Zelenyi Hai; UA_A3, grazed grasslands of Rohozianka; UA_A4, grazed grasslands of Kamianka; UA_B1, grasslands of Nesterivka shortly after abandonment; UA_B4, grasslands of Kamianka shortly after abandonment; UA_B5, grasslands of Novomlynsk shortly after abandonment; UA_C1, long-abandoned grasslands of Nesterivka; UA_C5, long-abandoned grasslands of Novomlynsk; KZ_0, north locality: roadsides; KZ_1, north locality: arable fields; KZ_2, north locality: grazed steppe; KZ_3, north locality: grazed former croplands; KZ_4, north locality: ungrazed former croplands; KZ_5, south locality: grazed steppe; KZ_6, south locality: ungrazed steppe; KZ_7, south locality: grazed former croplands; KZ_8, south locality: ungrazed former croplands, and UA_A1_ig, grazed grasslands of Nesterivka, ignored vegetation.

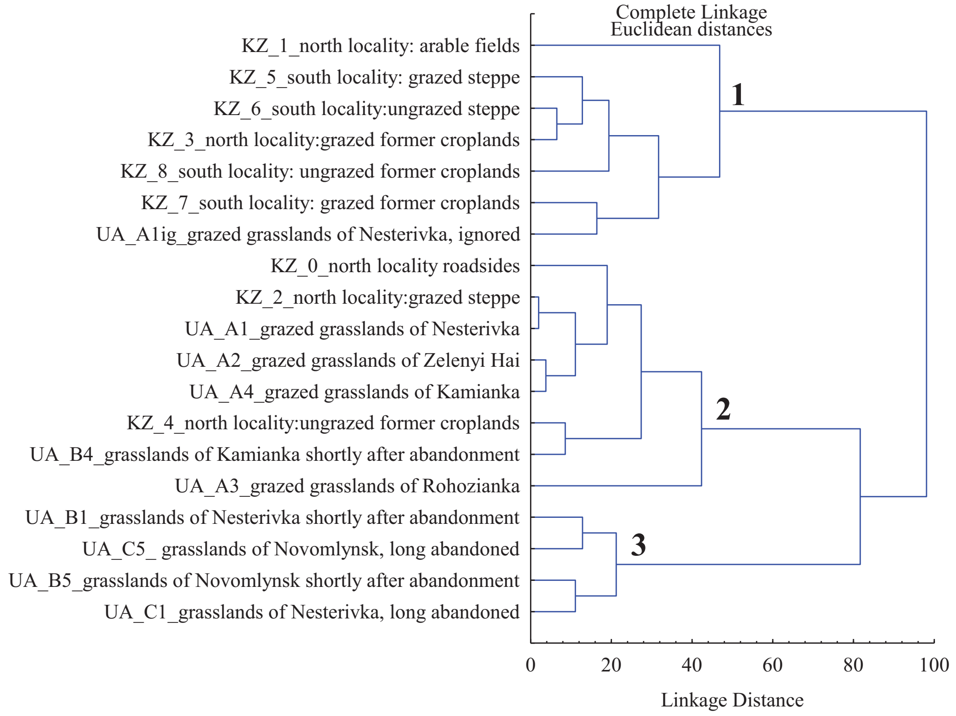

FIGURE 4 | The clusters based on mean general vegetation parameters (living cover of vascular plants, cover of litter, cover of forbs, cover of grasses, and herbage height) for different marmot habitats of Northeastern Ukraine (UA) and Northern Kazakhstan (KZ).

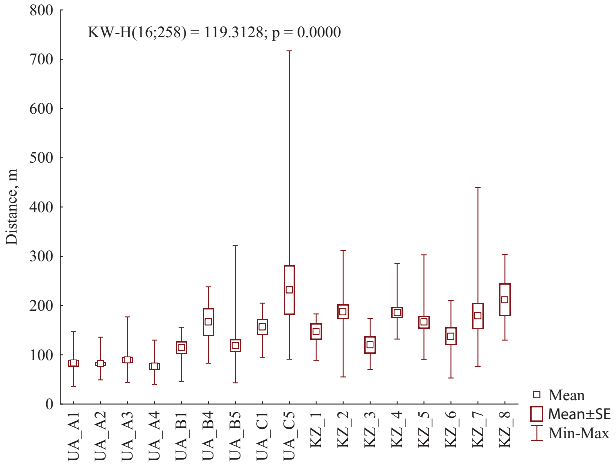

FIGURE 5 | Mean distances between the nearest winter burrows of the neighboring families in the studied settlements of *M. b. bobak* and *M. b. schaganensis*. UA_A1, grazed grasslands of Nesterivka; UA_A2, grazed grasslands of Zelenyi Hai; UA_A3, grazed grasslands of Rohozianka; UA_A4, grazed grasslands of Kamianka; UA_B1, grasslands of Nesterivka shortly after abandonment; UA_B4, grasslands of Kamianka shortly after abandonment; UA_B5, grasslands of Novomlynsk shortly after abandonment; UA_C1, long-abandoned grasslands of Nesterivka; UA_C5, long-abandoned grasslands of Novomlynsk; KZ_1, north locality: arable fields; KZ_2, north locality: grazed steppe; KZ_3, north locality: grazed former croplands; KZ_4, north locality: ungrazed former croplands; KZ_5, south locality: grazed steppe; KZ_6, south locality: ungrazed steppe; KZ_7, south locality: grazed former croplands; KZ_8, south locality: ungrazed former croplands.

marmot settlements: normal population, population decrease, and population collapse. The short herbage height (5–9 cm) and the low cover of litter (1–5%) accompanied normal population density. The current anthropogenic effect that provides these parameters is grazing. On the contrary, both population decrease and collapse were accompanied by the high herbage height (Kruskal–Wallis test, *Í* = 67.3, *df* = 3, *P* < 0.001) and the dense cover of litter (*Í* = 63.3, *df* = 3, *P* < 0.001) (**Figures 6A,B**). Such vegetation changes were observed shortly after pasture abandonment. Differences in both abovementioned parameters in the foraging and ignored areas of the normal population were statistically insignificant.

The vegetation associated with the normal population differed significantly from that associated with the population decrease and the population collapse due to the low cover of uneaten species together with litter (Kruskal–Wallis test, *Í* = 49.2, *df* = 3, *P* < 0.001) (**Figure 6C**). It was also different from the ignored vegetation (Kruskal–Wallis test, *Í* = 38.5, *df* = 3, *P* < 0.001) due to the high cover of food species (**Figure 6D**). The cover range of food species in the settlements with normal population included the whole variability of this cover in the settlements with both population decrease and collapse.

The Response of *M. b. bobak* Population to Pasture Abandonment

According to our observations, the normal population grade is accompanied by a stable settlement structure; one marmot family uses the same winter burrow for many years in a row. We counted

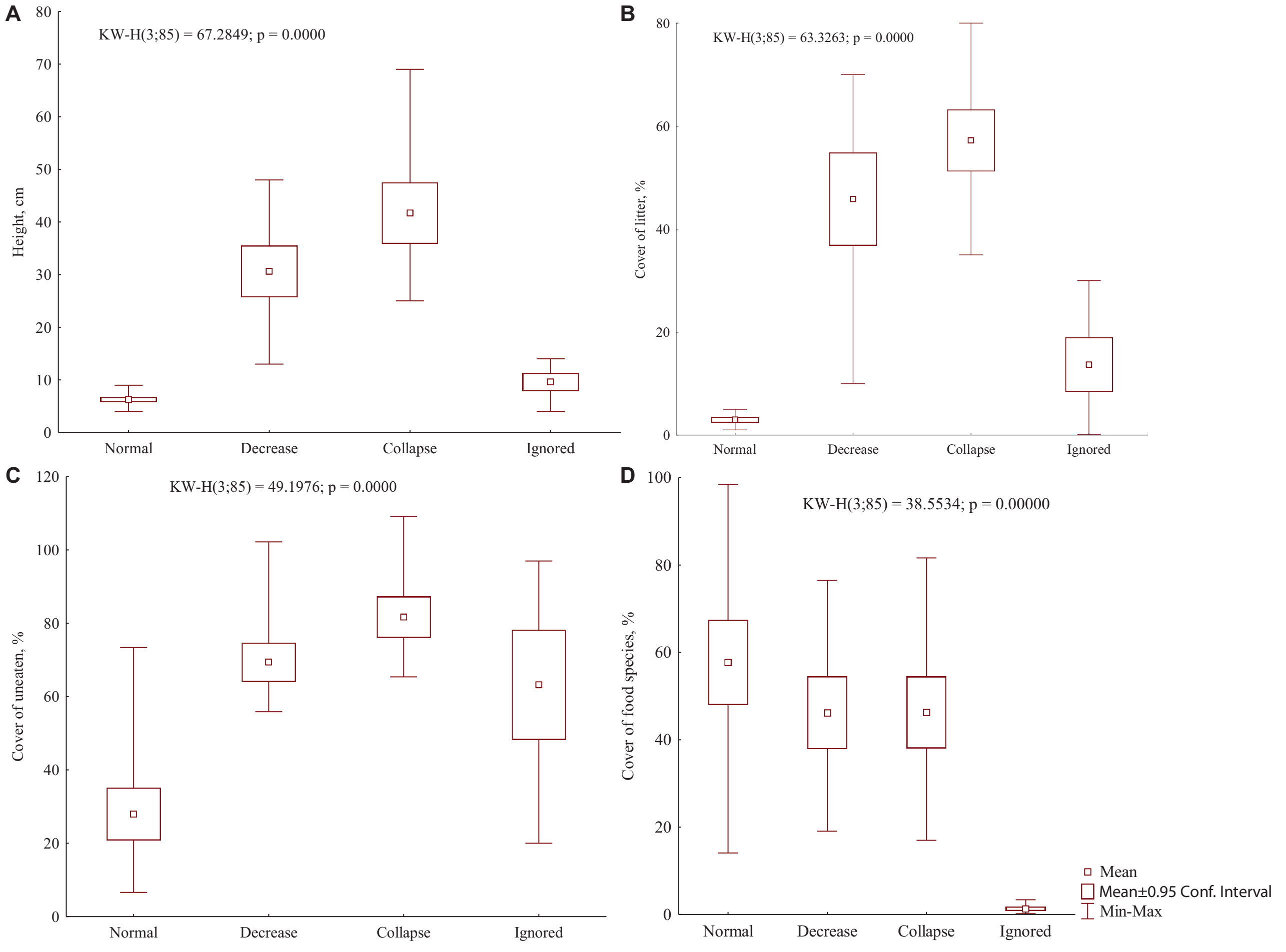

FIGURE 6 | Vegetation characteristics of *M. b. bobak* habitats: height of main herb layer **(A)**, cover of litter **(B)**, cover of uneaten species together with litter **(C)**, cover of food species **(D)** in foraging areas with three grades of population density (normal, decrease, and collapse) and in ignored vegetation with the normal population density.

the total number of all types of burrows in the central part of the Nesterivka settlement (25.5 ha) (**Supplementary Table S2**). On average, there were 1.50 ± 1.2 burrows (mean ± SD, *n* = 647) per 20 × 20 m quadrate. The confidence limits for mean (95%) are quite narrow (1.41, 1.60), which indicates a fairly uniform distribution of burrows in the central part of the settlement. According to our estimates, the population uses about half of available burrows per year.

In 2001, cattle grazing was the main type of anthropogenic effect. The pastures covered nearly the whole area of the Nestrivka settlement. From 2004 to 2014, the grazing area decreased gradually, and finally, it was abandoned in 2015. Before the grazing cessation, the marmot population density corresponded to the normal grade (0.81 families/ha). An average number of burrows per family varied from 24.1 ± 8.1 (median: 24.0, *n* = 27, 2001) to 23.0 ± 6.4 (median: 21.0, *n* = 23, 2014). In 2017, the number of families dropped to 12. The population density was described as decreasing (0.48 families/ha). An average number of burrows used by each survived family grew to 27.1 ± 4.2 (median: 26.5). In the spring of 2019, there were only seven families left. The population density decreased to the grade of collapse (0.24 families/ha). A tendency to increase the number of burrows per family reversed: each family used on average 17.0 ± 2.2 burrows (median: 16.5, *n* = 7). Data ranking showed that the most frequent was rank of 15–21 burrows per family, and the rarest was one with more than 35 burrows per family (**Figures 7A,B**).

We also investigated the other 15 marmot settlements in Northeastern Ukraine, and a sharp decrease in population density after grazing cessation was registered in all of them. Along with the population decline, the same above-described changes were recorded: (1) multiple increase in herbage height and (2) increase in the home ranges of remaining families due to occupation of the winter burrows of neighboring extinct families as well as the area around these burrows. In 2017, the cattle grazing occurred only in two monitored settlements, and its intensity decreased compared to 2001. The food base of the most families did not change, and the population density remained at the normal population grade (Zelenyi Hai: 0.95 families/ha, 2017; Rohozianka: 0.89 families/ha, 2017; in total 19 and 25 families,

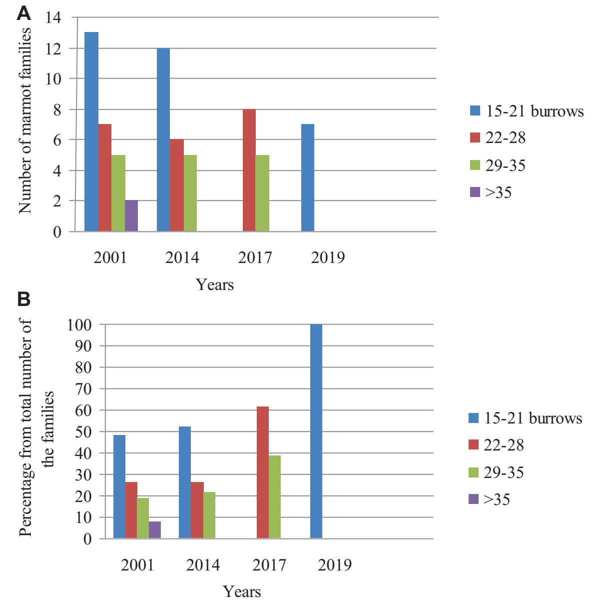

FIGURE 7 | The ratio of marmot families using a certain number of burrows. Central part of the Nesterivka settlement. Population density grades: 2001 and 2014 – normal, 2017 – decrease, 2019 – collapse. **(A)** Absolute number of marmot families and **(B)** percentage of families.

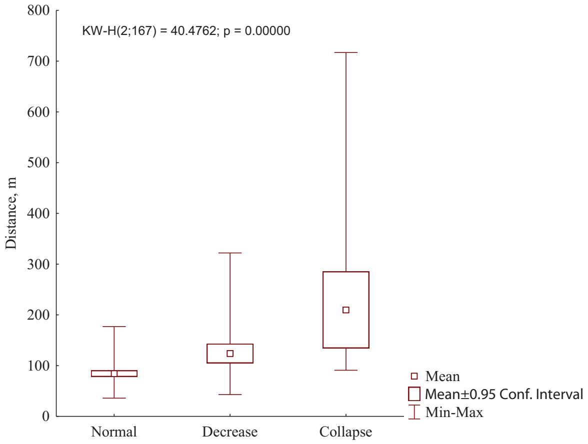

FIGURE 8 | Distances between the nearest inhabited winter burrows in marmot settlements in Northeastern Ukraine depending on the grades of population density (normal, decrease, and collapse).

respectively). This proportion of abandoned and grazed marmot settlements (18 to 2) corresponds approximately to the current state of pastoral activity in Northeastern Ukraine, but there is still a tendency toward a decrease in pasture areas.

One more trait of the marmot settlement, which depends on the population density, is a distance between the nearest inhabited winter burrows. In the case of the normal population, it equaled 84.4 ± 30.9 m ($n = 100$). With a decrease in population density, this distance increased significantly (**Figure 8**): In normal populations, this parameter was 124.1 ± 64.3 m ($n = 46$) while in collapsed populations it was 210.0 ± 165.5 m ($n = 21$) (Kruskal–Wallis test, $H = 40.5$, $df = 2$, $P < 0.001$).

DISCUSSION

General Vegetation Parameters of the Habitats of *M. bobak*, and the Structure of Its Settlement

The cluster analysis (**Figure 4**) shows that most Kazakhstan habitats are joined into one cluster with the ignored areas of the Ukrainian grazed grasslands. The second cluster was formed by the Ukrainian grazed grasslands and a bulk of habitats in the northern sampling sites in Kazakhstan: roadsides, grazed steppes, and ungrazed former croplands. We noted earlier that the roadside vegetation responds well to the feed requirements of the European steppe marmot (Ronkin et al., 2009). Pastures of the northern sampling sites in Kazakhstan, which lay in a moister climate, are similar to the Ukrainian pastures in both appearance and basic characteristics. The ungrazed former croplands of the northern sampling sites in Kazakhstan are similar to the Ukrainian pastures as to the cover of forbs and living cover of vascular plants. The third cluster was formed by the Ukrainian shortly and long-abandoned grasslands. They are united by the large height of herbage and the dense cover of litter.

Most authors consider that peculiarities of the settlement structure reflect specificity of local foraging conditions and site topography (Sludsky et al., 1969; Bibikov, 1989; Mashkin, 1997). It was found that *M. b. bobak* has higher population density and lower distance between the winter burrows than *M. b. schaganensis* (Sludsky et al., 1969). Our data are in agreement with these observations. However, in recent decades, we have seen a decline of *M. b. bobak* settlements in Northeastern Ukraine that results in the increase of distances between inhabited winter burrows. Data analyses of density grading (**Figure 8**) revealed a sharp irregularity in the distribution of inhabited winter burrows. The low average value obtained for the Ukrainian samples is explained by the predominance of measurements in the settlements with normal density. In the studied Kazakhstan settlements, these distances were less variable, which can be addressed to greater homogeneity of living conditions in different habitats. A small number of burrows per *M. b. schaganensis* family, compared to a family of *M. b. bobak*, can also be explained by homogeneity of the environmental conditions in the habitats of the Kazakhstan subspecies. In our study, the distances between the burrows of the Kazakhstan subspecies were greater than those recorded in the first half of the 20th century: 70–440 m versus 50–80 m (Sludsky et al., 1969). The settlements with normal population density in Ukraine were the most different from all the settlements studied in Kazakhstan (e.g., UA_A1–UA_A4 and KZ_1–KZ_8, **Figure 5**), which reflects the difference between two types of marmot settlements: concentrated and dispersed, respectively (after Bibikov, 1989).

Interestingly, the greatest distances between inhabited winter burrows in Northern Kazakhstan were recorded in the grazed steppe (north locality, KZ_2, **Figure 5**) and ungrazed former croplands (south locality, KZ_8, **Figure 5**). In Northeastern Ukraine, the grazed habitats were characterized by the smallest distances (UA_A1–UA_A4, **Figure 5**). This may be partly explained by possible competition for fodder resources between marmots and livestock in the dry steppes of Kazakhstan. As a rule, distribution, quality, and abundance of the food resource determined an average distance between the nearest winter burrows. According to Seredneva (1986), the better food conditions in the marmot settlement, the less the average distance between the burrows.

Diverse habitats with differences in vegetation parameters as well as the differences between the settlement structures of *M. b. bobak* and *M. b. schaganensis* demonstrate a high ecological plasticity of the steppe marmot at the species level.

Forage Preferences of Marmots

The absence of large differences in the ranges of general vegetation characteristics (herbage height and cover of litter) in the foraging and ignored areas of the European steppe marmot confirms the high food selectivity of this subspecies. When analyzing the food and uneaten plant species, the difference between the forage and ignored vegetation becomes highly significant. On the contrary, the Kazakhstan subspecies does not demonstrate high selectivity in its diet (Sludsky et al., 1969; Zimina and Isakov, 1980).

According to our data, the European subspecies ignored areas with the total cover of its food species of about 1–3%. The ignored vegetation is either dry steppe vegetation dominated by bunch grasses (*Stipa capillata*, *Stipa pennata*, and *Festuca valesiaca*) and xerophytic forbs (*Galatella villosa*) or chalky vegetation dominated by xerophytic *Artemisia hololeuca* and *Thymus cretaceus*. On the contrary, many food species in the habitats of the European steppe marmot belong to the group of mesophytic forbs. The species *Lactuca serriola*, *Lactuca tatarica*, *Cirsium arvense*, *Sonchus arvensis*, and *Trifolium sativum* have succulent leaves. These plants grow only in the pastures, croplands, and on the roadsides in both Ukraine and Kazakhstan.

However, the steppe marmot does not specialize in feeding on mesophytic forbs only. Its food plasticity is very wide compared to the mountain marmot species. Dry steppe grasses, xerophytic forbs, and even leaves of steppe shrubs serve as fodder plants to the Kazakhstan subspecies (Sludsky et al., 1969; Zimina and Isakov, 1980). In grazed steppes and ungrazed former croplands dominated by *Stipa lessingiana*, *Festuca valesiaca*, and *Artemisia* ssp., we registered families with offspring, and the juveniles looked well fed. Most of those Kazakhstan habitats are combined in one cluster with ignored vegetation of *M. b. bobak* (**Figure 4**). All of the above confirms the general opinion about the lower food selectivity of *M. b. schaganensis* (Sludsky et al., 1969; Bibikov et al., 1990) that provides its stable existence in the conditions unsuitable for *M. b. bobak*.

The Population Density of *M. bobak* and the Food Base of Its Habitats

In 2016–2017, the current population size of *M. b. schahanensis* was estimated at 6.1 ± 2.4 million individuals (Koshkina et al., 2019). In Kazakhstan, its range has remained almost unchanged since the 1950s despite several drastic episodes of the land-use change (Koshkina et al., 2019). Comparison of the lists of *M. b. schaganensis* food plants, including crops (Sludsky et al., 1969; Zimina and Isakov, 1980; Mashkin, 1997) with a list of weeds of Kazakhstan croplands suggests that in the absence of regular herbicide treatments, forage conditions in the arable fields may be better for the marmot than that in ungrazed former croplands and dry grazed steppes. For instance, *Lactuca tatarica*, *Cirsium arvense*, and *Sonchus arvensis*, willingly eaten by marmots, are common weeds of the arable fields (**Supplementary Figure S5**). Urakchintseva (2005) estimated the weed cover in the agricultural fields not treated with herbicides as minimum as 34–48%. In our opinion, life in arable fields cannot be considered a new ecological adaptation since the field vegetation corresponds to the food requirements of the Kazakhstan subspecies, and plowing is not able to destroy their deep winter burrows (Rumyantsev, 1991). An increase in the use of herbicides in agriculture can make Kazakhstan fields unsuitable for marmots in the near future (Koshkina et al., 2019).

According to many authors, the Kazakhstan subspecies is the most independent from mesophytic forbs compared to other marmot species (Sludsky et al., 1969; Zimina and Isakov, 1980; Bibikov, 1989). It has adapted to feeding on grasses and xerophytic forbs in the Kazakhstan dry steppes (Bibikov, 1989). Currently, the European subspecies needs to adapt to changes of its habitats. Otherwise, its settlements are doomed to further decline despite a large cover of food plants. In the absence of grazing, the vegetation coarsens and gains senescence quickly. Moreover, the marmot food base is worsening due to increased herbage height and cover of uneaten plant species together with litter. A dramatic decline of the European subspecies population under total pasture abandonment encouraged us to seek possible measures alternative to grazing to restore the marmot's habitat. Frequently repeated mowing can maintain vegetation parameters similar to those in grazed habitats during the whole season of marmot activity (Savchenko and Ronkin, 2018). It can be an effective management measure, but it requires strong and consistent dedication and enthusiasm from volunteers to mow the marmot foraging areas (Valkó et al., 2018). We are studying the possibility to preserve the gene pool of the European subspecies in Northeastern Ukraine in seminatural conditions. For this purpose, we test plants for compliance with the main forage components similar to marmot's natural diet.

A wide range of vegetation parameters in the foraging areas of the European subspecies refers to the current period when both grazed and abandoned grasslands can be observed. However, only the range that accompanies normal population grade is suitable for animals of all ages. Vegetation transformation after grazing cessation does not meet the nutritional needs of juveniles (Savchenko and Ronkin, 2018) and results in population decrease and then collapse even though the adult nonbreeding marmots fully satisfy their food needs. Such a feature, when the needs of adults have a wider satisfaction range, corresponds well to the climate peculiarities of the steppe areas with periodic droughts. A vast fluctuation amplitude of the absolute values of environmental factors in the Kazakhstan steppes was shown in climatic data by Mordkovich (2014). Unfortunately, the food conditions become stably unsuitable for juvenile animals, which we are currently observing in Northeastern Ukraine. The range of vegetation parameters accompanying the normal population grade of the European subspecies is quite narrow. Thus, it has low ecological plasticity in food choice. A rapid population collapse after pasture abandonment does not allow the marmot to acquire adaptations to the changing food conditions.

The Response of *M. b. bobak* Population to Pasture Abandonment

The European subspecies formed compact dense and numerous settlements in the steppe gullies of limited size giving an example of environmental adaptation to excess food resources with limited of habitat area. According to Seredneva (1986), the number of home ranges and burrows used in a stable settlement is more or less constant. Our data concurs with this author. Lenti Boero (2003) shows that such parameters as the most used summer burrows and hibernacula, the core foraging and the feeding area, and the home range dimension are permanent and stable structural components of the alpine marmot (*M. marmota*) territories, and they can be inherited by different and unrelated families. Our investigation of the steppe marmot shows that, under the deterioration of food conditions and a reduction in the number of families, the deserted territories and burrows are used by the neighbors. For example, the presence of two families using more than 35 burrows in 2001 (**Figure 7**) is explained by the extinction of four neighboring families. The absence of the range of 15–21 burrows per family in 2017 resulted from the death of 11 out of 23 families and the use of their territories by the neighbors. Thus, with deteriorating feed conditions, the home ranges of the remaining families increase. Perhaps, it is due to the fact that the European steppe marmot retained its adaptation to occupying excess territories to ensure seasonal movements to the areas with mesophytic forbs as is described for mountain species (Bibikov, 1989).

In the conditions when undergrazing makes the herbage unsuitable for marmots, a more social behavior model, similar to the large family groups of black-tailed prairie dogs (*Cynomys ludovicianus*) (Hoogland, 1981, 2013; Hoogland and Brown, 2016), would suit the European steppe marmot. The large number of family groups enables prairie dogs to change the vegetation cover within their settlements. The Kazakhstan subspecies can also significantly change the vegetation around the mounds of winter burrows as the family resources include one winter burrow and one or five foraging ones (Mashkin et al., 2010; Koshkina et al., 2019). The vegetation far around the mounds is short cut due to the constant biting by the animals (**Supplementary Figure S6A**). Obviously, this contributes to producing a larger number of vegetative shoots, which are most valuable in terms of food quality. We did not observe any signs of the European subspecies' ecological adaptations that resulted in concentration of their foraging activity in a small area. The vegetation remained relatively tall since the marmots did not concentrate their foraging activity around the mound (**Supplementary Figure S6B**).

The Steppe Marmot's Status in the Plant–Herbivore Interaction System in the Grasslands of Northeastern Ukraine and Northern Kazakhstan

In the climatic conditions of Northeastern Ukraine, the peak of vegetation activity occurs in May. Then, the grass matures, and the water content in the aboveground plant biomass decreases (Savchenko and Ronkin, 2018). Such course of vegetation growth is unfavorable for the European subspecies since it feeds on the succulent, constantly regrowing parts of plants. The marmot is not able to ensure plant regrowth in a sufficient part of its foraging areas. It is provided by cattle grazing. Vegetation changes under grazing impact result in alteration of dominant species. Uneaten species of *Festuca* and *Stipa* are replaced by the food species of *Poa* and *Elytrigia*. In addition, pasture herbage includes a large number of mesophytic forbs. Cessation of cattle grazing adversely affects survival of juveniles (Savchenko and Ronkin, 2018). Settlements are declining because the marmot has no time to develop new ecological adaptations. Consequently, the European steppe marmot was and continues to be a secondary pasture user. This specialization enabled it to occupy the European grasslands. According to Dinesman (1977), these grasslands were evolutionarily formed under the impact of large herbivores. After the extirpation of wild herding ungulates, the steppe marmot continued thriving in the European grasslands owing to cattle grazing. Moreover, the most favorable feeding conditions for the steppe marmot were formed at the gully bottoms, which are more humid than the steppe slopes. Both vegetation and invertebrate assemblages (Polchaninova et al., 2016) confirm the steppe-meadow character of this habitat.

On the contrary, the Kazakhstan subspecies can be both a secondary and a primary user of the Asian dry steppes. We assume that other Marmot species are also divided into primary and secondary grassland users. For example, *M. camtschatica* (Tokarsky and Valentsev, 1994) can stably live in the mountain meadows of Kamchatka on conditions of the absence of grazing ungulates due to a wide range of plants with succulent leaves and the long-lasting vegetative period of these meadows. The most preferred foraging areas of the marmots in the Tatra Mountains (*M. marmota latirostris*) are the communities of tall forbs and grasses found mainly in mesophilous habitats (Ballová and Šibík, 2015). On the contrary, ungulate grazing is necessary in *M. menzbierii* habitats in the Tien Shan Mountains to prevent rapid vegetation maturation and senescence (Mashkin et al., 2010). Nikol'skii and Ulak (2006) observed that *M. himalayana* in the Manaslu mountain range fed exclusively on livestock pastures.

In the process of evolution, marmots adapted to living in diverse countries (Bibikov, 1989; Armitage, 2014). They control the temperature of hibernation (Nikol'skii and Savchenko, 2002; Nikol'skii et al., 2005; Nikol'skii, 2009) and have a peculiar circannual rhythm (Bibikov, 1989; Armitage, 2014). Social thermoregulation acquired in the course of evolution helps the marmots to increase juvenile survival during hibernation (Arnold, 1990). However, are the marmots capable of a quick adaptation acquirement in response to sharp deterioration of the food base of their habitats? In the case of *M. b. bobak*, there is an additional risk that reduces its chances of a successful outcome. We suggest that, in the beginning of the 20th century, *M. b. bobak* genetic diversity was reduced dramatically due to a sharp population decrease and isolation of the remaining populations. Further conditions of its rebound were very similar throughout its range. Perhaps, we are now witnessing a scenario that was predicted by Gossmann et al. (2019): "If low genetic variation is a contributory factor to extinction risk, not only small but also large populations can be at risk, if their life history traps them permanently in a state of low genetic diversity."

The food base of the European subspecies has quickly deteriorated in vast areas within the marmot range that does not give the marmot any chance to adapt to the changing conditions and to become the primary grassland user. The only real measure capable of the European subspecies conservation is a government policy aimed at restoring livestock grazing in the marmot habitats. For the Kazakhstan subspecies, we recommend avoiding herbicide use along the field margins in the areas inhabited by marmots.

CONCLUSION

Geographical peculiarities of *M. bobak* ecological conditions within its vast geographic range are reflected in different ecological adaptations of its subspecies. The European subspecies has taken the place of a secondary user of the pastures of herding ungulates. This allowed it to thrive in the moist pasture ecosystems after the extinction of wild herding herbivores as long as the ecosystem functioning was providing by livestock. Abandonment of the pastoral cattle breeding in the mid-1990s resulted in practically concurrent grazing cessation. Now, the forage conditions in *M. b. bobak* habitats got their pessimum nearly everywhere, which caused the collapse of families. The high-speed, large-scale changes of their habitats leave *M. b. bobak* no chance for adaptation. The Kazakhstan subspecies is capable of sustainable existence under conditions similar to the pessimum that causes extinction of the European subspecies. Adaptation to the constant environmental rigidity allows the Kazakhstan subspecies to survive in the habitats plowed in the middle of the 20th century during the virgin lands campaign. In our opinion, that was not a new ecological adaptation, but a consequence of the fact that the living conditions remained in accordance with *M. b. schahanensis* requirements. Modern agricultural technologies can quickly deprive the Kazakhstan subspecies of this type of habitat.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by the Committee on Bioethics of Kharkiv National University named after V. N. Karazin.

AUTHOR CONTRIBUTIONS

VR and GS: study design, data collection, data analysis, and manuscript writing. VT: study design and data collection. NP: manuscript writing. AA: data collection. AK: data collection and organizing of the Kazakhstan expedition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00219/full#supplementary-material

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Think Before They Squeak: Vocalizations of the Squirrel Family

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Squirrels (Sciuridae) are a diverse group in behavior, morphology, and ecology. This variation is typified by the wide range of vocalizations spanning ground squirrels (Marmotini and Xerini), tree squirrels (Callosciurinae and Sciurini), and flying squirrels (Pteromyini). Squirrels produce calls that range in frequency, modulation, and function, with a complex set of social calls occurring across the family. We review the history of recording methods used in the development of squirrel vocalization repertoires, with emphasis on how the ecology and methodology impact the frequency values reported. The fundamental (F_0 – the mean frequency of the fundamental harmonic), dominant (F_{Dom} – the frequency of maximum energy or amplitude), minimum (F_{Min} – the minimum frequency of the fundamental harmonic), maximum (F_{Max} – the maximum frequency of the dominant harmonic), and highest harmonic (F_{Harm} – the mean frequency of the highest visible harmonic) frequencies were considered against popular hypotheses that have attempted to explain the evolution of vocal frequency characteristics in terrestrial mammals. These hypotheses include body size, predator avoidance, habitat type, and diel activity pattern. Phylogenetic generalized least squared modeling revealed that body mass and the frequency limits of the methods were the strongest drivers of high-frequency communication. Consistent with popular hypotheses, social squirrels exhibited a broader range of F_0, F_{Dom}, and F_{Max} than solitary squirrels while habitat openness promoted higher F_{Dom} and F_{Harm}. Additionally, nocturnality was significantly associated with higher F_0, F_{Dom}, and F_{Max}, suggesting that flying squirrels, the only nocturnal squirrels, commonly use high-frequency acoustic signals, a finding that merits further investigation. In conclusion, our review provides a unique insight into the role of behavioral ecology on vocal repertoires and the importance of accurate equipment selection for sampling across a diverse taxon.

Keywords: Sciuridae, squirrels, vocalizations, alarm call, ultrasonic vocalizations, frequency characteristics

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INTRODUCTION

Mammalian bioacoustics is an expansive field in which biology, physics, and psychology are used to study the taxonomic diversity of signalers and interpret the complexity of vocal communication. Over the last century or so, the bioacoustic community has seen the development, implementation, and specialization of their research technology. Ever-improving recording devices have allowed advancements from phonetic descriptions of non-human animal calls to the first spectrographic visualizations to the development of software allowing quantitative analysis of calls in minute detail (Terhune, 2011). While phonetic descriptions of the calls served an important purpose at one point in time, the recording of calls provided the first opportunity for a detailed comparison of the differences between species and the evolution of vocal production in mammals through spectrographic analyses. The first acoustic spectrograph was developed in 1946 to study human speech, though the authors suggested that this new device could be used to study environmental sounds, musical instruments and orchestral composition, and potentially animal sounds under the right conditions (Koenig et al., 1946).

Spectrographs were, and continue to be, important because they allowed researchers to study the shape and structure of the sounds being produced, but they also allowed the visualization of calls that cannot be heard by the human ear. Humans are limited to a hearing frequency range of about 20 Hz to a maximum of 20 kHz, though 15–18 kHz is the cut-off for the average human (Masterton et al., 1969). Given that it is difficult for even the best human ears to hear sounds near the upper limit (Masterton et al., 1969), some have argued that 15 kHz should be the cut-off for differentiating between the audible range (what can be heard by humans) and the ultrasonic range (calls that exceed the upper limit of human hearing). However, most publications describe 20 kHz, the absolute maximum of human hearing, as the frequency at which calls can be described as ultrasonic.

Ultrasonic vocalizations (USVs), calls that completely lie in the ultrasonic range (>20 kHz herein), were first studied in cetaceans and bats that had been observed navigating and hunting without visual cues. USVs were first recorded with the aid of frequency-altering heterodynes in bats (Griffin and Galambos, 1941) and were first visualized in porpoises (Wood, 1952). Bats and cetaceans largely remained the focus of ultrasonic studies for decades, due in particular to interest in echolocation. The intensive echolocation focus has likely overshadowed ultrasound-production in many other wild species such as shrews (*Blarina* and *Sorex*: Gould et al., 1964; *Sorex*: Tomasi, 1979), singing mice (*Baiomys* and *Scotinomys*: Miller and Engstrom, 2007; *Scotinomys*: Fernández-Vargas et al., 2011), and even several species of squirrel (*Urocitellus richardsonii*: Wilson and Hare, 2004; *Glaucomys*: Murrant et al., 2013). Additionally, the widespread availability of lab mice and rats has led to many studies investigating the function and biomechanical processes involved with USV production. The study of USV production in these rodents has been quite active since the early 1970s (Oswalt and Meier, 1975; Geyer and Barfield, 1978; Geyer et al., 1978; Hofer and Shair, 1978, 1980; Corrigan and Flannelly, 1979; Geyer, 1979; Smith, 1979), leading to a wealth of publications, but also, along with bats, a taxonomic bias in publications about ultrasound use. While still crucial in understanding the physiological and cognitive processes associated with USVs, captive rats and mice have been isolated from natural processes for generations and are not known to produce well-studied wild calls such as predator alarm calls (Blanchard et al., 1991; Hahn and Lavooy, 2005; Sirotin et al., 2014; Kalcounis-Rueppell et al., 2018).

Ultrasound can be a useful communication tool because the short sound waves are highly directional and are not readily heard by most predatory birds, reptiles, and amphibians (Knudsen, 1981; Yamazaki et al., 2004). It has been proposed that the main drivers of ultrasound use for all terrestrial vertebrates are body size, predator avoidance, and increasing the signal-to-noise ratio (Arch and Narins, 2008). Several studies support the hypothesis that body size has a strong relationship to the frequency of many vocalizing animals (avian mass: Ryan and Brenowitz, 1985; mammalian mass: Charlton and Reby, 2016; Martin et al., 2016; mass of mole rats: [*Cryptomys, Heterocephalus, Spalax*], Credner et al., 1997; length of ground squirrels: *Spermophilus*, Nikol'skii, 2017). The mass-call frequency relationship is influenced directly by the physical mechanisms of vocal production (i.e., lung capacity, larynx size) and through indirect effects like species ecology (e.g., larger animals using different environments than smaller animals; Ryan and Brenowitz, 1985). In contrast, predator-avoidance is more difficult to quantify and this hypothesis seems to be more supported by anecdotal evidence of vocal shifts toward ultrasonic frequencies when animals are in the presence of predatory birds (Kotler, 1984; Tamura and Yong, 1993; Digweed and Rendall, 2009). Finally, the signal-to-noise ratio hypothesis has been supported by only a few examples, such as the Kihansi spray toad (*Nectophyrynoides asperginis*) which has shifted all vocal production to USVs to reduce interference from nearby roaring, low-frequency waterfalls (Arch et al., 2011). Similarly, the acoustic adaptation hypothesis, a version of the signal-to-noise idea that focuses on sound propagation relative to environmental characteristics (e.g., open versus closed) instead of ambient sound, postulates that higher frequencies will be employed in open habitats where the shorter sound waves are less likely to be attenuated by the environment before reaching the receiver (Ey and Fischer, 2009). In contrast to the acoustic adaptation hypothesis, it has also been shown that some mammals inhabiting forested habitats have improved hearing sensitivities to continue to use high-frequency calls in closed habitats (Charlton et al., 2019).

Our review will focus on literature covering the recording and reporting of the highly vocal family, Sciuridae. With spectrographic reports of squirrel vocalizations beginning in the 1960s, squirrel genera have been well sampled, and many vocal libraries have been developed and updated with improving technologies. We apply popular and novel hypotheses related to vocal characteristics (**Table 1**) to observe which behavioral and ecological traits drive frequency use in squirrels. We also challenge the currently established repertoires in light of new reports of USVs in flying squirrels and call for future research to employ equipment that is intended for non-human animals, and not subject to the limitations of equipment designed for use with humans.

SQUIRREL VOCALIZATIONS – A SHORT HISTORY

The first published spectrographic analyses of squirrels appeared in 1966; the authors used these visualizations to describe a variety of calls of the Uinta ground squirrel (*Urocitellus armatus*; Balph and Balph, 1966) and the yellow-bellied (*Marmota flaviventris*) and hoary marmots (*M. caligata*; Waring, 1966).

TABLE 1 | Summary of parameters used in the phylogenetic least square (PGLS) analyses and how they relate to popular or novel vocal range hypotheses.

Parameter	Hypothesis	Citation
Body mass (-)	Body size controls the size of vocal producing structures; body size estimated through body mass.	Charlton and Reby, 2016; Martin et al., 2016; Arch and Narins, 2008
Sociality: social (+)	Social species communicate more often and rely on conspecifics for predator avoidance; higher frequencies are less likely to detected by predatory birds, reptiles, and amphibians.	Ramsier et al., 2012; Arch and Narins, 2008
Habitat: openness (+)	Acoustic adaptation hypothesis: species will alter call structure to maximize transmission in different environments; closed habitats attenuate high-frequency sound waves rapidly while open habitats do not inhibit transmission.	Ey and Fischer, 2009
Diel activity pattern: nocturnality (+)	Gliding and nocturnality are intertwined for Sciuridae; nocturnality facilitates the use of echolocation as visual cues are greatly reduced.	Novel
Method limits (+)	The frequency limits of the microphone and spectrograph restrict the detection range of the equipment.	Novel

The predicted direction of the relationship to frequency is indicated as positive (+) or negative (–).

While the authors published separately, they used the same equipment to record the squirrels: a unidirectional microphone with a relatively narrow frequency bandwidth (Electrovoice-644 Sound Spot, frequency response of ∼0.05–12 kHz; Balph and Balph, 1966; Waring, 1966) attached to a relatively large portable tape recorder. Waring (1966) used a W-610-EV battery-operated tape-recorder (weight ∼7.7 kg; Amplifier Corporation of America, 1959) whereas Balph and Balph (1966) used an 11.8 kg Wollensak T 1700 tape recorder (Revere-Wollsenak Division, 2010). The fundamental frequency (F0 – the vibration frequency of the vocal folds which is determined by the shape of the larynx as well as airflow; Fitch et al., 2002; Charlton and Reby, 2016; Riede et al., 2017) and the dominant frequency (F_{Dom} – the frequency with the maximum amplitude or energy; **Figure 1**) of the calls produced by the ground squirrels and marmots were captured within this narrow frequency range as demonstrated on the spectrographs. However, Waring noted at least one call produced by the yellow-bellied marmot had strong harmonics (resonant frequencies produced at intervals above F_0) exceeding 40 kHz, well beyond the dynamic range of the microphone and only detectable with a heterodyne. The dynamic range of the recording microphone is limited by the physical shape of the transducer; this element vibrates when sound is produced, translating the physical sound wave into an electronic signal (Obrist et al., 2010). Similarly, heterodynes (handheld ultrasonic detectors that transpose USVs down to the human hearing range so that researchers can listen to USVs in real-time) can detect a broad range of frequencies with a specialized transducer. However, the transposer (which translates the vibration into an audible sound) is restricted to a narrow bandwidth and the researcher must tune the transducer to listen to different frequencies (Ahlén, 2004).

As Waring (1966) demonstrated, harmonics can lead to vocalizations that partially exceed human hearing abilities (i.e., ultrasonic harmonics that exceed 20 kHz). F_0 is produced as air exits the lungs (called the glottal flow) and without any interruptions, this tone would only consist of F_0 (Titze, 2009). However, if the glottal flow collides with the vocal tract (vocal folds, upper larynx, tongue, mouth, etc.), infinite harmonics will be produced, decreasing in strength as they increase in frequency (Monsen and Engebretson, 1977; Titze, 2009). Harmonics can be strengthened or dampened, depending on the shape of the vocal tract (like the addition of harmonic resonance chambers; Riede et al., 2017) and the presence of strong high-frequency harmonics can be indicative of active manipulation of the vocal tract (Titze, 2009; Fenton et al., 2011). Harmonics can allow animals to extort a larger bandwidth (as in bats; Kingston and Rossiter, 2004; Fenton et al., 2011) or to communicate at a higher frequency (as in anurans and birds; Narins et al., 2004; Arch et al., 2011), though the function of harmonics in most squirrels remains unclear. The yellow-bellied marmot is not the only squirrel for which ultrasonic harmonics have been described. Other genera include *Callospermophilus* (Eiler and Banack, 2004), *Glaucomys* (Gilley, 2013; Murrant et al., 2013; Gilley et al., 2019), *Sciurus* (Zelley, 1971; Lishak, 1982), *Spermophilus* (Koshev and Pandourski, 2008; Schneiderová, 2008, 2012; Matrosova et al., 2011, 2012, 2016; Schneiderová and Policht, 2012; Schneiderová et al., 2020), *Tamias* (Blake, 1992), and *Urocitellus* (Melchior, 1971; Manno et al., 2007). The function of harmonics has been discussed in the context of a few species of ground squirrel (*Spermophilus*). California ground squirrels (*Spermophilus beecheyi*) will dampen the strength of higher harmonics to increase the clarity of F_0 and improve the ability for receivers to locate the sender (Leger et al., 1980). As well, California ground squirrels habituating to noisy environments have developed stronger harmonics than their counterparts inhabiting quieter environments, improving the signal-to-noise ratio of their calls (Rabin et al., 2003). In contrast, some ground squirrels with larger geographic ranges or with multiple subspecies may alter the strength or number of harmonics to accommodate different habitat or social constraints, resulting in distinct dialects (Eiler and Banack, 2004). So, while all species would have infinite ultrasonic harmonics if recorded under ideal conditions (i.e., facing the recorder directly, maintaining close proximity to reduce attenuation of higher frequencies, remove all background noises, microphone with infinite sensitivity), we consider it of some interest if strong ultrasonic harmonics have only been detected in a subset of free-roaming squirrels.

Since the first spectrographic publications, the calls of over 70 extant squirrel species have been reported. There has been a strong emphasis on alarm calls (reported in 74/89 publications), likely because these are often the highest-amplitude calls, they

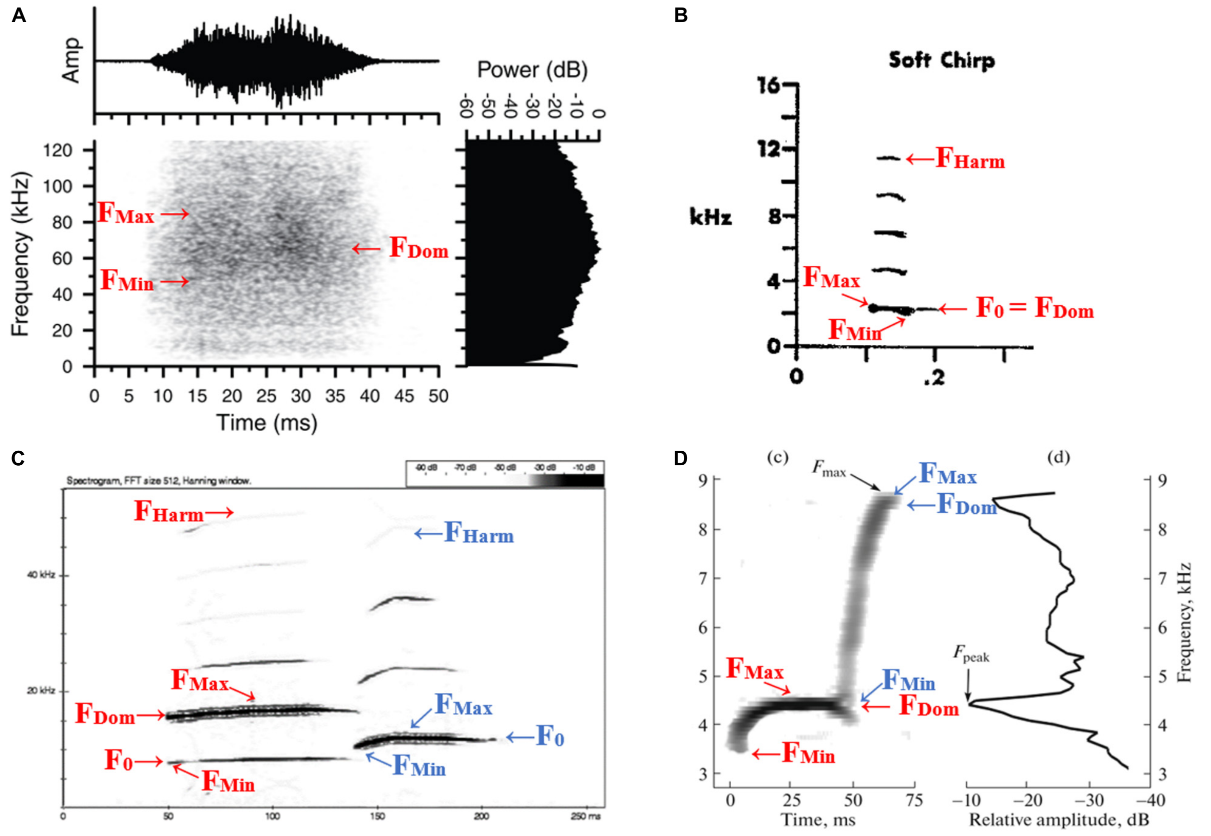

FIGURE 1 | Examples highlighting the variation of how Sciuridae calls are presented as well as how frequency characteristics were measured on different spectrographs and call types. Freqeuncy characteristics measured for all call types: $\mathbf{F_0}$ (fundamental frequency: the mean frequency of the primary vibrational frequency of the vocal membrane; when the dominant frequency occurs on the fundamental harmonic, $F_0 = (F_{Max} + F_{Min})/2$), $\mathbf{F_{Dom}}$ (dominant frequency: the frequency with the greatest energy, power or amplitude), $\mathbf{F_{Min}}$ (minimum frequency: the minimum frequency of the fundamental frequency), $\mathbf{F_{Max}}$ [maximum frequency: the maximum frequency of the fundamental frequency (or of harmonic on which $\mathbf{F_{Dom}}$ is measured)] and $\mathbf{F_{Harm}}$ (frequency of highest visible harmonic: mean frequency of the highest complete harmonic visible on the spectrograph). **(A)** Broadband noise produced by a female southern flying squirrel (*Glaucomys volans*; Murrant et al., 2013). **(B)** Alarm call ("shrill chirp") recorded in a colony of Columbian ground squirrels (*Urocitellus columbianus*; Betts, 1976). **(C)** Alarm call (two call elements identified by the initial researchers and therefore recorded separately, red = element 1, blue = element 2) recorded in a population of European ground squirrels in the Ponor Mountains (*Spermophilus citellus*; Koshev and Pandourski, 2008). **(D)** Alarm call (also with two distinct elements identified by red and blue) emitted by a female little ground squirrel (*Spermophilus pygmaeus*; Nikol'skii, 2019). Attribution details for previously published figures available in **Supplementary Table 3**.

are produced in response to human observers, and they are most obvious to humans given our own sensory bias (Masterton et al., 1969). Therefore alarm calls are likely the easiest to record. Alarm calls function to notify conspecifics of the presence of a predator and usually prompt a behavioral response from the receivers. Blumstein (1999b) spent over a decade looking at the role of alarm calls in marmots (*Marmota* spp.), noting that several species can communicate the threat level of a predator by modifying the speed or repetitive pattern of the alarm calls (Blumstein and Armitage, 1997a; Blumstein, 1999a). Additionally, many authors have reported that squirrels will produce different alarm calls (distinct in structure and frequency) to identify unique predator types and to elicit a specific behavioral response in the receivers (Rendall et al., 2009). For example, Richardson's ground squirrels (*Urocitellus richardsonii*) produce a short chirp (lasting only 75 ms and rapidly decreasing in frequency from 8 to 3.5 kHz) to warn of aerial predators and evoke an immediate retreat response in conspecifics. In contract, a terrestrial predator is identified by a long whistle (lasting around 400 ms with a constant frequency of 10 kHz) which provokes conspecifics to become for alert stand erect (Davis, 1984). Additionally, these squirrels can add a chuck syllable (only 22.5 ms long and decreasing in frequency from about 6 to 2 kHz) to the end of either the chirp or whistle when predators are nearby, eliciting increased vigilance from receivers after their initial behavioral response (Sloan et al., 2005) and like marmots, Richardson's ground squirrels will increase the repetition of their calls as the threat level increases (Warkentin et al., 2001). When predator-specific alarm calls are used in a species, the caller most often only distinguishes between terrestrial and aerial predators [*Callosciurus* (Tamura and Yong, 1993; Tamura, 1995), *Cynomys* (Ackers and Slobodchikoff, 1999; Loughry et al., 2019), *Marmota* (Taulman, 1977; Davis, 1991; Blumstein and Arnold, 1995; Blumstein, 1999a), *Otospermophilus* (Owings and Virginia, 1978;

Owings and Leger, 1980), and *Urocitellus* (Balph and Balph, 1966; Melchior, 1971)], though some Asian tree squirrels have a third call which elicits snake mobbing behavior in conspecifics (Tamura and Yong, 1993).

While alarm calls have been the focus of most studies, squirrels have many distinct call types (**Table 2**) varying in function from mating (male courtship, pre- and postcopulatory calls, and female mating calls), territoriality, facilitating aggressive (threat, combat) or amicable (maternal, group-cohesion) interactions, and communicating internal states (distress). Several species of flying squirrel have also been observed producing calls during their glides and while exploring their environment (*Glaucomys sabrinus* and *G. volans*: Murrant et al., 2013; *Petaurista leucogenys*: Ando and Kuramochi, 2008), suggesting this nocturnal tribe (Pteromyini; Brandt, 1855) may be using echo-based orientation (Siemers et al., 2009). While this hypothesis has been explored once (Chattin, 1969), the recent discovery of ultrasonic vocalizations in North American flying squirrels (Gilley, 2013; Murrant et al., 2013; Eisinger et al., 2016; Gilley et al., 2019) and the vast improvement in ultrasonic recording since the 1960s warrants a re-examination of the hypothesis.

It was not until 2004 that a USV (i.e., a call where F_0 entirely exceeds 20 kHz) was first reported in a squirrel species: the Richardson's ground squirrel (*Urocitellus richardsonii*; Wilson and Hare, 2004). Richardson's ground squirrels are known to both produce predator-specific calls that differentiate between terrestrial and aerial predators (Davis, 1984) and vary the repetition and call structure to communicate perceived risk (Warkentin et al., 2001; Sloan et al., 2005). USVs were found to be another way for this species to convey the perceived predation risk by varying their calls between weaker, ultrasonic vocalizations and louder, audible vocalizations as the threat imposed by the presumptive predator increases (Wilson and Hare, 2006). When a predator is farther away, it is less likely to detect highly directional USVs and therefore prey individuals can call altruistically while remaining inconspicuous. As the predator becomes more of a threat, the colony benefits from louder, more omnidirectional alarm calls to ensure that the maximum number of receivers are notified. In addition to varying USV use between perceived threats, it was found that there is a significant increase in the frequency of ultrasonic calls when the emerging juveniles reach their highest density, implying that ultrasonic communication may be most beneficial for social cohesion (Wilson and Hare, 2006). It is key to note that the authors did not use a secondary microphone in the audible range (<20 kHz) to confirm that the calls were not biphonic (two unrelated fundamental frequencies are produced at the same time; Matrosova et al., 2012) with one fundamental in the audible range and a second in the ultrasonic range. However, the authors published a follow-up report in which they attempted to quantity biophonic versus ultrasonic calls in the same population by noting ultrasonic call behaviors (thoracic cavity expanding and mouth held in communicative position, sometimes a very faint whistle can be heard by the observer) while recording with an audible microphone and they were able to differentiate between ultrasonic and biphonic calls (termed "mixed calls" by the authors; Wilson and Hare, 2006).

Wilson and Hare's (2004) publication challenges the squirrel vocal literature because most of the published repertoires have used microphones that are not sensitive to the ultrasonic range (85% of studies reviewed in our report did not use equipment that encompassed ultrasonic frequencies). By constraining the microphones to ranges that maximize human voice recording, the published repertoires have been unintentionally biased by methodology. While the detection of ultrasonic calls can be extremely rare even when the correct equipment is being used (as in some *Spermophilus*; Matrosova et al., 2012), since 2004, three new Sciuridae species have been observed to produce USVs. USVs have now been reported in northern (*Glaucomys sabrinus*: Murrant et al., 2013) and southern flying squirrels (G. volans: Murrant et al., 2013; Eisinger et al., 2016) and noted, though rarely, in the speckled ground squirrel (*Spermophilus suslicus*: Matrosova et al., 2012). Calls where F_0 partially extend into the ultrasonic range have been also been noted in both the southern (G. volans: Gilley, 2013; Murrant et al., 2013; Eisinger et al., 2016; Gilley et al., 2019) and northern flying squirrels (*G. sabrinus*: Gilley, 2013; Gilley et al., 2019) as well as the Carolina flying squirrel (*G. sabrinus coloratus*: Gilley, 2013).

Despite the underrepresentation of USVs in published squirrel vocal repertoires owing to technological limitations and difficulties associated with recording free-ranging individuals, squirrels are a useful taxonomic group to preliminarily evaluate factors controlling the frequency limits of mammalian vocal signals. Squirrels have been well sampled across the taxa and represent a diverse range of social structure, habitat use, and behavior. We used phylogenetic methods to investigate the following five popular and novel hypotheses relating to different frequency characteristics of the vocalizing members of Sciuridae (**Table 1**). (1) The role of body size, which has been shown to drive frequencies higher as body size decreases, will be explored (Arch and Narins, 2008; Martin et al., 2016). However, it has already been noted in Sciuridae that body size differences attributed to age class (pup versus adult) do not result in within-species differences in vocal characteristics (Matrosova et al., 2007; Swan and Hare, 2008), therefore this relationship is only expected to be significant when making cross-species comparisons. (2) To investigate the potential role of predator avoidance (prey species will favor acoustic ranges that exceed the detection of common predators; Arch and Narins, 2008), we used sociality as a proxy because species that exhibit social complexity are more likely to exhibit increased vocal complexity (Blumstein and Armitage, 1997b; Blumstein, 2007). Primates that exhibit more complex social systems are sensitive to higher frequencies (Ramsier et al., 2012) and mammals with complex social systems are more likely to produce higher frequency vocalizations associated with submissiveness and appeasement (Charlton and Reby, 2016). Additionally, Wilson and Hare (2006) suggest that squirrel social systems in which kin are more clustered, either spatially or temporally from juvenile emergence, are more likely to favor USV production which is less likely to be detected at a distance by an eavesdropping predator. Therefore, we predicted that more socially complex squirrels will vocalize at higher

TABLE 2 | Summary of call functions used by all reported vocalizing squirrel genera of Sciuridae (n = 73).

Genus	Alarm	Territorial	Distress	Threat	Friendly	Mating	Movement
Callosciurus	X*	–	–	–	–	X	–
Callospermophilus	X	–	–	–	–	–	–
Cynomys	X*	–	X	X	X	X	–
Glaucomys	–	–	–	–	–	–	X
Ictidomys	X	–	X	X	X	–	–
Marmota	X*	–	X	X	X	X	–
Otospermophilus	X*	–	X	X	–	–	–
Petaurista	X	X	–	X	–	X	X
Sciurus	X	X	X	X	–	X	–
Spermophilus	X	–	X	X	–	X	–
Tamias	X	X	X	X	–	X	–
Tamiasciurus	X	X	X	–	X	–	–
Urocitellus	X*	–	X	X	X	X	–
Xerospermophilus	X	–	X	–	–	–	–
Xerus	X	–	–	–	–	–	–

**Indicates predator-specific vocalizations.*

frequencies. (3) We explored the acoustic-adaptation hypothesis (terrestrial animals will modify call structure depending on their environment; Blumstein, 2007; Ey and Fischer, 2009) by comparing the frequency limits of squirrels in open versus closed habitats, with open habitats expected to promote the use of higher frequencies. (4) The role of diel activity patterns is interesting as the only nocturnal squirrels are the flying squirrels which might be using vocalizations for echolocation as echo-based orientation (or "echonavigation") is associated with reduced visual cues in other rodents (Ancillotto et al., 2014; Panyutina et al., 2017). Therefore, we predicted that nocturnal species would have higher frequencies than diurnal species. (5) Finally, the relevance of the equipment used will be analyzed by incorporating the method detection limits (defined as the limits of either the microphone or spectrographic analysis, hereinafter "method limits") of the publication methods into the models defined below. We predicted that method limits, particularly the upper-frequency limit, will constrain the perceived vocal ranges of recorded species.

REVIEWING FREQUENCY CHARACTERISTICS

We developed a database beginning with a list of publications that described the vocalizations of squirrels (summarized in **Supplementary Table 1**). The minimum requirement for each publication was the description of at least one call with either a spectrographic analysis or numerical data, though the majority of publications described multiple calls per species or described multiple species per publication (493 calls from 72 species represented in 89 publications; summarized in **Supplementary Table 1**). The databases used to search for these publications were Google Scholar, JSTOR, Web of Science, and Wiley Online Library. We used the keywords acoustics, acoustic repertoire, calls, frequency, Hz, vocalizations, and ultrasound paired with Sciuridae, squirrel, or an exhaustive list of currently valid and invalid genera (the most updated nomenclature was taken from the Integrated Taxonomic Information System[1]). For each call described in the selected publication, the following characteristics were taken: the fundamental frequency (F_0: the mean frequency of the primary vibrational frequency of the vocal membrane; kHz), dominant frequency (F_{Dom}: the frequency with the greatest energy, power or amplitude; kHz), minimum frequency (F_{Min}: the minimum frequency of the fundamental frequency; kHz), maximum frequency (F_{Max}: the maximum frequency of the fundamental frequency or of harmonic on which F_{Dom} is measured; kHz), and the highest visible harmonic (F_{Harm}: mean frequency of the highest complete harmonic visible on the spectrograph; kHz) (**Figure 1**).

Once our review of vocalization publications was complete, we searched for the body mass (g), diel activity pattern (diurnal or nocturnal), social complexity, and habitat openness of the dominant habitat (open or closed) of each species from the relevant vocalization papers. If not provided, other resources including Mammalian Species accounts, PanTHERIA (Jones et al., 2009), and the Animal Diversity Web (Myers et al., 2020) were reviewed. Both male and female body masses were initially recorded, but male body size could not be found for *Spermophilus taurensis*. Male and female body mass were strongly correlated (r = 0.98, p < 0.001), therefore female body mass was chosen to represent squirrel body size. Because we could only assign an adult female body mass to all species, calls that are exclusively produced by males or pups were removed from the dataset before analysis. We pooled all other calls (calls produced by both sexes or females only as well as calls produced by juveniles and adults) as there is little evidence to suggest that juveniles and adults produce acoustically distinct calls across the family (Matrosova et al., 2007, 2011; Volodina et al., 2010; Schneiderová, 2012; but

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[1] http://www.itis.gov/

see: Nikol'skii, 2007). While the initial database included a five-tiered social classification ranging from solitary to colonial [based on the social grades of ground squirrels described by Matějů et al. (2016)], social classes were reduced to social or solitary living to reduce model parameters. Species that exhibit dynamic social structures, such as flying squirrels that engage in social nesting to a greater extent during one portion of the year (Garroway et al., 2013), were treated as socially living. Two subspecies (*Marmota baibacina centralis* and *Tamias dorsalis dorsalis*) could not be used in the subsequent analyses because ecological data and body mass-specific to each subspecies could not be found; similarly, the species *Spermophilus pallidicauda* could not be included as body mass for either sex could not be found.

Phylogeny

VertLife, an online resource that allows the user to extract pruned trees from vertebrate supertrees, was used to produce 100 pruned trees from the Mammalian supertree (**Figure 2**; Upham et al., 2019). Three subspecies had to be incorporated under their parent species, so branch tips were broken in two and subspecies were treated as equivalent to parent species, with branch lengths identical between the parent and subspecies (the addition of a subspecies did not create any polytomies in the tree). Three species are represented by subspecies only: *Sciurus aberti kaibensis, Sciurus niger rufiventer*, and *Callosciurus erythraeus thaiwanensis*.

Statistical Analysis

Phylogenetic generalized least square (PGLS) modeling was used to account for the variation in acoustic repertoire that may be explained by phylogenetic relatedness. PGLS models produce a lambda parameter, λ, that represents the degree to which the variance of traits is explained by the phylogenetic relationships in the model. The λ parameter varies between 0 and 1, with 0 representing no phylogenetic trace and 1 representing absolute Brownian motion (Freckleton et al., 2002; Martin et al., 2016).

PGLS modeling restricts each species to a single observation (i.e., no subsampling of species permitted). Therefore, the numerous data entries per species had to be reduced. For the fundamental, dominant, maximum, and highest harmonic frequencies, the absolute maximum value for each characteristic reported among all publications was chosen. Likewise, for minimum frequency, the absolute minimum reported frequency was chosen. We use maximum and minimum values rather than the median for a more rigorous test of our hypothesis about method limits.

Body mass and all frequency characteristics were log-transformed to achieve normal distributions. Additive models were built for each frequency type [β_0 + body mass (β_{Mass}) + diel activity pattern (β_{Diel}) + sociality ($\beta_{\mathrm{Sociality}}$) + habitat openness ($\beta_{\mathrm{Open}}$) + method limits ($\beta_{\mathrm{Lim}}$)] using the caper package in R (ver 3.6.2). We reported the test statistics of the regression to evaluate significance and effect size (*F*-statistic, *p*-value, and adjusted R^2).

EVALUATING CORRELATES OF FREQUENCY CHARACTERISTICS

The effect of body mass and method limits were significant for all five frequency characteristics (F_0, F_{Dom}, F_{Min}, F_{Max}, and F_{Harm}) whereas habitat openness only had a significant effect for F_{Dom} and F_{Harm} (**Table 3**). The PGLS models fit the data well for most frequency characteristics, with effect sizes (adjusted R^2) of 0.64 ($p < 0.001$), 0.57, ($p < 0.001$), 0.61 ($p < 0.001$), and 0.41 ($p < 0.001$) for F_0, F_{Dom}, F_{Max}, and F_{Harm} accordingly. The minimum frequency PGLS model did not fit the data well, yielding an effect size of only 0.12 ($p < 0.05$), however, it was the only model that yielded a significant phylogenetic signal (Pagel's λ with 95% confidence intervals) of 0.65 [0, 0.88]. In contrast, the phylogenetic signal was estimated to be 0 for F_0 (0 [0, 0.96]), F_{Dom} (0 [0, 0.32]), F_{Max} (0 [0, 0.95]), and F_{Harm} (0 [0, 0.43]). A summary of model parameters for secondary candidate models (ΔAICc < 2; Mazerolle, 2004) selected through stepwise regression can be found in the **Supplementary Material** (**Supplementary Table 2**). Notably, harmonic frequency values could only be found for 43 of the 73 species, with species reported in only one publication being significantly less likely to have reported harmonics (Chi-square = 17.1, $p < 0.001$) than those appearing in multiple publications.

Body Mass

Body mass (**Figure 3**) had a significant effect on F_0 (slope = −0.22 (±SE = 0.05; **Table 4**), $F_{1,58} = 23.14$, $p < 0.001$), F_{Dom} (slope = −0.20 (±0.05), $F_{1,60} = 12.37$, $p < 0.001$), F_{Min} (slope = −0.19 (±0.09), $F_{1,58} = 6.24$, $p < 0.05$), F_{Max} (slope = −0.25 (±0.05), $F_{1,59} = 37.61$, $p < 0.001$), and F_{Harm} (slope = −0.29 (±0.08), $F_{1,37} = 9.18$, $p < 0.01$). These results are consistent with our prediction that increased body mass results in the production of lower frequencies as already shown in mammals (Charlton and Reby, 2016) and birds (Ryan and Brenowitz, 1985). The avian mass-frequency relationship focused on the "emphasized" frequency, similar to F_{Dom} reported here (Ryan and Brenowitz, 1985). Our results are somewhat consistent with Martin et al. (2016) who also found that F_{Min} was constrained by mammalian body mass, but the mass-F_{Min} relationship reported was twice as strong as reported here (slope = 0.41). This stronger relationship likely arose because their dataset ranged from microbats (weighing less than 0.1 kg) to whales (larger than 100,000 kg), representing a much larger range of body sizes and therefore showing a stronger effect of body size on frequency types. In comparison, this dataset covered only a range of about 36 g (*Tamias alpinus*) to 8.0 kg (*Marmota sibirica*). In contrast to our results, Martin et al. (2016) found that F_{max} was not constrained by body size, though the lack of relationship was largely attributed to the interaction between environment and body size. They suggest that because sound waves travel differently in the water, aquatic species are freed from the constraints of body size on the maximum frequency compared to their terrestrial relatives. Like Martin et al. (2016), Ryan and Brenowitz (1985) also found that there was an interaction between environment and body size in avian song frequency, but

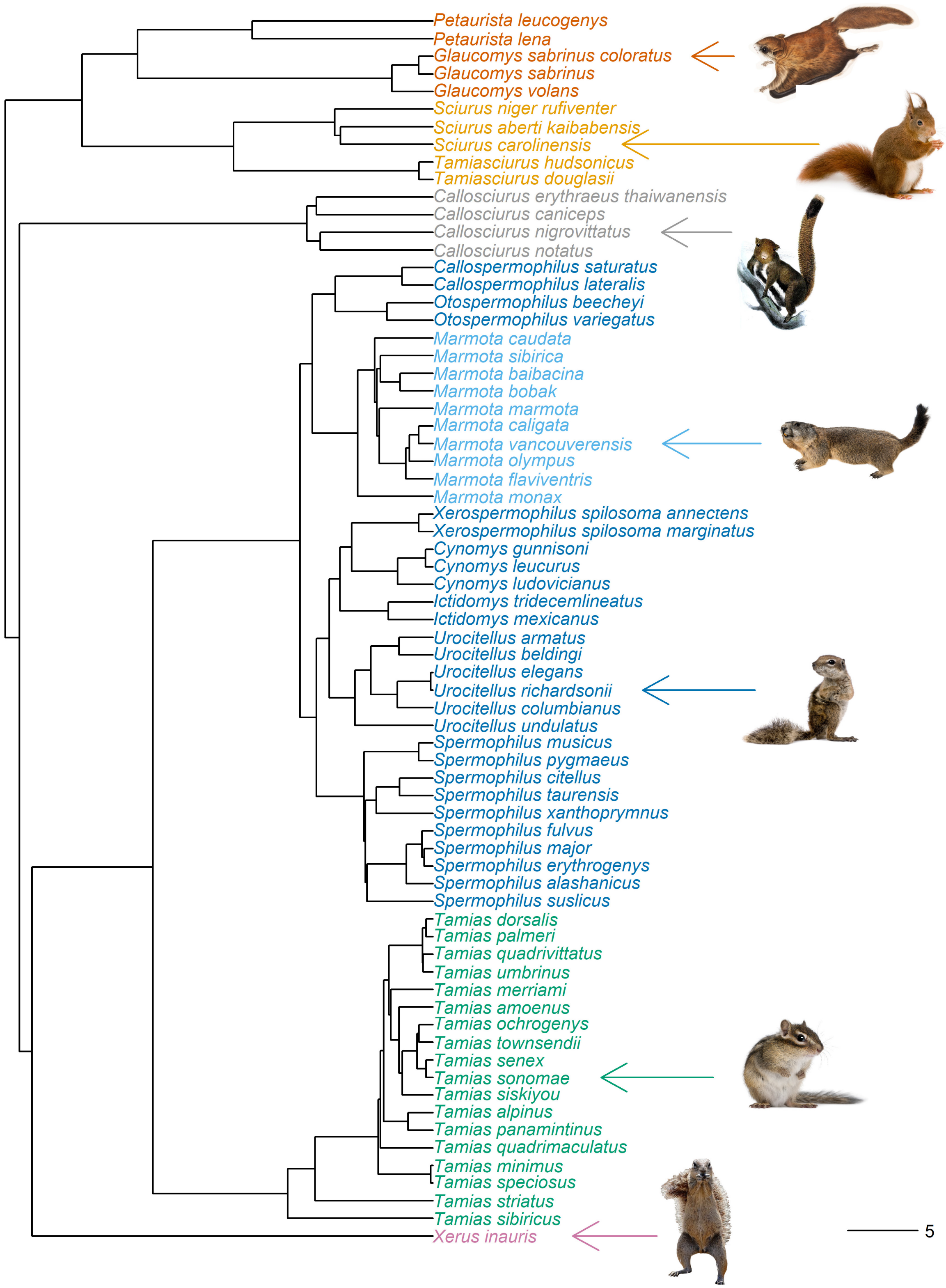

FIGURE 2 | Phylogeny of Sciuridae reduced to all species used in analysis (*n* = 70). Species present represent the subfamily Callosciurinae (gray: southern Asian tree squirrels), Sciurinae (orange: Pteromyini, flying squirrels; yellow: Sciurini, Holarctic tree squirrels tree squirrels) and Xerinae (pink: tribe Xerini, African ground squirrels; Marmotini, Holarctic ground squirrels). Marmotini is subdivided into the genera *Tamias* (green: chipmunks), *Marmota* (light blue: marmots) and the remaining Holarctic ground squirrels (dark blue). Edge lengths to scale; scale bar represents 5 million years from present. Tree downloaded from Vertlife.org. Attribution details for the animal images available in **Supplementary Table 3**.

TABLE 3 | Phylogenetic generalized least square (PGLS) results for the effects of all factors on the frequency characteristics (fundamental, dominant, minimum, maximum, and highest harmonic frequency; kHz) of Sciuridae.

Variable	Fundamental	Dominant	Minimum	Maximum	Highest harmonic
Body mass	**$F_{1,58}$ = 23.14*****	**$F_{1,60}$ = 12.37*****	**$F_{1,58}$ = 6.24***	**$F_{1,59}$ = 37.61*****	**$F_{1,37}$ = 9.18****
Sociality	**$F_{1,58}$ = 9.54****	**$F_{1,60}$ = 8.60****	$F_{1,58}$ = 1.14, p = 0.29	**$F_{1,59}$ = 8.57****	**$F_{1,37}$ = 5.31***
Habitat	$F_{1,58}$ = 1.87, p = 0.18	**$F_{1,60}$ = 4.22***	$F_{1,58}$ = 0.32, p = 0.57	$F_{1,59}$ = 0.22, p = 0.64	**$F_{1,37}$ = 5.65***
Diel activity pattern	**$F_{1,58}$ = 51.22*****	**$F_{1,60}$ = 40.84*****	$F_{1,58}$ = 1.63, p = 0.21	**$F_{1,59}$ = 33.10*****	$F_{1,37}$ = 2.96, p = 0.09
Method limits	**$F_{1,58}$ = 37.91*****	**$F_{1,60}$ = 23.78*****	**$F_{1,58}$ = 4.63***	**$F_{1,59}$ = 25.16*****	**$F_{1,37}$ = 11.01****

*Significant effects are given in bold. *p < 0.05; **p < 0.01; ***p < 0.0001.*

they compared habitat openness are we did here. Interestingly, adding the interaction between habitat openness and body mass did not change the model outcome (AICc values or variable effects) for most frequency characteristics, but it did yield a significantly better model for F_{Harm} (ΔAICc = −9.78). The effect size of the model increased (adjusted R^2 = 0.55, $p < 0.001$), the effect of habitat openness increased [slope = 3.70 (±0.91), $F_{1,36}$ = 6.80, $p < 0.05$] and the interaction between habitat openness and body mass was significant [slope = −0.55 (±0.16), $F_{1,36}$ = 12.51, $p < 0.01$]. However, the slope estimate of body mass was reduced [slope = −0.06 (±0.12), $F_{1,36}$ = 12.04, $p < 0.01$] and the effect of sociality became non-significant [slope = −0.10 (±0.19), $F_{1,36}$ = 1.67, p = 0.21]. By observing an interaction plot, we determined that as body size increased in the closed habitat, there was little change in F_{Harm}, whereas there was a large decrease in F_{Harm} with increasing body size in the open habitat.

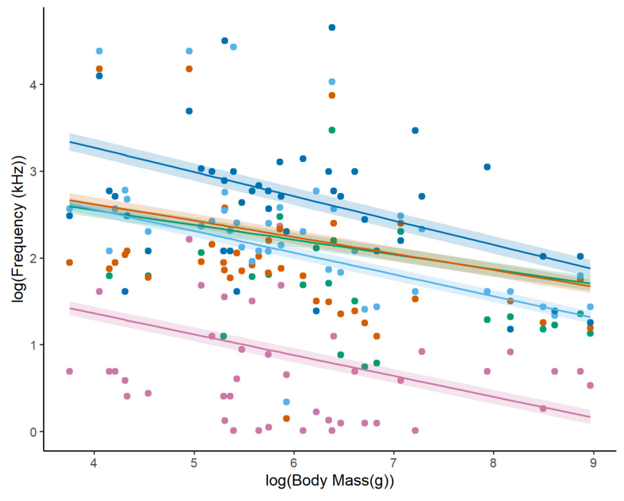

FIGURE 3 | Absolute minimum (pink), fundamental (green), dominant (orange), maximum (light blue), and highest harmonic (dark blue) frequencies of squirrel (Sciuridae, n = 42) vocalizations as a function of body mass (all variables log-transformed for normality; SE of slopes represented as shaded ribbons). Phylogenetic generalized least square (PGLS) models were used to describe the relationships between the variables and are represented by the following linear trend line: minimum [pink, $\log(Y) = -0.24*\log(X) + 2.3$], fundamental [green, $\log(Y) = -0.17*\log(X) + 3.2$], dominant [orange, $\log(Y) = -0.19*\log(X) + 3.4$], maximum [light blue, $\log(Y) = -0.18*\log(X) + 3.6$], and highest harmonic [dark blue, $\log(Y) = -0.28*\log(X) + 4.4$].

Sociality

Sociality [social (0) or solitary (1)] had a significant effect on F_0 [slope = −0.007 (±0.13), $F_{1,58}$ = 9.54, $p < 0.01$], F_{Dom} [slope = −0.04 (±0.14), $F_{1,60}$ = 8.60, $p < 0.01$], and F_{Max} [slope = −0.07 (±0.13), $F_{1,59}$ = 8.57, $p < 0.01$]. Notably, sociality had a significant effect on these models while yielding a non-significant slope; investigating the models further, there was no evidence of collinearity between sociality and other variables. However, there was a larger variation in frequencies used by social squirrels compared to solitary squirrels. These results are consistent with Blumstein and Armitage (1997b) who found that social complexity was related to increased use of alarm calls in the repertoire in marmots. In our results, we found that alarm calls are widespread across the taxa, appearing in 64 of the 73 vocalizing Sciuridae species. While social squirrels might not be more likely to have alarm calls in their vocal repertoire than solitary squirrels (Chi-Square = 3.51, p = 0.06), they likely maintain shorter distances between the sender and receiver and increase their production of alarm calls (e.g., *Urocitellus beldingi*; Sherman, 1985). This reduced distance could facilitate the incorporation of higher frequencies into the repertoire as these frequencies are highly directional but are more likely to be attenuated over longer distances (Smith, 1979). As well, the increased proximity not only results in more altruistic alarm calling (Sherman, 1985) but also the production of more vocalizations to facilitate group cohesion and maintain social hierarchies, particularly between emerging juveniles and established adults. For example, calls in adult female yellow-bellied marmots (*Marmota flaviventris*) are directly correlated to the emergence of juveniles (Blumstein et al., 1997). Primates, another group of highly communicative and socially variable mammals, also trend toward increased vocal complexity and overall improved hearing sensitivities in more social species and larger groups (Ramsier et al., 2012). To compensate for increased exposure via sound, social animals benefit from communicating in ranges less readily heard by predatory species (Arch and Narins, 2008). In contrast, solitary squirrels would benefit from louder calls if they are warning surrounding conspecifics of potential danger, especially if the species holds relatively large home ranges. However, the maintenance of these territories would likely lead to vocal production that maximizes the effectiveness of territorial displays (which we only found reports of in solitary and socially alternating species), meaning that lower frequencies would be more beneficial to maximize amplitude over longer distances and to increase omnidirectionality. In addition

TABLE 4 | Summary of slope (±SE) and phylogenetic signal (λ [95% CI]) estimates for all variables taken from phylogenetic generalized least square (PGLS) models of the frequency characteristics (fundamental, dominant, minimum, maximum, and highest harmonic frequency; kHz) of Sciuridae.

Variable	Fundamental	Dominant	Minimum	Maximum	Highest harmonic
Intercept	−1.33 (±0.87), *p* = 0.13	−0.88 (±0.91), *p* = 0.34	**1.46 (±0.65)***	−0.19 (±0.90), *p* = 0.84	−1.50 (±1.87), *p* = 0.43
Body mass	**-0.22 (±0.05)*****	**-0.20 (±0.05)*****	−0.19 (±0.09), *p* = 0.05	**-0.25 (±0.05)*****	**-0.29 (±0.08)****
Sociality	−0.01 (±0.13), *p* = 0.96	−0.04 (±0.14), *p* = 0.75	0.19 (±0.17), *p* = 0.26	−0.07 (± 0.13), *p* = 0.61	−0.06 (±0.22), *p* = 0.78
Habitat	0.18 (±0.13), *p* = 0.17	**0.31 (±0.14)***	−0.16 (±0.29), *p* = 0.59	0.07 (±0.13), *p* = 0.61	**0.59 (±0.25)***
Diel activity pattern	**0.79 (±0.22)*****	**0.96 (±0.24)*****	0.43 (±0.56), *p* = 0.45	**0.61 (±0.23)***	0.31 (±0.38), *p* = 0.43
Method limits	**0.45 (±0.08)*****	**0.39 (±0.08)*****	**0.12 (±0.05)***	**0.40 (±0.08)*****	**0.51 (±0.15)****
λ	0 [0, 0.96]	0 [0, 0.32]	0.65 [0, 0.88]	0 [0, 0.95]	0 [0, 0.43]

*Significant estimates are given in bold. *p < 0.05; **p < 0.01; ***p < 0.0001.*

to social structure, mating systems may be complicating the results observed. We grouped monogamous and polygynous species. However, Charlton and Reby (2016) suggests that F_0 is a sexually selected trait and is higher in polygynous species and lower in monogamous species. While we excluded calls produced exclusively by males, we kept calls produced by both sexes, therefore not eliminating calls produced by both sexes that are also under sexual selection. We suggest further research into the role of sociality and mating systems in vocal complexity across Sciuridae.

Habitat

The habitat type [open (1) or closed (0)] only had a significant effect on F_{Dom} [slope = 0.31 (±0.14), $F_{1,60} = 4.22$, $p < 0.05$] and F_{Harm} [slope = 0.59 (±0.25), $F_{1,37} = 5.65$, $p < 0.05$], with open habitats facilitating higher dominant and higher maximum harmonic frequencies than closed habitats. We expected that the F_{Max} would be most affected by habitat type because higher frequencies are attenuated more rapidly in closed environments (Smith, 1979) resulting in a shift of vocal range (minimum to maximum frequency). Instead, we found that squirrels in open habitats spend their maximum acoustic energy (F_{Dom}) on higher frequencies than squirrels in closed habitats, with little effect on range, as F_{Min} and F_{Max} were not significantly influenced by habitat openness (**Tables 3**, **4**). Charlton et al. (2019) have shown that forested animals may not be hindered by their ability to produce or detect high frequencies as many have been shown to co-evolve improved acoustic structures to not lose their access to these frequencies in a closed environment. They suggest that forested animals may boost the amplitude and increase the frequency band of their calls to help counteract the increased attenuation of their environment and improve the ability of receivers to locate the sender (Charlton et al., 2019). This hypothesis would explain why squirrels in closed habitats continue to use high frequencies, despite the limitations. Based on this hypothesis, we would also predict F_{Harm} would not be affected by habitat openness as increased harmonic strength would improve the bandwidth of calls in closed environments and high harmonics would be unhindered in open habitats, making them favorable in both conditions. We found instead that higher harmonics are detected in open habitats, supporting the more traditional acoustic adaptation hypothesis (Blumstein, 2007; Ey and Fischer, 2009). However, we think it is important to note that the detection of the highest harmonic is largely dependent on the recording techniques of the researcher and how the calls have been displayed on spectrographs. It may likely be that high harmonics are easier to record in open environments, whether because the acoustics are better for recording (reduced attenuation of harmonics before reaching the microphone as in bats; Fenton et al., 2011) or the individual squirrel ecology lends to better study subjects (i.e., congregated ground squirrels allow microphones to be placed close to predictable burrow holes compared to free-ranging tree squirrels which have many temporary nests and retreats across a forest canopy). While not explored in this study, environments with greater humidity transmit higher frequencies better than arid environments where calls are attenuated faster (Nikol'skii, 1984). The importance of habitat ariditity was demonstrated in ground squirrels (*Spermophilus*; Nikol'skii, 2017) when the strength of the relationship between the F_{Dom} and body mass was significantly improved when habitat aridity was incorporated as a factor into the models. Overall, our findings suggest that higher dominant frequencies are more easily incorporated and recorded in squirrels in open habitats, but high frequencies are not necessarily unused or completely inhibited by closed habitats.

Diel Activity Pattern

As predicted, nocturnality resulted in significantly higher values for F_0 [slope = 0.79 (±0.22), $F_{1,58} = 51.22$, $p < 0.001$], F_{Dom} [slope = 0.96 (±0.24), $F_{1,60} = 40.84$, $p < 0.001$], and F_{Max} [slope = 0.61 (±0.23), $F_{1,59} = 33.10$, $p < 0.001$]. Though the frequency traits between diel activity patterns was not significant for F_{Min} [slope = 0.43 (±0.56), $F_{1,58} = 1.63$, $p = 0.21$] and F_{Harm} [slope = 2.96 (±), $F_{1,37} = 2.96$, $p = 0.09$], all frequency characteristics trend toward nocturnal species using higher frequencies than diurnal species (**Figure 4**). Unfortunately, the lack of behavioral observations associated with the published flying squirrel repertoires, the only nocturnal squirrels, means that interpretation of these results is mostly hypothetical (as nocturnal patterning is only represented by 5 different (sub)species). Both species of Asian giant flying squirrel (*Petaurista lena* and *P. leucogenys*) stand out as the only squirrels with movement-associated vocalizations that were recorded while observing free-ranging individuals (Ando and Kuramochi, 2008; Shen, 2013). Additionally, one call associated with the exploration of a novel environment was reported in

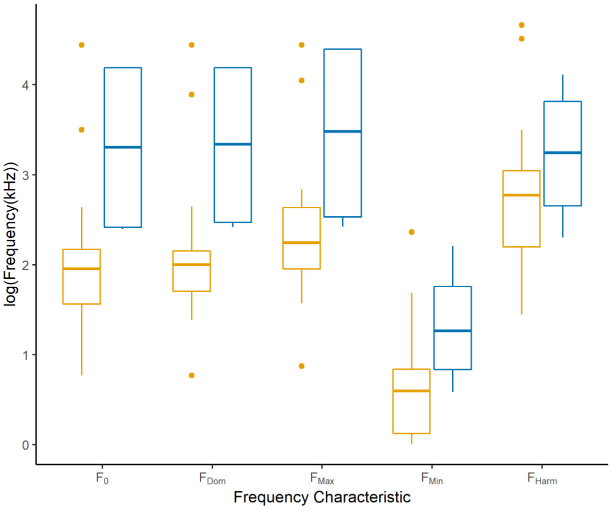

FIGURE 4 | The frequency characteristics of calls produced by diurnal (orange) versus nocturnal (blue) squirrels (species of the family Sciuridae; $n = 43$). Frequency characteristics presented are the fundmanetal (F_0), dominant (F_{Dom}), maximum (F_{Max}), minimum (F_{Min}), and maximum harmonic (F_{Harm}) frequencies and all are log-transformed for normality.

the southern flying squirrel (*Glaucomys volans*; Murrant et al., 2013). While there has yet to be any direct evidence of echo-based navigation (Siemers et al., 2009) or echolocation in flying squirrels (Chattin, 1969), we propose that the strong effect of nocturnality on multiple frequency characteristics, correlated to calls in higher frequencies, as well as the recent discovery of USVs in North American flying squirrels (Gilley, 2013; Murrant et al., 2013), warrants a re-examination of the function of USVs in these squirrels with specific echo-based navigation experiments included in the research.

Method Limits

As we predicted, the limits of the methods (microphone or analysis ranges) did significantly influence all the frequency characteristics as shown in the PGLS models. As equipment limits increased, so did the F_0 [slope = 0.45 (±0.08), $F_{1,58} = 37.91$, $p < 0.001$], F_{Dom} [slope = 0.39 (±0.08), $F_{1,60} = 23.78$, $p < 0.001$], F_{Max} [slope = 0.40 (±0.08), $F_{1,59} = 25.16$, $p < 0.001$], and F_{Harm} [slope = 0.51 (±0.15), $F_{1,37} = 11.01$, $p < 0.01$]; similarly, lower method limits were related to a lower F_{Min}, though this relationship was not as strong [slope = 0.12 (±0.05), $F_{1,37} = 4.63$, $p < 0.05$] Additionally, the AICc of all secondary PGLS models were improved by about 10% when method limits were included (note that all secondary candidate models include β_{Lim}; **Supplementary Table 2**). We conclude that the type of equipment being used is crucial for detecting all frequency characteristics and that using equipment with large bandwidths (the entire range of frequencies that could be detected) is important for all squirrel acoustics research as we continue to discover novel USV production in Sciuridae.

Summary

We predicted that body mass, sociality, habitat openness, diel activity pattern, and method limits would influence the

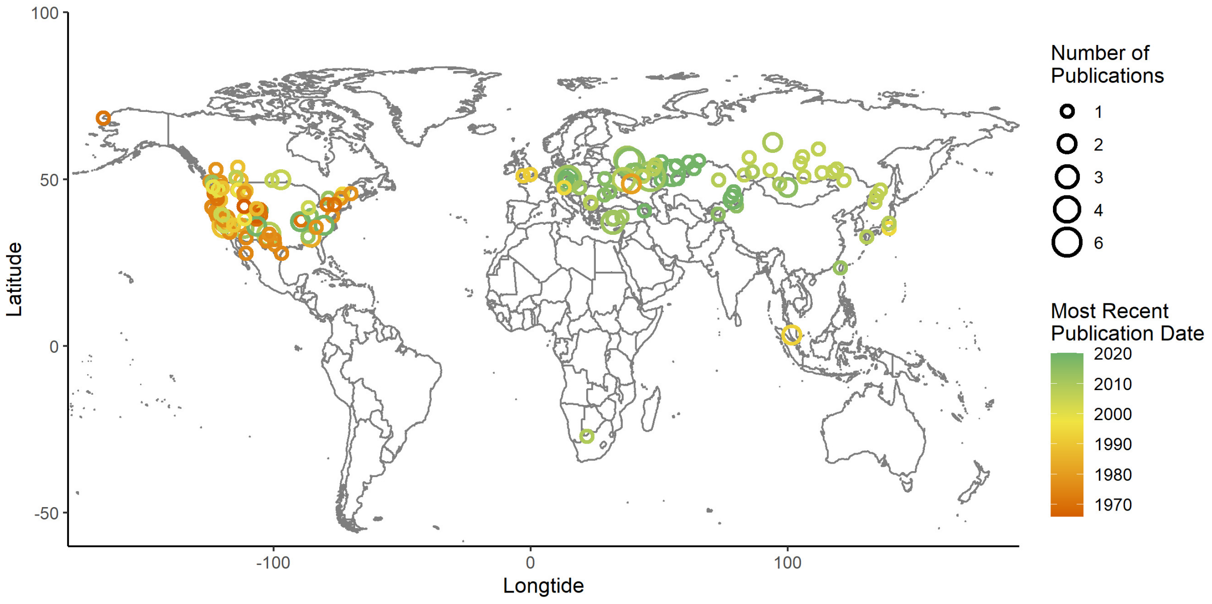

FIGURE 5 | The recording locations of squirrel vocal repertoires published between 1966 and 2020. The year of publication is represented along a color gradient and the number of studies recorded at each location is represented along a size gradient. The epicenter of each circle represents the location; circles are translucent to minimize overlap. The United States alone has hosted 52% of all published studies; Russia, Canada, and the Czech Republic are also notable contributors representing 18, 11, and 9% each. North America, Europe, Asia, and Africa represent 61, 30, 13, and 1% respectively, with no record of South America, India, or the majority of Africa hosting a repertoire study.

fundamental, dominant, minimum, maximum, and highest harmonic frequencies (kHz; **Table 1**). We found at least some support for all of these hypotheses. We found that across the squirrel family, body mass significantly affected all frequency characteristics, a finding that is mostly consistent with other literature (Arch and Narins, 2008; Charlton and Reby, 2016; Martin et al., 2016). Social squirrels used higher fundamental, dominant, and maximum frequencies which aligns with the predator-avoidance hypothesis (Arch and Narins, 2008). Social squirrels also used a larger range of frequencies which can be partially attributed to increased communication amongst kin (Blumstein and Armitage, 1997b) or sexual selection acting on vocal traits (Charlton and Reby, 2016). As predicted by the acoustic adaptation hypothesis (Blumstein, 2007; Ey and Fischer, 2009), we found that species inhabiting open habitats used higher dominant and harmonic frequencies than those in closed habitats where high frequencies are more rapidly attenuated by the environment (Smith, 1979); however, the maximum frequency was not affected by habitat type which suggests that squirrels in closed habitats may have co-evolved structures to still maintain access to USVs despite the environmental complications (Charlton et al., 2019). Nocturnality was associated with higher frequencies for the fundamental, dominant, and maximum frequencies, suggesting that nocturnal, gliding squirrels are under unique selection pressures for using USVs. An explanation for this general pattern of higher frequencies in nocturnal squirrels remains elusive. Finally, the significant effect of method limits on all frequency characteristics and the presence of method limits in all candidate models (**Supplementary Table 2**) suggest that method limits are important for framing the ranges detected during recording sessions.

THINK BEFORE THEY SQUEAK – CONCLUSION

Squirrels are vocally complex taxa, displaying a wide range of call frequencies and functions. While there has been a representative sampling across the genera, there are large gaps in the geographic sampling of squirrels (**Figure 5**). The United States alone accounts for over 50% of the published vocal repertoires. In contrast, only a single study has occurred in Africa and no repertoires have been published from Central or South America. In the face of global extinction crises and habitat loss, it is more important than ever to archive the traits of rare and cryptic species so that we can maximize the utility of all available monitoring and conservation tools. Bioacoustic monitoring is an effective tool for cryptic species (Walker, 1964; Gilbert et al., 1994; Tripp and Otter, 2006) and has been shown to improve the monitoring of flying squirrels which are notoriously difficult to detect (Diggins et al., 2016). Considering these analyses, we encourage researches to record squirrel vocalizations in under-sampled locations. We further suggest the use of recording equipment that can resolve frequencies that extend into the ultrasonic range so that repertoires accurately reflect the ranges used by the species being studied. Additionally, we caution that researchers studying the vocalizations of previously reported squirrels should consider the accuracy of previous literature, especially when there is possible evidence of partial USVs or ultrasonic harmonics present. Squirrels that are socially complex or inhabiting open areas are of interest for studies focused on high-frequency vocalizations; we also strongly encourage the examination of the role of USVs in nocturnal, flying squirrels. We caution that while USV research requires specialized technology, this work also requires ideal recording conditions and patience as USVs can be rare and difficult to detect in some Sciuridae species (Matrosova et al., 2012). In conclusion, while rodent bioacoustic studies are typified by controlled environments and lab animals, studying the vocalizations of free-ranging rodents can reveal new information about the natural world, providing informative findings and new insights that apply to real-time conservation efforts.

DATA AVAILABILITY STATEMENT

The data and R scripts that support the findings of this study are openly available on Dryad at https://doi.org/10.5061/dryad.vt4b8gtpm.

AUTHOR CONTRIBUTIONS

JB and SN conceptualized the manuscript. SN collected data, conducted analyses, and prepared the manuscript with guidance and critical feedback from JB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00193/full#supplementary-material

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Black-Tailed Prairie Dog (*Cynomys ludovicianus*) Reintroduction Can Limit Woody Plant Proliferation in Grasslands

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Tree and shrub proliferation has been widespread in grasslands worldwide, and has altered ecosystem function and wildlife habitat. Several causes have been proposed for the woody plant encroachment phenomenon. The widespread eradication of a native keystone herbivore in North American grasslands, the prairie dog (*Cynomys* spp.), is one potential contributing factor that has received relatively little attention. We hypothesized prairie dogs would have historically suppressed woody plants by creating "browse traps" through their systematic clipping of vegetation. We tested this hypothesis by conducting surveys and experimentally manipulating shrub accessibility via exclosures and artificial saplings on and around recently reestablished black-tailed prairie dog (*Cynomys ludovicianus*) colonies in southeastern Arizona, United States. Shrubs were common on the nascent colonies (mean ± SE = 132 ± 32.7 plants ha^{-1}), but at substantially reduced densities compared to off colonies (305 ± 94.9 plants ha^{-1}). Among branches placed on colonies to simulate "saplings" 89% were damaged within 3 days of "planting," whereas those placed off colonies were virtually untouched. This was true for both a deciduous, N_2-fixing shrub (velvet mesquite, *Prosopis velutina*) and an evergreen non-N_2-fixing shrub (creosote bush, *Larrea tridentata*). Prairie dogs on newly established colonies did not extirpate woody plants over the time-frame of our study, but reduced their abundance and suppressed their growth, which would ostensibly prevent them from achieving dominance. Implications for extending the longevity of widely practiced "brush management" grassland restoration treatments are discussed in the context of perceptions of prairie dogs as rangeland pests. Prairie dogs represent an enigma in keystone conservation. Whereas the reintroduction of large or charismatic keystone species [e.g., sea otters (*Enhydra lutris*)] are conducted to restore critical ecological function, reintroductions of other keystone species, such as gray wolves (*Canis lupus*) and in our case, prairie dogs, are highly controversial. Our findings suggest reintroductions of this negatively perceived small herbivore could function as a tool to locally suppress woody plant proliferation that is widely regarded as an impediment to livestock production. Accordingly, prairie dogs could promote restoration efforts to re-establish and maintain habitat for grassland endemics while promoting biological diversity and other ecosystem services.

Keywords: black-tailed prairie dog, browse trap, ecosystem engineer, exclosure, grassland, keystone species, reintroduction, woody plant encroachment

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INTRODUCTION

Woody plant encroachment, the proliferation of trees and shrubs within grasslands and savannas, is a phenomenon occurring in arid and semiarid grasslands and savannas worldwide. This proliferation may involve increases in the density, stature and cover of native trees and shrubs within their historic ranges or the introduction of non-native woody plants (Archer et al., 2017). Shifts from grass to woody plant dominance affects a variety of ecological processes and the allocation of numerous ecosystem goods, and provisioning (e.g., forage production), supporting (e.g., primary production, biodiversity), and regulating (e.g., carbon sequestration) services (Eldridge et al., 2011; Archer and Predick, 2014). Increases in woody cover also substantially alters wildlife habitat, favoring some species and adversely affecting others (Fulbright et al., 2018). Increases in atmospheric CO_2 and changes in climate and fire regimes have been proposed as causal factors; however, the direct and indirect effects of intensification of livestock grazing over the past century appears to be a primary driver (Archer et al., 2017). Perspectives on shrubs in grasslands vary widely (Archer et al., 2017). Commercial ranching enterprises based on cattle have traditionally viewed shrub proliferation as an impediment to livestock production; pastoralists raising goats, camels or other domesticated browsers see shrubs as a potentially important forage resource and may look favorably on increases in their abundance; conservation biologists have biodiversity concerns that shrub proliferation will threaten grassland and savanna ecosystem types and the plants and animals endemic to them (Archer et al., 2017). These contrasting perspectives pose challenges for managing lands for multiple uses.

Coincident with the intensification of livestock grazing in North America was the widespread eradication of the prairie dog (*Cynomys* spp.), a rodent traditionally viewed as an impediment to livestock production (Vermeire et al., 2004; Derner et al., 2006; Detling, 2006). Although prairie dogs have been recognized as contributing to the maintenance of grassland ecosystems (Hoogland, 1995; Kotliar et al., 1999), the consequences of their removal have received relatively little attention in the context of regulating grass-woody plant interactions. In Africa, herbivores of varying sizes mediate woody plant abundance through browsing activities [e.g., elephants (*Loxodonta* spp.), dik-diks (*Madoqua* spp.), impala (*Aepyceros melampus*); Sankaran et al., 2013]. Systematic browsing maintains woody vegetation at a small stature, thereby creating a "browse trap" that also contributes to a "fire trap" (Bond and Keeley, 2005; Staver et al., 2009; Sankaran et al., 2013). For the purpose of this manuscript, "browse trap" and "fire trap" refer to the maintenance of woody vegetation at small stature by herbivores and frequent, low-intensity wildfires, respectively. Woody plants caught in a fire or browse trap are unable to achieve dominance until the suppressing pressure is removed. We suggest that prairie dogs in North America historically prevented woody plant recruitment or created and maintained browse traps that suppressed shrubs and trees. In the former case, prairie dog eradication would have opened the door for woody plant establishment. In the latter case, prairie dog removal would have released established, but diminutive, woody plants from the browse trap. In both cases, removal of the suppressing pressure, i.e., prairie dogs, would have permitted woody plants to reach adult stature in grasslands throughout the prairie dog's former range.

Prairie dogs, once widely distributed across the North American west (Proctor et al., 2006), were deemed a rangeland pest in competition with livestock, leading to widespread and highly successful eradication efforts beginning in the early 1900s (Andelt, 2006; Forrest and Luchsinger, 2006; Reeve and Vosburgh, 2006). Historic population sizes are hard to pin-point (Vermeire et al., 2004), but at the most conservative estimate, active eradication, habitat loss, and sylvatic plague (*Yersinia pestis*; Luce et al., 2006) appear to have reduced prairie dog populations to 2% of historic numbers (Whicker and Detling, 1988; Miller et al., 1994), and range occupancy to $\leq$1% (Luce et al., 2006). Prairie dogs are grazers, but systematically gnaw, clip, and girdle other vegetation to maintain plants on their colonies at small stature (<30 cm; King, 1955; Hoogland, 1995; Weltzin et al., 1997) and thereby maximize predator detection. Accordingly, their removal would have removed an impediment to woody plant establishment and growth.

Woody plant proliferation in grasslands has led to the implementation of "brush management" practices involving heavy equipment, herbicides, and/or prescribed fire to reduce the cover of trees and shrubs (Hamilton et al., 2004; Collins et al., 2015). Such techniques are typically expensive with short-lived effects, and hence are seldom cost-effective (Archer et al., 2011). We sought to investigate whether the black-tailed prairie dog (*Cynomys ludovicianus*; hereafter "prairie dog") might constitute a natural, potentially long-term, sustainable "biocontrol" solution for deterring woody plant encroachment and proliferation in grasslands. We hypothesized that reintroduced prairie dogs would suppress woody plant growth on their colonies, and create a "browse trap" through their systematic felling of vegetation. If our hypothesis was supported, we predicted that woody plants on newly established prairie dog colonies would be less abundant than those off-colony, that shrubs occurring outside of exclosures on prairie dog colonies would have slower growth and higher mortality rates than protected shrubs, and that disturbance to introduced artificial "simulated saplings" would be greater on colonies than off colonies.

From a broader perspective, our study was also intended to determine whether the reintroduction of a keystone herbivore following a prolonged absence could restore the browse trap that historically excluded woody plants or prevented them from expressing dominance in grassland systems. Studies involving keystone species typically seek to assess ecosystem effects while the species of interest is present or absent from the landscape (e.g., Reisewitz et al., 2006); however, studies assessing the ecosystem-level effects of keystone species reintroductions are more rare (Hale and Koprowski, 2018). A study in northwestern Mexico investigated independent and interactive effects of existing prairie dogs and cattle on woody encroachment in an elegant field experiment (Ponce-Guevara et al., 2016), wherein plots received one of four treatments: prairie dogs only, cattle only, prairie dogs + cattle, and neither prairie dogs nor cattle.

Results indicated that prairie dogs played an important role in suppressing woody encroachment, and that their role was amplified in the presence of cattle. The study we report here similarly assesses the effects of black-tailed prairie dogs on shrubs in an arid grassland, but differs in that we had the unique opportunity to ascertain whether a *reintroduced* population of prairie dogs could influence shrub encroachment. Our study is among the first to investigate consequences of the reintroduction of a keystone species, and the extent to which that reintroduction might be justified as part of an integrated, comprehensive plan to more effectively address ecosystem management challenges in grasslands.

MATERIALS AND METHODS

Study Site

Las Cienegas National Conservation Area (hereafter Las Cienegas) is a 17,000 ha working cattle ranch managed by the Bureau of Land Management, and is located 72 km southeast of Tucson, AZ, United States in Santa Cruz County. The study area is a representative of the natural regional grasslands occurring across central and southern Arizona, southwestern New Mexico, and northern Mexico (Gori and Enquist, 2003; Gori and Schussman, 2005; Bodner and Simms, 2008). Mean annual precipitation is 405 mm, mostly falling during the summer monsoon months (July–September); mean annual temperature is 15.7°C (Bodner and Robles, 2017). During our study (2013–2015) annual precipitation and temperature (±SE) averaged 350 ± 47 mm and 16.2 ± 0.2°C (Empire Remote Area Weather Station; QEMA3)[1]. Grasslands at Las Cienegas are located between 1300 and 1500 m in elevation, and soils are primarily gravely, sandy, and clay loam (Bodner and Robles, 2017).

Semi-desert grasslands in Arizona support a variety of herbivores, including peccaries (*Pecari tajacu*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), pronghorn (*Antilocapra americana*), and historically, supported black-tailed prairie dogs, which were extirpated by 1960 (Bock and Bock, 2000; Underwood and Van Pelt, 2000). The Arizona Game and Fish Department began reintroducing black-tailed prairie dogs at Las Cienegas in 2008 using animals obtained from populations in New Mexico, United States, and Sonora, Mexico. We conducted our experiments on four of these colonies.

Colonies were situated on sites with similar soils (fine, deep, well-drained), slopes (0–15%), and elevations (1367–1412 m). One colony was initiated per year starting in 2008 and ending in 2011. The Arizona Game and Fish Department prepared sites via mechanical removal of large ($>\sim$1 m height) shrubs, mowing, and installation of 25 artificial burrows within a 4 ha area (see Hale et al., 2013 for details). Managers re-mowed colonies in 2011 to facilitate prairie dog establishment.

Throughout our study, three of the four colonies remained occupied by prairie dogs; however, one colony (hereafter referred to as the "control site") failed and was unoccupied during 2014 and 2015. The Arizona Game and Fish Department initially prepared this site in the same manner as the other colonies, but the colony lacked prairie dogs throughout the majority of our study period ($<$10 prairie dogs present in May 2013; the site was vacant by October 2013). We only used the control site for our simulated sapling experiment.

Woody Plant Surveys

We surveyed woody plants on each occupied colony in May of 2013 and 2014. We conducted eight belt (5 m width) transect surveys, wherein transects originated at the center of each colony and extended to and slightly beyond the colony boundary. We considered colony boundaries to be where the junction of short, prairie dog- defoliated grass and burrows met tall, unclipped grass and an absence of burrows. Off-colony transects extended up to 100 m beyond the colony perimeter for four transects at colonies A and B, and six transects at colony C. Some off-colony transects did not extend to the full 100 m due to logistical or topographic constraints (e.g., one transect was cut short by a deep, wide wash). We counted shrubs with stems originating within the transect belts, and noted the presence and degree of disturbance in the form of clipping, chewing, or girdling of their stems on a scale of 0–3 (0 = no disturbance, 1 = few stems disturbed, 2 = many stems disturbed, 3 = majority of stems disturbed). We considered colony to be the experimental unit (n = 6; two surveys at three colonies).

Exclosure Experiments

Among the woody plants encountered in our 2013 survey, we tagged plants of a random subset of the two most abundant species (velvet mesquite, *Prosopis velutina* and catclaw acacia, *Acacia greggii*; n = 40) for monitoring on each occupied colony (typically n = 20 on-colony and 20 off-colony). We installed exclosures around half of the plants at each on-off colony location (n = 10), with the other half remaining unprotected. We used garden fencing (mesh size 5.1 cm × 7.6 cm) to exclude prairie dogs and larger mammals (e.g., lagomorphs) for half of the exclosures (n = 5), and hardware cloth (mesh size 0.6 cm × 0.6 cm) for the remainder of the exclosures (n = 5) to exclude prairie dogs and smaller mammals [e.g., northern pygmy mice (*Baiomys taylori*), pocket mice (*Chaetodipus* spp., *Perognathus* spp.), kangaroo rats (*Dipodomys* spp.), woodrats (*Neotoma* spp.), grasshopper mice (*Onychomys* spp.), deer mice (*Peromyscus* spp.), harvest mice (*Reithrodontomys* spp.), and cotton rats (*Sigmodon* spp.)]. We left exclosures in place for ~3 months (mid-May through August) in 2013 and 2014 and for ~5 months (mid-May through late October) in 2015. We measured the canopy diameter of plants along their longest axis and perpendicular to their longest axis at the time of exclosure installation and again at the time of exclosure removal. We standardized change in shrub canopy area (CA), (m^2, computed as an ellipse) to a monthly basis (30 days). We followed the same plants each year; if a plant measured on the previous year had died, we made note of it and replaced it with the nearest live individual of the same species.

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[1] http://mesowest.utah.edu

Simulated Sapling Experiment

We removed foliated branches [average (±SE) length = 47 ± 0.6 cm; diameter = 1.1 ± 0.02 cm] from mature shrubs in nearby off-colony areas, and inserted them ~10 cm into the ground along four transects that originated at the center of each colony and extended 100 m beyond the colony boundary in randomized directions. We also installed transects of "simulated saplings" on the unoccupied control site that had been prepared in the same manner as the occupied colonies (see section "Study Site").

We hypothesized that prairie dog responses to artificial "simulated saplings" would indicate how they would react to the appearance of new woody plants. We spaced saplings ($n = 10$) evenly along transect segments on the colonies (the spacing distance varied for each transect segment, depending on its absolute length), and at 10 m intervals along the off-colony transect segments ($n = 10$). We considered three simulated saplings on each transect to be "periphery" saplings: the sapling installed on the colony boundary, and the closest sapling inside and outside of the colony boundary (**Supplementary Figure S1**). We also sought to ascertain whether prairie dogs would respond to familiar vs. novel shrubs in the same manner, so we used two woody species: velvet mesquite (*P. velutina*; hereafter "mesquite"), a native shrub common on and around the prairie dog colonies, and creosote bush (*Larrea tridentata*), also a native shrub, but one that did not occur on or near the colonies on the Las Cienegas. Both species are abundant across the Sonoran Desert and on landscapes within the study area. Mesquite is a thorny, N_2-fixing deciduous shrub (Fabaceae), whereas creosote bush is thorn-free, non-N_2-fixing evergreen shrub (Zygophyllaceae) that dominates many landscapes in each of the three North American hot deserts.

We measured saplings for height (cm) and recorded the number of secondary stems arising from the primary stem (typically several for mesquite and none for creosote bush). After 3 days of exposure to prairie dogs, we re-measured saplings and recorded the extent of damage (i.e., chewing, clipping, or girdling). We quantified damage as the proportion of stems disturbed on mesquite saplings and the relative proportion of height change of creosote bush saplings (since there were rarely secondary stems present). We conducted the experiment with mesquite in May 2014, and repeated it in late July/early August 2014. We conducted the experiment with creosote bush saplings in early September 2015, and repeated it in late September 2015. We generated different sets of random transects for each trial, so considered temporal replicates independent of one another for statistical analysis. For our 2014 experiment, we used branches of various heights (11–156 cm) and basal diameters (2.2–24.5 mm) to ascertain if prairie dog treatment of small, less visible plants would differ from that of larger more prominent plants.

Data Analysis

We performed statistical analyses in JMP (1989-2007). We assessed shrub density on and off colonies using a Student's t-test, with location (on-colony, off-colony) as the explanatory variable. We removed outliers (points > ± 2 SD's from the mean; $n = 7$ of 320 shrub canopy area data points) from analysis based on examination of histograms and residual plots. We log transformed changes in shrub canopy area (m^2/month) in exclosure experiments to normalize the data and assessed relationships using a full factorial two-way ANOVA with exclosure mesh size (prairie dogs and larger mammals excluded, or all mammals excluded) and location (on-colony, off-colony) as main effects. Additionally, we assessed change in shrub canopy area using a four-way ANOVA with location (on-colony, off colony), exclosure presence (yes, no), colony (A, B, or C), and year (2013, 2014, 2015) as main effects.

To assess the simulated sapling experiment, we performed a full factorial three-way ANOVA with trial number (first or second), species (mesquite or creosote bush), and location (on-colony, colony periphery, off-colony, on-control, control periphery, off-control) as independent variables, and proportion of total plants disturbed or degree of disturbance as the dependent variables. We considered colony to be the experimental unit ($n = 16$; four temporal replicates per colony).

When significant differences were indicated by ANOVA ($\alpha \leq 0.05$), we used Tukey's honest significant difference (hsd) tests (where statistical significance was set at 0.05 divided by the total number of comparisons in each test) to evaluate differences among means. In cases where no interactions occurred between effects having only two levels, we performed Student's t-tests on each effect with $\alpha \leq 0.05$.

RESULTS

Woody Plant Surveys

Mean (±SE) shrub density (plants ha^{-1}) was greater off of colonies (305 ± 94.9) than on colonies (132 ± 32.7; $P = 0.07$; **Figure 1**). The frequency of disturbance was greatest for shrubs on colonies [85% ($n = 329$ plants)] compared to only 9% ($n = 279$) in off-colony locations.

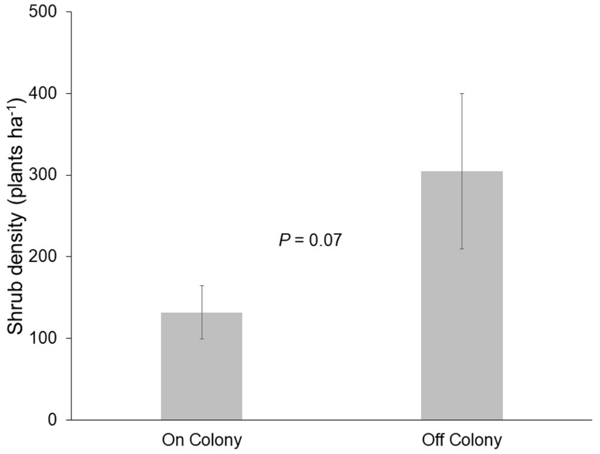

FIGURE 1 | Mean (±SE) density of shrubs (*Prosopis velutina* and *Acacia greggii*) on and off of black-tailed prairie dog (*Cynomys ludovicianus*) colonies ($n = 6$; two surveys at three colonies).

Exclosure Experiments

Shrub mortality during our study was low: only three of the 116 tagged mesquite plants died. Survival of mesquite was comparable on (95 ± 2.0%) and off of colonies (100 ± 2.0%; $P = 0.10$), and inside (95 ± 2.0%) and outside of exclosures (100 ± 2.0%; $P = 0.10$).

Changes in the canopy area of individual shrubs (m^2/month) were statistically comparable in exclosures of differing mesh size ($F_{1,159} = 0.01$, $P = 0.918$; **Table 1**) so we pooled these data for subsequent analysis. Additionally, changes in shrub canopy area were statistically comparable among colonies ($F = 0.412$, $P = 0.663$; **Table 1**), so we also pooled these data for subsequent analysis. We examined data to determine if there might be a size threshold above which shrubs might be more consistently damaged by prairie dogs, but we found no indication of such.

Change in canopy area was affected by location ($F_{2,297} = 20.06$, $P < 0.001$; **Table 1**), year ($F_{2,297} = 9.67$, $P < 0.001$; **Table 1**), exclosure presence ($F_{2,297} = 29.68$, $P < 0.001$; **Table 1**), and the interactive effects of location × year ($F_{2,297} = 10.48$, $P < 0.001$; **Table 1**) and exclosure presence × year ($F_{2,297} = 8.14$, $P < 0.001$; **Table 1**). On average, (i) canopies of shrubs protected by exclosures expanded more than those of unprotected shrubs ($P < 0.001$), (ii) levels of canopy expansion of shrubs on colonies (0.14 ± 0.03 m^2/month) was greater than that of shrubs off colonies (0.00 ± 0.02 m^2/month; $P < 0.001$), and (iii) canopies of shrubs expanded more in 2013 (0.15 ± 0.02 m^2/month) than in 2014 (0.03 ± 0.02 m^2/month; $P < 0.001$) or 2015 (0.03 ± 0.02 m^2/month; $P < 0.01$). Interaction effects were largely driven by year, with shrubs on colonies in 2013 expanding significantly more than all other groups (**Table 1**), and shrubs inside exclosures in 2013 expanding more than all other groups (**Table 1**).

Simulated Sapling Experiments

Size of simulated saplings did not affect whether or not they were disturbed [X^2 (df = 3, $n = 638$) = 1.309, $P = 0.73$], so we pooled size classes for subsequent analysis. Colony had no effect on the proportion of saplings disturbed or degree of disturbance, so we pooled these data across occupied colonies as well. Neither trial number (first or second; $F_{1,47} = 0.17$, $P = 0.688$) nor shrub species (creosote or mesquite; $F_{1,47} = 0.19$, $P = 0.671$) influenced the proportion of saplings disturbed, but location (on-colony, colony periphery, or off-colony, on-control, control periphery, off-control) did ($F_{1,47} = 61.63$, $P < 0.001$). The proportion of saplings disturbed was greatest on colonies (89 ± 4.0%; see **Figure 2** for illustration of disturbance), followed by colony peripheries (39 ± 4.0%; Tukey's hsd, $P < 0.001$); disturbances off-colony were essentially nil (0–2%; **Figure 3A**). Expressed as a spatial continuum, the proportion of saplings disturbed was consistently high (74–100%) among on-colony transect positions, decreasing sharply at the colony periphery locations, and was consistently low to nil for off-colony positions and the control site (**Figure 4**).

Similarly to the proportion of plants disturbed, neither trial number (first or second; $F_{1,47} = 1.43$, $P = 0.243$) nor shrub species (creosote or mesquite; $F_{1,47} = 2.27$, $P = 0.145$) influenced the degree of sapling disturbance. Conversely, location (on-colony, colony periphery, or off-colony, on-control, control periphery, off-control) was significant ($F_{1,47} = 44.35$, $P < 0.001$). Degree of sapling disturbance was greatest on colonies (0.54 ± 0.03), intermediate on colony peripheries (0.22 ± 0.03), and least off of colonies (0.05 ± 0.03) and on control sites (on: 0.06 ± 0.05, periphery: 0.05 ± 0.05, off: 0.05 ± 0.05; **Figure 3B**).

DISCUSSION

Woody plant encroachment is a threat to grasslands and their endemic plants and animals (Archer et al., 2017). Chemical, mechanical and pyric approaches to reducing woody plant abundance and restoring shrub-encroached grasslands have been widely practiced (collectively known as "brush management"), but are typically expensive and their effects are short-lived (Archer and Predick, 2014). Our results suggest that the reintroduction of prairie dogs subsequent to "brush management" could prolong the effectiveness of those treatments while concurrently resuscitating ecosystem processes that would sustain long-term success and promote biodiversity. In this respect, prairie dog re-introductions represent another example of how "rewilding" may revive ecosystem structure, function, and health in a cost-effective manner (e.g., Guyton et al., 2020).

Our exclosure experiments indicated that herbivory was reducing shrub growth both on and off the colony, but more so off-colony than on-colony. One hypothesis to account for this counter-intuitive result would be that above- vs. belowground allocation patterns differed for shrubs on- and off-colony, wherein on-colony shrubs were preferentially allocating more resources to aboveground growth. Another hypothesis would be that growth of protected on-colony shrubs was enhanced by reductions in competition resulting from the felling of surrounding plants by prairie dogs, while growth of protected off-colony shrubs was muted or suppressed owing to the relatively higher levels of competition from surrounding plants. In any case, our observations, surveys, and sapling experiments collectively suggest that benefits gained by potential release from competition or increased allocation to aboveground growth were more than offset by high levels of damage to on-colony shrubs. We directly observed prairie dogs gnawing and clipping shrubs (including those that escaped the broad-scale mechanical treatment and those that re-sprouted following mechanical treatment) and our simulated saplings, consistent with their well-documented habit of systematically felling plants to maintain vegetation in a short-statured state to aid in visual detection of predators (King, 1955; see Figure 5.18 in Hoogland, 1995). Small-statured shrubs that remained on colonies after the mechanical removal of adult shrubs were disturbed more frequently than those located on colony peripheries and off of colonies, as substantiated by observational results (surveys), and experienced the greatest degree of disturbance as indicated by our simulated sapling experiments. Prairie dogs were thus able to substantively mitigate shrub abundance and stature on these nascent colonies. The introduced prairie dogs did not eliminate the small shrubs remaining on their colonies over the time-frame of this study,

TABLE 1 | Summary of ANOVA outcomes for exclosure experiments and coefficients for main effects and significant interactions.

Main effects	*F*-value Coefficient	df	*P*	Interactions	*F*-value Coefficient	df	*P*
Exclosure mesh size two-way ANOVA							
Location (L)	14.18	1, 159	<0.001	L × ES	0.99	1, 159	0.321
On colony	0.22						
Off colony	0.07						
Exclosure mesh size (ES)	0.01	1, 159	0.918				
Small	0.15						
Large	0.14						
Four-way ANOVA							
Location (L)	20.06	1, 297	<0.001	L × E	1.10	1, 297	0.296
On colony	0.13			L × C	3.41	2, 297	0.035
Off colony	0.01			Off, A	0.03		
Exclosure (E)	29.68	1, 297	<0.001	Off, B	0.05		
Yes	0.14			Off, C	−0.05		
No	0.00			On, A	0.14		
Colony (C)	0.41	2, 297	0.663	On, B	0.08		
A	0.08			On, C	0.16		
B	0.06			L × Y	10.48	2, 297	<0.001
C	0.06			Off, 2013	0.01		
Year (Y)	9.67	2, 297	<0.001	Off, 2014	0.01		
2013	0.15			Off, 2015	0.01		
2014	0.03			On, 2013	0.30		
2015	0.03			On, 2014	0.04		
				On, 2015	0.04		
				E × C	1.00	2, 297	0.370
				E × Y	8.14	2, 297	<0.001
				Yes, 2013	0.30		
				Yes, 2014	0.07		
				Yes, 2015	0.05		
				No, 2013	0.01		
				No, 2014	−0.02		
				No, 2015	0.01		
				C × Y	0.18	4, 297	0.951
				L × E × Y	0.80	2, 297	0.452
				L × E × C	0.21	2, 297	0.812
				L × Y × C	3.10	4, 297	0.016
				Off, 2013, A	0.08		
				Off, 2013, B	0.10		
				Off, 2013, C	−0.16		
				Off, 2014, A	−0.01		
				Off, 2014, B	0.02		
				Off, 2014, C	0.02		
				Off, 2015, A	0.02		
				Off, 2015, B	0.02		
				Off, 2015, C	0.00		
				On, 2013, A	0.26		
				On, 2013, B	0.22		
				On, 2013, C	0.41		
				On, 2014, A	0.09		
				On, 2014, B	−0.02		
				On, 2014, C	0.04		
				On, 2015, A	0.05		
				On, 2015, B	0.05		
				On, 2015, C	0.03		
				E × Y × C	0.47	4, 297	0.760
				L × E × Y × C	0.25	4, 297	0.911

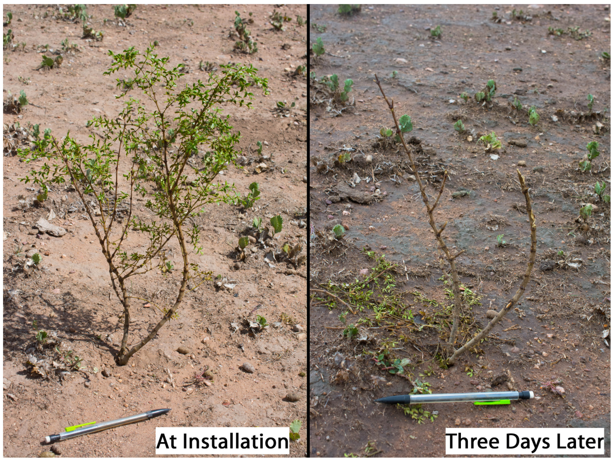

FIGURE 2 | Simulated velvet mesquite (*Prosopis velutina*) sapling (approximately 30 cm in height) upon installation on an active black-tailed prairie dog (*Cynomys ludovicianus*) colony **(left)** vs. after 3 days of exposure to black-tailed prairie dogs **(right)**.

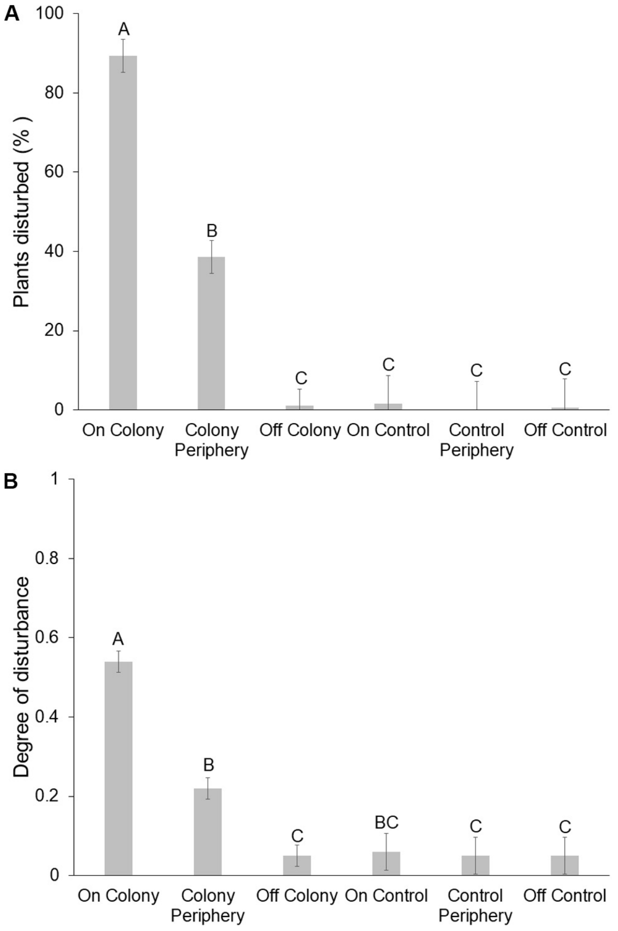

FIGURE 3 | Mean (±SE) percentage of "simulated" *Prosopis velutina* and *Larrea tridentata* saplings disturbed by black-tailed prairie dogs (*Cynomys ludovicianus*) **(A)** and scalar (0 = none; 1 = max) assessments of the degree of disturbance **(B)** on, on peripheries, and off of active colonies and a control site (n = 16; see section "Materials and Methods"). Different letters above bars indicate significant ($P < 0.05$) differences between means within a panel.

but the density of shrubs on colonies was 40% that of off-colony sites (**Figure 1**). This suggests that prairie dogs may reduce recruitment and, over longer time-scales, induce shrub mortality on their colonies.

Prosopis velutina and *A. greggii*, the two main shrubs on our site, have the ability to vegetatively regenerate from basal meristems following removal of aboveground biomass (Glendening and Paulsen, 1955; Cross and Wiedemann, 1997). Accordingly, prairie dogs on these nascent colonies did not affect shrub survival, demonstrating that shrubs at this site are capable of persisting even under heavy levels of herbivory. This is reminiscent of the "browse trap" phenomenon. We would expect that the loss of prairie dogs from this site, which had been mechanically cleared of adult woody plants, would release small shrubs or those recruiting from the seed bank from the browse trap. These expectations are consistent with those from studies on well-established prairie dog colonies (Weltzin et al., 1997; Ceballos et al., 2010; Ponce-Guevara et al., 2016). Such a release would allow shrubs to re-gain dominance and thus neutralize the "brush management" effort to restore this former grassland. The fact that simulated saplings placed on a recently failed prairie dog colony received no damage (**Figures 3**, **4**), despite their being highly conspicuous to lagomorphs and other rodents, suggests that prairie dogs, not other small mammals, were regulating shrubs within colonies. This interpretation is consistent with another study showing that small rodents on prairie dog colonies did not influence rates of shrub seed disappearance (Weltzin et al., 1997).

There was little evidence that large herbivores contributed to a browse trap for shrubs in this region. Native (e.g., American bison, *Bison bison*) and non-native ungulates, such as domestic cattle (*Bos taurus*) have diets that consist primarily of grasses and forbs (Meagher, 1986; Beck and Peek, 2005) so their preferential foraging on prairie dog colonies (e.g., Coppock et al., 1983) would not be likely to constrain the development of communities dominated by woody plants prior or subsequent to prairie dog removal. This would be particularly so for highly unpalatable shrubs such as mesquite and creosote bush (Campbell and Taylor, 2006; Brock et al., 2014; Bovey, 2016). Pronghorn (*Antilocapra* spp.) are also known to preferentially forage on prairie dog colonies (King, 1955; Krueger, 1986; Hoogland, 1995), but given the abundance of shrubs in other portions of their range [e.g., sagebrush (*Artemesisia* spp.) in the Intermountain West and northern Great Plains] it does not seem likely they exert sufficient browsing pressure to keep shrubs in check. The browse trap in this system thus appears to center around prairie dogs and their activities, which could, in turn, be enhanced by cattle grazing (Ponce-Guevara et al., 2016).

A coincidence between the elimination of small native grazers and woody encroachment is not restricted to North America. In Australia, native granivorous rodents may have historically limited shrub recruitment following fire events, but rodent

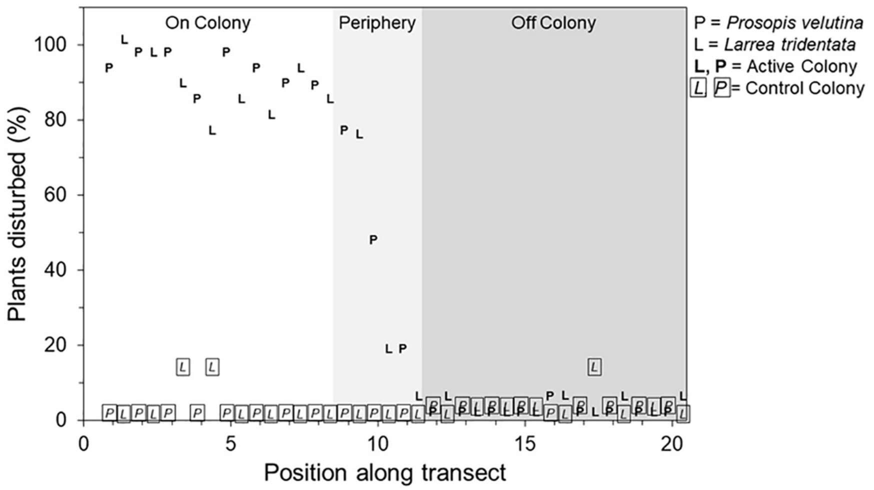

FIGURE 4 | Mean percentage of simulated *Prosopis velutina* (denoted "P") and *Larrea tridentata* "saplings" (denoted "L") disturbed along transects originating at the centers (position = 0) of active black-tailed prairie dog (*Cynomys ludovicianus*) colonies (bold font) and a control colony (italic font, boxed). Transects varied in length, depending upon the size of the colony, so positions are relativized such that 1–8 represent locations on colony, 9–11 represent locations on the colony periphery, and 12–20 denote the off colony locations (see **Supplementary Figure S1**).

populations have declined due to pastoral activity, and their declines have coincided with shrub encroachment (Gordon and Letnic, 2019). Plains vizcacha (*Lagostomus maximus*) in Argentina and burrowing bettong (*Bettongia lesueur*) in Australia exhibit similarities to prairie dogs not only in their ecology and behavior, but also in their pest status, which has led to elimination from parts of their range (Jackson et al., 1996; Noble et al., 2007). Likewise, plateau pikas (*Ochotona curzoniae*) in China play a keystone role analogous to that of prairie dogs, but have also been subjected to eradication efforts (Smith and Foggin, 1999; Delibes-Mateos et al., 2011; Fahong et al., 2012). As with the elimination of prairie dogs in North America, elimination of these perceived small mammalian pests has had the unintended consequence of promoting woody plant proliferation (Brandt et al., 2013; González-Roglich et al., 2015). Such trade-offs should be considered in rangeland management and conservation plans where the maintenance of grasslands and the organisms endemic to them are a priority.

Woody plant proliferation in grasslands has prompted the implementation of "brush management" practices aimed at reducing shrub cover to enhance livestock production (Hamilton et al., 2004) and restore habitat for grassland wildlife (Fulbright et al., 2018). These practices often involve herbicides, heavy equipment (e.g., cutting, shredding, or chaining), and prescribed burning alone or in combination (Collins et al., 2015). Such endeavors are costly, short-lived, and require repeated application (Archer et al., 2011). Prairie dogs rely on short vegetation for visual detection of predators (Hoogland, 1995; King, 1955), so are unlikely to colonize areas with dense woody vegetation and would not be likely to persist if introduced into a landscape with well-developed woody cover (Milne-Laux and Sweitzer, 2006). However, our study suggests that reintroductions of prairie dogs following brush management can succeed and could extend the shrub treatment longevity and reduce or perhaps eliminate the need for follow-up treatments, while simultaneously promoting the restoration of grassland habitats and the plants and animals endemic to them (Whicker and Detling, 1988; Kotliar et al., 1999, 2006; Ceballos et al., 2010).

Our sapling experiments suggest that prairie dogs are not limited by previous experience with woody species, as the Las Cienegas prairie dogs felled both a species they encountered regularly (*P. velutina*) as well as a species they had never encountered (*L. tridentata*). This suggests that (i) prairie dogs can restore the browse trap even when source and reintroduction sites differ in woody species composition, and (ii) prairie dog reintroduction within areas of their former range could simultaneously function as a natural, cost-effective, and sustainable option for limiting woody plant encroachment in present-day grasslands and for restoring grassland structure, function and biodiversity where shrub encroachment has occurred. These positive facets of prairie dogs may compensate for or more than off-set the traditional negative perspectives associated with prairie dog-livestock competition for forage and declines in forage and livestock production (O'Meilia et al., 1982; Derner et al., 2006). A comprehensive assessment of these complexities in livestock-prairie dog interactions and the various trade-offs that emerge would require a landscape-scale accounting of livestock grazing intensity, seasonality, and patterns and prairie dog colony locations, sizes and ages. When prairie dog re-introductions are contemplated, where best to locate them and how colonies might subsequently expand should be given careful consideration.

The conservation and reintroduction of large or charismatic keystone species [e.g., gray wolves (*Canis lupus*) and sea otters (*Enhydra lutris*)] can restore critical ecological function to ecosystems (Estes and Palmisano, 1974; Callan et al., 2013). However, similar to the historical perception of wolves, small keystone herbivores are often viewed as pests that are targeted for eradication rather than conservation (Delibes-Mateos et al., 2011). Accordingly, the ecological implications of reintroducing small keystone herbivores has not been widely investigated. We add to this understanding, and highlight the ability of small keystone herbivores to address significant ecological issues, such as woody encroachment, immediately following their reintroduction and in conjunction with other land management practices. Our results indicate the important role(s) that small keystone herbivores can potentially play in maintaining diverse, healthy ecosystems, and the substantial ecological consequences that can result from their removal (Weltzin et al., 1997; Davidson et al., 1999, 2012, 2014; Miller et al., 2000; Kotliar et al., 2006; Brandt et al., 2013; González-Roglich et al., 2015). A better understanding of such species and their reintroduction after extirpation will be important for the restoration and conservation of grassland ecosystems throughout the world.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee (protocol 11-251).

AUTHOR CONTRIBUTIONS

SH contributed to the research design, collected data in the field, performed the experiments, analyzed the data, and wrote the manuscript. JK and SA contributed to the research design, data analysis, and provided valuable edits and revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00233/full#supplementary-material

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Spatiotemporal Diversification of Tree Squirrels: Is the South American Invasion and Speciation Really That Recent and Fast?

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Tree squirrels (Sciurinae, Sciurini) represent a diverse radiation that successfully colonized Europe, Asia and the Americas during the Miocene-Pliocene, but information on their evolutionary history remains unclear. In the Neotropics, they have been shown to exhibit the highest rate of diversification amongst all arboreal squirrels, with strikingly high species accumulation rates in the past 3 Mya. In this study, we investigated the tempo and mode of diversification of tree squirrels using a mitogenome dataset that includes 43 Sciurini species. Our results corroborate the date of origin of the tribe Sciurini around 14 Mya (13.4–15.5) but suggest that their ancestral area was most likely in North America. This is in contrast to previous findings that suggested that the ancestors of this tribe occupied Eurasia. We estimated that cladogenetic events leading to the Eurasian lineages occurred twice at 10.5 and 9.7 Mya. Current North American genera originated in a temporal window from 6.2–2.3 Mya, and the origin of the Neotropical radiation was estimated to have occurred around 6 Mya in northwestern South America, in the Pacific dominion. Remarkably, our results indicate that tree squirrels entered South America at an earlier date than previously estimated. This could have happened either through a land corridor connecting the Caribbean islands or through the Panamanian land bridge. Most cladogenetic events in Eurasia and North America appear to have occurred either late in the Miocene or in the Pleistocene, while the majority of Neotropical cladogenetic events occurred along the Pliocene—right after the South American invasion. We found a fairly constant speciation rate for tree squirrels (averaging 0.29), which contrasts with the peak of lineage accumulation observed in the Pliocene. The absence of fluctuations in the diversification rate may be the result of several extinction events that were responsible for equalizing the number of lineages maintained over time. Finally, we conclude that the South American invasion was not as recent as previously inferred, but the diversification there was indeed very fast.

Keywords: ancestral range, founder-event speciation, Isthmus of Panama, mitogenome, Neotropics, Sciurini

INTRODUCTION

Tree squirrels (Sciurinae, Sciurini) represent a diverse radiation that successfully colonized forested landscapes of Europe, Asia and the Americas during the Miocene and Pliocene (Mercer and Roth, 2003; Pečnerová et al., 2015). The timing of the origin of the tribe is imprecise and has been estimated to have occurred from 19 to 13 million years ago (Mya; Mercer and Roth, 2003; Fabre et al., 2012; Pečnerová et al., 2015; Zelditch et al., 2015). In addition, the uncertainties regarding the location of their ancestral geographic range have also contributed to the blurred inferences of the evolutionary history of tree squirrels. Pečnerová and Martínková (2012) suggested that Sciurini originated in the northern hemisphere, not distinguishing between Eurasia and North America. A similar result was found by Rocha et al. (2016), who proposed a Holarctic distribution for the ancestor of tree squirrels. Subsequently, Pečnerová et al. (2015) estimated a more restricted ancestral range of Sciurini, confined to the Palearctic region.

Discrepancies on inferences about the spatiotemporal diversification of Sciurini are likely a result of the limited datasets, in terms of geographic and taxonomic coverage, employed by previous analyses. None of the aforementioned studies included more than a third of the South American tree squirrel species and also missed some Central and North American taxa (sensu Thorington et al., 2012; Vivo and Carmignotto, 2015); therefore, the lack of so many taxa and presumed lineages might mislead phylogenetic and, consequently, biogeographic inferences. A recent comprehensive phylogenomic study conducted by Abreu-Jr et al. (2020), that included samples from 40 of the 43 recognized species of Sciurini (sensu Thorington et al., 2012; Vivo and Carmignotto, 2015; Hope et al., 2016), revealed an underestimated diversity on the genus and species group levels within the tribe and clarified several important points with regards to the phylogenetic relationships and systematics of tree squirrels. This study did not find support for the recognition of two species previously considered as valid—*Sciurus richmondi* sensu Thorington et al. (2012) and *Microsciurus venustus* sensu Vivo and Carmignotto (2015)— and found evidence for the recognition of six additional lineages that might represent species to be named or revalidated. This new taxonomic proposal suggested a diversity of 46 species within Sciurini which were tentatively organized in 14 genera coined by previous authors (see Abreu-Jr et al., 2020). The mitogenome dataset produced in that study is highly suitable to investigate the tempo and mode of evolution of tree squirrels, especially across the Neotropical region, where the taxonomic and geographic sampling were strengthened.

In the Neotropics, tree squirrels have been shown to exhibit the highest rate of diversification amongst all arboreal squirrels (a non-phylogenetic ecological category that includes members of four subfamilies; Roth and Mercer, 2008), with strikingly high species accumulation rates in the past three million years, presumably after the final closure of the Panamanian land bridge and the establishment of the Great American Biotic Interchange (GABI). The GABI is one of the most significant events of transcontinental fauna dispersal that occurred during the temporal window from the late Pliocene to the late Pleistocene, wherein several mammal lineages migrated from North America to South America and the other way around (Simpson, 1983; Stehli and Webb, 1985; Woodburne, 2010). Among rodents, tree squirrels and sigmodontine rodents arguably shared a very successful history of diversification and colonization of South America accompanying the GABI (Patterson and Pascual, 1972; Simpson, 1983).

However, alternative scenarios proposed either a diversification at the generic level in tropical portions of North and Central America, followed by an invasion of South America, after the Panamanian land bridge arose; or an early entrance in South America, sometime in the Miocene, prior to the establishment of the Panamanian land bridge and the GABI (see detailed explanation in D'Elía, 2000). For sigmodontine rodents, current hypotheses suggest an early arrival in South America (Smith and Patton, 1999; Steppan et al., 2004; Parada et al., 2013), in the Miocene, supporting the scenario proposed by Hershkovitz (1966, 1969) and Reig (1984). For squirrels, current hypotheses sustain the entrance of this group in South America during the GABI, after the Panamanian land bridge arose (Mercer and Roth, 2003), but results so far are still inconclusive, as most of the South American squirrel diversity remained unstudied.

These rodents are an ideal group of mammals that can help to address a wide range of biogeographic questions, such as the timing of the dispersal events across the Americas and colonization within South America, and the potential relevance of specific areas as sources of diversity within the Neotropics. The South American continent has a history of major geological (such as the Andes uplift and the Amazon basin formation) and climatic (such as the Pleistocene climate fluctuations) changes that occurred in the past 10 million years. These events gave rise to a great variety of environments and new habitats that leveraged the rapid diversification of several lineages of the tree of life (Hoorn et al., 2010).

In this study, we test current hypotheses on the tempo and mode of diversification of tree squirrels (Pečnerová and Martínková, 2012; Pečnerová et al., 2015) employing a comprehensive dataset (mitogenome data) and a diverse taxonomic coverage (including 43 Sciurini putative species; Abreu-Jr et al., 2020). We provided a time-scaled phylogeny to infer the timing of the origin and diversification of the main lineages, and performed biogeographic analyses to estimate ancestral ranges and evaluate the drivers of diversification at a global scale. In addition to the broad-scale analysis that includes all Sciurini species—and since the Neotropics is home of the most diverse assemblage of species—we also performed a finer-scale analysis focused exclusively on the diversification processes of Neotropical taxa, to unravel their colonization history across Central and South Americas. Finally, we tested the hypothesis that tree squirrels represent a rapid-diversified radiation (Roth and Mercer, 2008) by estimating speciation rates and investigating diversification rate fluctuations through time.

MATERIALS AND METHODS

Dataset Taxonomic Composition

Our dataset is composed of 14 genera and 43 species of Sciurini delimited in a recent contribution (Abreu-Jr et al., 2020). Taxonomic identifications at the genus and species levels follow Abreu-Jr et al. (2020). At the species level, those authors kept the museum identifications for several specimens, especially those housed at the American Museum of Natural History (AMNH) and at the Smithsonian National Museum of Natural History (USNM), which had been made by some of the main authorities on tree squirrel taxonomy (e.g., R. W. Thorington and M. de Vivo). For material not previously identified, they performed identifications by examining the morphology of the vouchers, consulting original descriptions and other relevant literature. In cases of unviability to examine vouchers, original museum identifications were accepted if (i) those identifications correspond to the known geographic distribution of the taxon in question, and (ii) phylogenetic analyses of their DNA sequences were consistent with the museum identification. The currently valid species of tree squirrels not included in this study are: *Tamiasciurus fremonti* sensu Hope et al. (2016), *Microsciurus santanderensis* and *M. simonsi* sensu Vivo and Carmignotto (2015). We used as outgroup two representatives of the tribe Pteromyini (the sister-tribe of Sciurini): *Glaucomys volans* (USNM 569823) and *Hylopetes phayrei* (USNM 584420). A complete list of specimens analyzed in this study accompanied by geographic information and GenBank accession numbers for complete mitogenomes is provided as (**Supplementary Table S1**).

DNA Matrix, Alignment and Saturation Test

We selected the 13 mitochondrial protein-coding genes (CDS) from one sample of each of the 43 putative species of Sciurini to perform the analyses. The CDS matrix was aligned using MUSCLE (Edgar, 2004), with up to eight interactions. We investigated the presence of saturation in our dataset by plotting transitions and transversions against nucleotide divergence in DAMBE7 (Xia, 2018).

Divergence Times Estimate

Divergence times were estimated using a Bayesian framework in BEAST 2.6.1 (Bouckaert et al., 2019). We applied a single nucleotide substitution model (GTR) with a gamma category of 4 to our dataset and implemented a lognormal relaxed clock with a Yule tree prior. The analysis was conducted by running 600 million generations of Markov chain Monte Carlo (MCMC), sampling every 60,000 generations. The results of MCMC runs were visualized in Tracer 1.7 (Rambaut et al., 2018) to confirm a minimum of 200 effective sample size for all parameters. A time-calibrated tree was generated with TreeAnnotator v2.6.0 (Bouckaert et al., 2019), considering 10% of burnin and selecting the maximum clade credibility as the target tree. We do not specify posterior limit for the nodes and we selected mean heights for the common ancestor heights. BEAST analysis was performed in the CIPRES Science Gateway (Miller et al., 2010).

We employed four calibrations points representing three distinct types of priors: (i) fossil record – the first known fossil record of *Tamiasciurus hudsonicus*, dated from the Irvingtonian (from 1.9 to 0.25 Mya; Steele, 1998), was implemented as uniform prior for the root of *Tamiasciurus*; (ii) fossil record – *Sciurus alleni* was first reported from the Late Pleistocene (from 0.126 to 0.0117 Mya; Jakway, 1958) and it was constrained as uniform prior for the split between *S. alleni* and *S. oculatus*; (iii) geological events plus fossil record – the crown of Nearctic *Sciurus* was constrained with uniform prior between 13.6 and 7.4 Mya, considering that *Sciurus* was present in North America since the late Miocene (Clarendonian; *Sciurus olsoni*; Emry et al., 2005) and this radiation supposedly arrived from Eurasia (Pečnerová et al., 2015), thus the colonization occurred before the initial opening of the Bering Strait about 7.4 Mya (Marincovich and Gladenkov, 1999); (iv) secondary estimation – we constrained the root of Sciurini with log-normal prior with offset of 13.4 Mya ($M = 0.5$ and $S = 1.0$), according to results of Upham et al. (2019), which is currently the most comprehensive phylogeny of mammals.

Ancestral Range and Diversification Events Estimate

We estimated the ancestral range of the phylogenetic nodes and investigated the historical events (e.g., vicariance, dispersal) that might be evoked to explain the diversification pattern of the tribe Sciurini. These analyses were performed employing a maximum likelihood framework using the R package BioGeoBEARS (Matzke, 2013). The time-scaled topology provided by BEAST with only one individual per species was used as the input tree. Six models of evolution—which distinctively incorporate vicariance, dispersal and extinction throughout the cladogenesis—were tested, as follows: (1) Dispersal-Extinction-Cladogenesis (DEC; Ree, 2005), (2) DEC + founder-event speciation ("jump"; DEC + J), (3) Dispersal-Vicariance Analyses (DIVALIKE; Ronquist, 1997), (4) DIVALIKE + J, (5) Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE; Landis et al., 2013), and (6) BAYAREALIKE + J. The best-fitting model was selected based on the Akaike Information Criterion weights (AICw; Wagenmakers and Farrell, 2004).

These biogeographic analyses were performed in two geographic scales: global and Neotropical. The first included all Sciurini species and we investigated their colonization history across large continental areas; species were coded as occupying: (1) Eurasia, (2) Borneo, (3) North America, (4) Central America, and (5) South America. The second focused exclusively on the diversification of Neotropical taxa. For this finer-scale analysis, we followed the Neotropical biogeographical dominions proposed by Morrone (2014) to categorize the current geographical range of species, which were coded as occurring at: (1) the Mexican transition zone, (2) the Mesoamerican dominion, (3) the Pacific dominion, (4) the Boreal Brazilian dominion, (5) the South Brazilian dominion, (6) the South-eastern Amazonian dominion, and (7) the Parana dominion.

Lineage Through Time and Speciation Rates

We investigated the diversification pattern of Sciurini by constructing a lineage through time (LTT) plot using the R package Ape (Paradis and Schliep, 2019). Speciation rates and the presence and temporal location of putative diversification rate shifts were explored by employing the Bayesian Analysis of Macroevolutionary Mixtures (BAMM; Rabosky, 2014; Rabosky et al., 2017). We implemented a speciation-extinction model, run for 10 million generations and sampled every 2,000 generations in BAMM v2.5.0. We incorporated a sampling frequency of 0.93, considering that we have sampled about 93% of the extant species of Sciurini (43 out of the 46 putative species; Abreu-Jr et al., 2020). Results of BAMM analysis were processed and visualized using the R package BAMMtools (Rabosky et al., 2014).

RESULTS

Phylogeny of the Tribe Sciurini

Our final mitogenome alignment was 11,278 bp long and included 45 species (43 ingroup and two outgroup). The mean sequence length per sample was 10,360 bp (ranging from 1,393 to 11,278 bp) and the overall matrix missing data (including gaps and undetermined characters) was 7.4%. We found no evidence for nucleotide substitution saturation in our sequences that could influence the phylogenetic signal (**Supplementary Figure 1**). The maximum clade credibility tree obtained from our BEAST analysis recovered similar relationships to those previously reported by Abreu-Jr et al. (2020), with all nodes strongly supported ($PP \geq 0.98$), excepted by the node of the genus *Echinosciurus*, which did not receive a significant support ($PP < 0.95$).

Model Selection in Ancestral Range Estimates

The analyses performed with BioGeoBEARS at the global scale recovered DEC + J as the most suitable model to explain the diversification of Sciurini (LnL = −47.61, AICwt = 0.82; **Table 1**), considering distinct evolutionary events, such as dispersal, extinction and vicariance. The ancestral range estimation focused on the Neotropical taxa recovered that the model BAYAREALIKE + J better fit our data (LnL = −68.79, AICwt = 0.85; **Table 2**), favoring dispersal-extinction scenarios rather than vicariance events.

Spatiotemporal Diversification of Sciurini at a Global Scale

The origin of the tribe Sciurini was estimated at 14.1 Mya (13.4–15.5; **Figure 1**; see also **Supplementary Figure 2**) and its ancestral geographic range was inferred with highest probability to North America (58.6%; **Figure 1**). An area including both North America and Eurasia was estimated with the second highest probability of occurrence for the ancestor of Sciurini (30.6%; **Supplementary Table S2**). The first lineage to diverge within the tribe was the genus *Tamiasciurus*. All of the known species of this genus are from North America and its ancestor was also estimated to have occurred in North America. The subsequent diversification event, leading to the Borneo endemic genus *Rheithrosciurus*, occurred at 10.5 Mya (9.5–11.7) and the ancestral range of this node was also in North America. Likewise, the ancestral ranges of the nodes leading to the Eurasian genus *Sciurus* (at 9.7 Mya), the North American genus *Hesperosciurus* (at 7.6 Mya), and the clade including both North American genera *Neosciurus* and *Parasciurus* (at 6.2 Mya), were most likely to be in North America. In summary, diversification events generating the major lineages from North America and Eurasia took place in the mid-late Miocene, whereas most speciation events involving extant species in those lineages occurred in the Pleistocene (**Figure 1**).

The large clade including exclusively Neotropical taxa (marked with a black star in **Figure 1**) originated with highest probability in South America at 5.9 Mya (5.2–6.5). Within this clade, the Central American radiation (including genera *Microsciurus*, *Syntheosciurus*, and *Echinosciurus*) originated at 4.6 Mya (3.8–5.3) and the South American lineage (including genera *Leptosciurus*, *Simosciurus*, *Guerlinguetus*, "*Microsciurus*," and *Hadrosciurus*) at 5.0 Mya (4.3–5.6). Therefore, these Central and South American radiations, as well as most Neotropical genera, originated in the early-mid Pliocene and experienced a rapid diversification, with speciation events occurring mostly in the Pliocene and in the Plio-Pleistocene transition, and only a few during the Pleistocene (**Figure 1**).

The ancestral range estimation of the tribe Sciurini on a global scale (**Figure 1**) allows the inference of seven founder-event speciation and three anagenetic dispersals, while it does not indicate the occurrence of vicariance events during the evolutionary history of tree squirrels (see **Supplementary Figures 3, 4**). The founder events (i.e., a jump of a lineage to a new area of occurrence outside of the ancestral range of the node; Matzke, 2013) were inferred for the ancestors of (1) the genus *Rheithrosciurus* from North America to Borneo, (2) the Eurasian genus *Sciurus* from North America

TABLE 1 | Results of the six biogeographic models implemented by BioGeoBEARS on the ancestral range estimation of Sciurini in a global scale.

Model	LnL	Parameters	d	e	j	AIC	AICwt
DEC	−52.54	2	0.018	0.007	0	109.1	0.016
DEC + J	**−47.61**	**3**	**0.007**	**1.00E-12**	**0.023**	**101.2**	**0.820**
DIVALIKE	−54.74	2	0.022	1.00E-12	0	113.5	0.002
DIVALIKE + J	−49.39	3	0.009	1.00E-12	0.025	104.8	0.140
BAYAREALIKE	−73.16	2	0.020	0.078	0	150.3	1.80E-11
BAYAREALIKE + J	−51.33	3	0.006	1.00E-07	0.031	108.7	0.020

Fits of alternative models were compared using AIC values and AIC weights (AICwt). The model that provided the best fit to our data is in bold.

TABLE 2 | Results of the six biogeographic models implemented by BioGeoBEARS on the ancestral range estimation of Sciurini in a Neotropical scale.

Model	LnL	Parameters	d	e	j	AIC	AICwt
DEC	−73.30	2	0.025	2.00E-08	0	150.6	0.025
DEC + J	−72.82	3	0.023	1.00E-12	0.008	151.6	0.015
DIVALIKE	−79.16	2	0.031	4.10E-09	0	162.3	7.20E-05
DIVALIKE + J	−77.19	3	0.026	1.00E-12	0.012	160.4	0.0002
BAYAREALIKE	−71.81	2	0.013	0.160	0	147.6	0.110
BAYAREALIKE + J	**−68.79**	**3**	**0.012**	**0.038**	**0.014**	**143.6**	**0.850**

Fits of alternative models were compared using AIC values and AIC weights (AICwt). The model that provided the best fit to our data is in bold.

to the Palearctic region, (3) the large Neotropical radiation from North America to South America, (4) the Central American clade from South America to Central America, (5) the clade *Parasciurus alleni* + *P. oculatus* from North America to Central America, (6) *Parasciurus nayaritensis* from North America to Central America, and (7) *Microsciurus* "species 1" from Central America to South America. Anagenetic dispersals included (1) the colonization of Central America by *Hesperosciurus aberti*—although this species occurs in Central America mainly in the Sierra Madre Occidental and there is a discussion whether this region is part of the Nearctic region (Holt et al., 2013) or it belongs to the Neotropical region (Morrone, 2014)—, (2) colonization of South America by *Syntheosciurus granatensis*, and (3) colonization of Central America by the ancestor of *Leptosciurus boquetensis* and *L. isthmius*.

Spatiotemporal Diversification of Sciurini in the Neotropics

Our biogeographic analyses focused on Neotropical Sciurini suggested with highest probability (92.2%) that this large clade originated in the northwestern portion of South America in the Pacific dominion (**Figure 2**; **Supplementary Table S3**). The Pacific dominion was also recovered with highest probability as the ancestral range of the Central American (93.6%) and South American (99.0%) major radiations. Within the Central American radiation, two genera originated in this same region: *Microsciurus* at 4.1 Mya (3.3–5) and *Syntheosciurus* at 3 Mya (2.1–3.7). The genus *Echinosciurus* originated at 3.7 Mya (3–4.4) in an area including the Mexican transition zone and the Mesoamerican dominion.

With regards to the South American radiation, the ancestors of the first two genera occupied the northwestern portion of the continent, west of the Andes, in the Pacific dominion: *Leptosciurus* that originated at 4.4 Mya (3.6–5.1) and *Simosciurus* that originated at 4 Mya (3.1–4.7). The following genera had their origins on the east side of the Andean cordillera, along the Amazon basin. The genus *Guerlinguetus* originated at 4.4 Mya (3.9–5.1) in the Boreal Brazilian dominion. The ancestor of the genera "*Microsciurus*" and *Hadrosciurus* occupied a large area including the Boreal Brazilian and the South Brazilian dominions. The origin of "*Microsciurus*" dated to 3.2 Mya (2.5–3.7) and the origin of *Hadrosciurus* was estimated to 3.9 Mya (3.6–4.9). In summary, the majority of the cladogenetic events observed for Neotropical tree squirrels took place in the northern portion of South America and southern Central America during the Pliocene. Two major dispersal events apparently led to the colonization of Mesoamerica by the genus *Echinoscirus* and the colonization of the east of the Andes by the most recent common ancestor of the genera *Guerlinguetus*, "*Microsciurus*" and *Hadrosciurus* (**Figure 2**).

The scenario recovered by this analysis allows the inference of four founder-event speciation and five anagenetic dispersal events during the diversification of the tribe Sciurini across the Neotropics, and, once again, no vicariance event was inferred (see **Supplementary Figures 5, 6**). The founder-event speciation corresponds to the jump of the ancestors of (1) the genus *Echinosciurus* from the Pacific dominion to the Mexican transition zone and Mesoamerican dominion, (2) the genera *Guerlinguetus*, "*Microsciurus*" and *Hadrosciurus* from the Pacific dominion to the Boreal Brazilian dominion, (3) (*Guerlinguetus aestuans* "b", (*G. aestuans* "c", *G. brasiliensis*) from the Boreal Brazilian dominion to the South Brazilian dominion, and (4) (*Guerlinguetus aestuans* "c", *G. brasiliensis*) from the South Brazilian dominion to Boreal Brazilian, South-eastern Amazon, and Parana dominions. Anagenetic dispersal

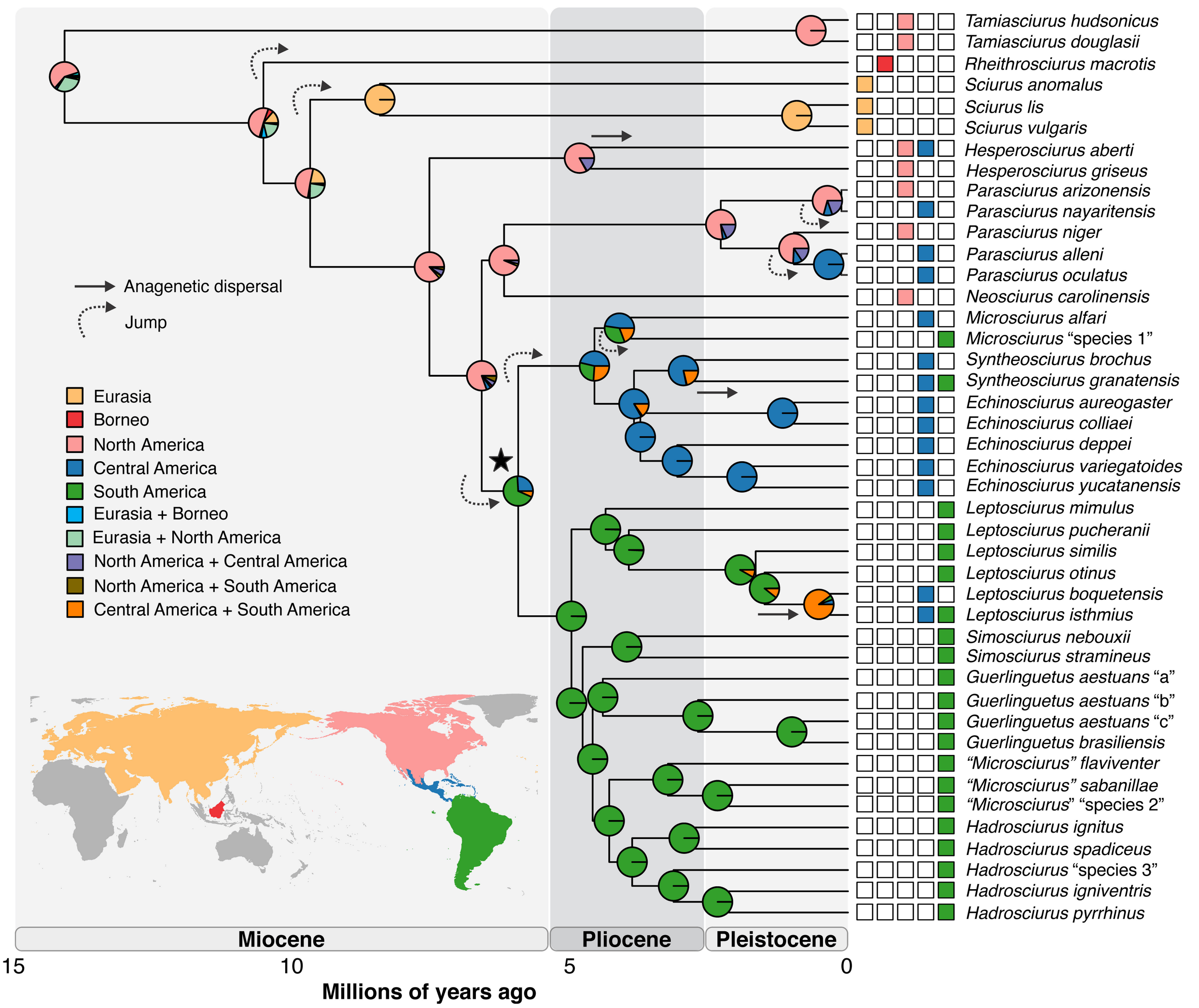

FIGURE 1 | Bayesian maximum clade credibility chronogram based on 13 mitochondrial protein-coding genes (11,278 bp) of 43 species of Sciurini and two species of Pteromyini used as outgroup (not shown). Ancestral ranges for Sciurini estimated by DEC + J model in the global scale analysis are illustrated. Colors in key boxes and map correspond to coloring of internal nodes (estimated probabilities of ancestral ranges) and terminal boxes (current distribution of species). Strait arrows denote anagenetic dispersal events and curved arrows designate founder-event speciation (jump). Black star identifies the node of Neotropical clade.

occurred with the colonization of (1) the Pacific dominion by *Echinosciurus variegatoides*, (2) the South Brazilian dominion by the ancestor of "*Microsciurus*" and *Hadrosciurus*, (3) the Boreal Brazilian and Parana dominions by the ancestor of *Guerlinguetus aestuans* "c" and *G. brasiliensis*, (4) the Pacific dominion by "*Microsciurus*" *sabanillae*, and (5) the Pacific dominion by *Hadrosciurus igniventris*.

Diversification Rates of the Tribe Sciurini

The LTT plot indicated an initial low and constant net diversification rate from about 14 to 10.5 Mya, followed by a moderate increase from 10.5 to 5 Mya, and an acceleration on the diversification rate from 5 Mya to the present (**Figure 3A**). This period of rapid inflation on the number of lineages, along the Pliocene and Pleistocene, coincides with the South American invasion and colonization by tree squirrels (**Figure 3A**; red star). The Bayesian approach, however, showed a pattern of constant speciation rate through time, with no inflection point and with a slight decrease on the magnitude of speciation over time (**Figure 3B**). The BAMM analysis also recovered the null model "no shifts" on speciation rate as the most probable (0.66) to represent the diversification of Sciurini. The mean speciation rate of Sciurini was 0.29.

DISCUSSION

The phylogenetic hypothesis presented here is concordant with the inferences depicted in Abreu-Jr et al. (2020), as we employed the same taxa and data source. We included in our analyses 43 of the 46 currently recognized species of tree squirrels (see Abreu-Jr et al., 2020). Previous biogeographic analyses have included up

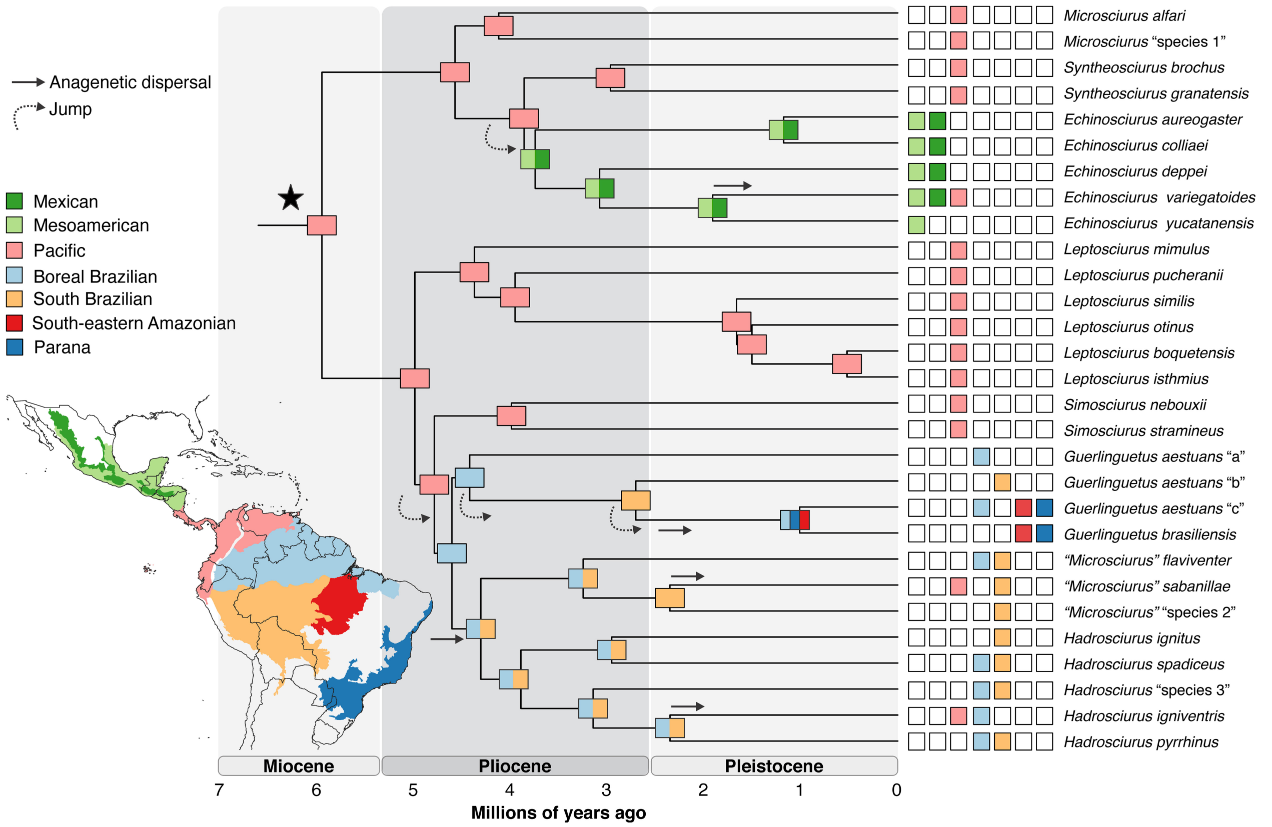

FIGURE 2 | The most probable biogeographical scenario for Neotropical Sciurini based on the set of ancestral ranges that received the highest likelihood in the Neotropical scale analysis, as inferred by BAYAREALIKE + J model. Colors in key boxes and map correspond to coloring of internal tree nodes (most probable ancestral ranges) and terminal taxa (current distribution of species). Strait arrows denote anagenetic dispersal events and curved arrows designate founder-event speciation (jump).

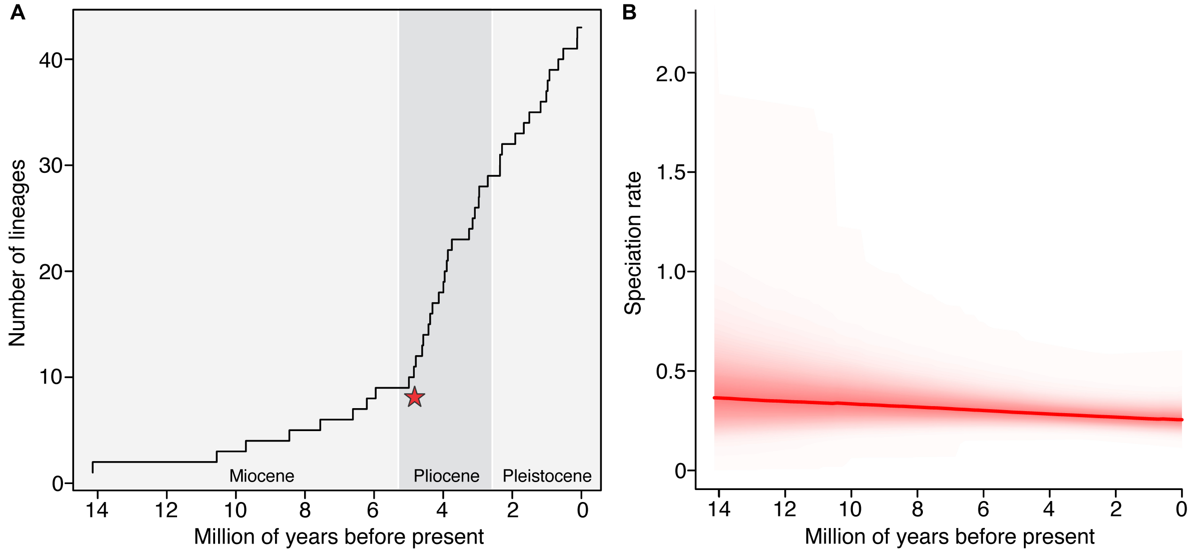

FIGURE 3 | Speciation plots of Sciurini. **(A)** Lineage-through-time (LTT) plot showing a rapid increase in lineage accumulation after the South American invasion (red star). **(B)** BAMM analysis demonstrating the mostly constant speciation rate of Sciurini through time; solid red line shows median estimate and thin red lines (cloud) indicate probability distribution.

to 28 Sciurini species, and less than a third of South American tree squirrel species (Pečnerová and Martínková, 2012; Pečnerová et al., 2015). Therefore, this is the most comprehensive study investigating the biogeographic history of the tribe Sciurini, both in number of species and geographic coverage.

The models with best fitting scores on each biogeographic analysis are distinct in their criteria, as one detects vicariant events (DEC) and the other (BAYAREALIKE) does not (Matzke, 2013). Both approaches employed to investigate the range of evolution of tree squirrels (analysis of the whole tribe and analysis focused on Neotropical taxa) did not recover vicariant events during the evolutionary history of Sciurini. These results suggest that vicariance was not a main driver of the diversification processes of tree squirrels at either global or local (Neotropical) scales (but extinctions might mask instances of vicariance; see discussion below). Dispersal events, on the other hand, were commonly recovered and founder-events were estimated to have happened more times than anagenetic dispersals in both approaches.

Our time estimation for the origin of Sciurini was similar to the result of Mercer and Roth (2003) and Upham et al. (2019), supporting a more recent date around 14 Mya compared to the estimation of Zelditch et al. (2015) and Pečnerová et al. (2015) around 18 and 19 Mya, respectively. This is also relatively more recent compared to the origin of the other tribes of the family Sciuridae that have been hypothesized to have happened before 18 Mya (Roth and Mercer, 2008). Our estimates suggested that the most recent common ancestor of Sciurini probably inhabited exclusively North America (near 60% of probability). This is in contrast to previous findings that suggested that the ancestors of this tribe originated in Eurasia or in a large area that included Eurasia and North America (Pečnerová and Martínková, 2012; Pečnerová et al., 2015; Rocha et al., 2016). Despite the higher probability for an exclusive North American origin, our results do not rule out the possibility of an origin that included both the Nearctic and Palearctic regions (supported as the ancestral range of Sciurini with about 30% of probability).

Pečnerová and Martínková (2012) suggested two alternative working hypotheses for the initial diversification and dispersal of tree squirrels. The first one postulates that the ancestor of the genus *Sciurus* dispersed from North America to Eurasia after diversification from *Tamiasciurus*. This ancestor diverged again in Eurasia and returned to North America via the Bering land bridge. The second hypothesis claims that ancestors of both *Tamiasciurus* and *Sciurus* originally occupied Eurasia and colonized the Americas in two distinct occasions, also crossing over Beringia. However, the results obtained by Pečnerová and Martínková (2012) do not allow them to choose conclusively between these two hypotheses and our results indicate a higher likelihood for a third distinct scenario. We found that the ancestors of *Tamiasciurus*, *Rheithrosciurus*, and the ancestor of the clade from which *Sciurus* diverged, occupied the Nearctic region with highest probability. Our results suggest that the ancestors of *Rheithrosciurus* and *Sciurus* dispersed from North America to Eurasia in two independent events, through subsequent jump dispersal events some thousand years apart (**Figure 4**). The cladogenetic events leading to *Rheithrosciurus* dated at 10.5 Mya and to the Eurasian *Sciurus* at 9.7 Mya, which supports the colonization of the Palearctic region by these two lineages before the opening of the Bering Strait around 7.4 Mya (Marincovich and Gladenkov, 1999).

Rheithrosciurus macrotis, endemic to Borneo (Thorington et al., 2012), possibly colonized the island overland from southeast Asia and has been isolated there since its arrival. Borneo is connected to mainland areas through the Sunda Shelf, and the distribution of shallow marine carbonates and the depths of sea water on the Sunda Shelf indicate the presence of past dispersal routes from the continent (perhaps via Java) to Borneo (Hall, 2001). Unlike *Rheithrosciurus*, the genus *Sciurus* successfully spread throughout Asia and Europe, and its species are currently widespread in the Palearctic region (Thorington et al., 2012).

Besides *Tamiasciurus*, the remaining Nearctic genera, *Hesperosciurus*, *Parasciurus*, and *Neosciurus*, also had their ancestors occupying North America. The estimated origin of *Hesperosciurus* dated to the late Pliocene (around 4.8 Mya), *Parasciurus* to the late Pleistocene (around 2.3 Mya), and *Neosciurus* diverged in the late Miocene (around 6.2 Mya). Thus, despite exhibiting the same ancestral range, the North American groups originated in distinct periods, during a relatively large temporal window (about 3.9 Mya) compared to the South American genera (see below).

The ancestral range of the large Neotropical clade was in South America, which indicates that the colonization of Central America by Neotropical taxa was via movement north back from South America (**Figures 1**, **4**). Remarkably, our estimates indicate that this clade originated around 6 Mya, an earlier date than ever suggested. Previous studies had estimated that tree squirrels colonized South America around 3 Mya (Mercer and Roth, 2003; Roth and Mercer, 2008; Pečnerová et al., 2015) and they argued that the South American invasion was conditioned by the closure of the Isthmus of Panama.

The date of the Panamanian land bridge formation—allegedly the moment that triggered the GABI—has gained a lot of attention and discussion in the scientific literature in the past decade. Current biological data suggest an early emergence of the Isthmus, around 6–8 Mya, and indicate that the biotic turnover associated with GABI was much longer and more complex than traditionally recognized, beginning perhaps as early as the Oligocene–Miocene transition (Bacon et al., 2015; Montes et al., 2015; Jaramillo et al., 2017; Molnar, 2017). However, comprehensive geological, paleontological, and also biological reviews pointed to scant evidence and analytical bias in some of the aforementioned studies, and sustained the conventional hypothesis of the formation of the Panamanian Isthmus *sensu stricto* around 3–2.8 Mya (Lessios, 2008, 2015; Leigh et al., 2014; Marko et al., 2015; O'Dea et al., 2016).

In addition to this ongoing discussion, Agnolin et al. (2019) proposed an alternative hypothesis to explain biotic interchanges between North and South America based on Pre-GABI land connections. The hypothesis from Agnolin et al. (2019) relies on paleontological and geological evidences for a large corridor of shallow waters and emergent spans of land connecting North and South America through the Caribbean islands during the

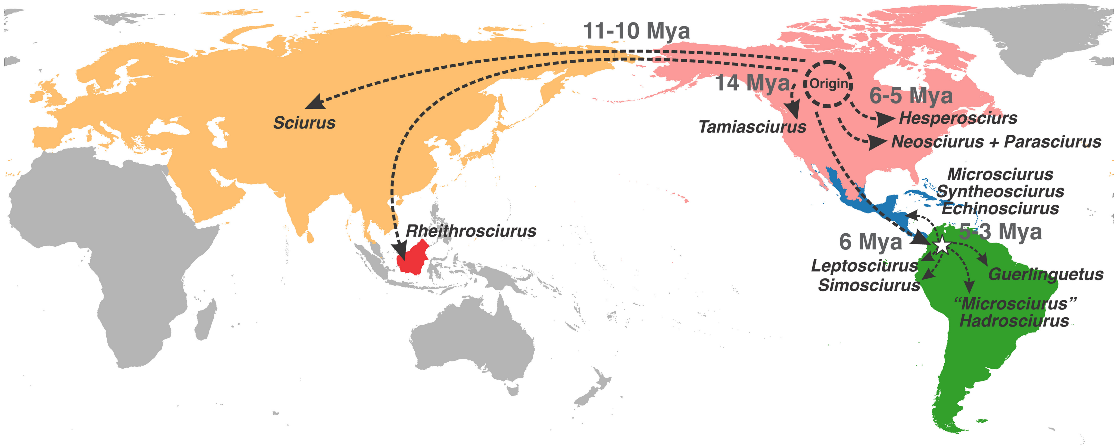

FIGURE 4 | Map summarizing the inferred colonization scenario for the tribe Sciurini showing the timing and the place of its origin and diversification in North America, the two independent dispersions into Eurasia, the invasion of South America, the re-invasion of Central America, and the diversification in South America.

Oligocene-Miocene, known as GAARlandia (Iturralde-Vinent and MacPhee, 1999). The presence of GAARlandia could help to explain the occurrence of early migrants (from the Miocene) in North and South America (see Pinto-Sánchez et al., 2012), considering the lack of strong geological evidence to sustain the early closure of the Panamanian Isthmus. Hershkovitz (1972); Savage (1974) and Reig (1984) also defended a much earlier entrance of land mammals in South America, in the early Miocene, prior to the GABI and the emergence of the land bridge, based on the patterns of diversity and endemism of sigmodontine rodents.

Our results for the first entrance of tree squirrels in South America could either fit this alternative route of colonization through GAARlandia or the traditional route recognized via the Panamanian Isthmus. Even if the complete establishment of the Isthmus occurred in the Pliocene, it might have been permeable to biota migration since the Oligocene (Molnar, 2008; Eizirik, 2012; Carrillo et al., 2014; Bloch et al., 2016). Marshall (1979) already defended that events of marine regression on Upper Miocene would have favored dispersion between South and Central/North America, via the Isthmus, suggesting its permeability. The timeframe of this invasion is also similar to what has been shown for sigmodontine rodents (Smith and Patton, 1999; Steppan et al., 2004; Parada et al., 2013). To the best of our knowledge, no previous hypothesis ever suggested that these two distinct lineages of rodents may have shared a parallel and similar history of invasion and dispersal throughout South America and re-invasion of Central America. During a similar lapse of time, sigmodontine rodents appear to have been more successful in adapting to a greater variety of ecosystems (including grasslands, shrublands, wetlands) and spreading as far as the temperate zone at the southern border of the continent, reaching a diversity of hundreds of species (Burgin et al., 2018). Squirrels, on the other hand, remained restricted to the mid-northern portion of the continent, inhabiting only dense and continuous forests (Vivo and Carmignotto, 2015), exhibiting much less species diversity.

The predominantly Central American radiation (including the genera *Microsciurus*, *Syntheosciurus* and *Echinosciurus*) is more likely to have originated in Central America around 4.6 Mya (probably southern Central America as suggested by the analyses focused on Neotropical taxa, see below), from a jump dispersal from South America. Also, most ancestors of the extant species within this radiation occurred in Central America. Two species, one from the genus *Microsciurus* (*M.* "species 1") and one from the genus *Syntheosciurus* (*S. granatensis*), colonized back to South America (**Figure 1**).

The major South American radiation is slightly older than the Central American one, with its date of origin estimated to around 5 Mya. Within this lineage all the ancestors occupied South America, except the most recent common ancestor of *Leptosciurus boquetensis* and *L. isthmius*, estimated to have inhabited Central America (**Figure 1**). Previous studies (Pečnerová and Martínková, 2012; Pečnerová et al., 2015) suggested a directional diversification process following the latitudinal gradient from North America to South America giving rise to all Neotropical taxa. Our results, supported by ancestral range estimation of Sciurini in both global and Neotropical scales, do not corroborate this hypothesis, and indicate a much more complex pattern, with distinct colonization events from South to Central America and vice-versa and perhaps through distinct dispersal routes.

Several Central American vertebrate lineages that originated along the Pliocene-Pleistocene boundary have been shown to exhibit their ancestral ranges in northern South America (Barrantes, 2009; Saldarriaga-Córdoba et al., 2017; Maestri et al., 2019). Savage (2002) proposed a two-step dispersal pulse hypothesis to explain amphibians and reptiles of South America inhabiting Middle America. The first colonization episode, according to him, occurred about 3.5 Mya when the sea level lowered and many taxa were able to invade Mesoamerica as far as Mexico. The second episode took place in the Pleistocene and the majority of taxa could not disperse further than southern Nicaragua or Costa Rica. The first episode described by Savage

(2002) coincides with the diversification and dispersion of the genus *Echinosciurus* to the Mesoamerican dominion and Mexican transition zone (see **Figure 2**), and the second episode coincides with the dispersal into Central America by *Leptosciurus boquetensis* and *L. isthmius* (see **Figure 1**). Both episodes of Central American colonization can be plausibly explained via the traditional dispersal route across the Isthmus of Panama.

The biogeographic analysis focused on Neotropical taxa recovered the origin of this clade in the Pacific dominion, which ranges from southern Central America to northwestern South America. This was estimated to have occurred in the late Miocene (around 6 Mya), when the Panamanian land bridge was still emerging and during the final uplift stages of the Andean Cordillera (Hoorn et al., 2010; Stange et al., 2018). This region was also the ancestral range of four genera: *Microsciurus* and *Syntheosciurus* (composed of Central and South American species), *Leptosciurus* (composed of species occupying mainly the northwestern portion of South America), and *Simosciurus* (composed of two species occurring in the coast of Ecuador and Peru). Within the mainly Central American radiation, a founder-event speciation is inferred, leading to the colonization of the Mexican transition zone and Mesoamerican dominion by the ancestor of the genus *Echinosciurus* around 3.7 Mya. Moreover, among these species, *E. variegatoides* colonized back to the Pacific dominion through anagenetic dispersal. Some groups of sigmodontine rodents also had their origin estimated to be in the late Miocene or Pliocene in northern South America, suggesting that this region has played an important role on the origin and diversification of forest rodents (Maestri et al., 2019). In the mid-Pliocene, when several of the cladogenetic events of the Central American radiation have been estimated, important geological events such as the development of the Sierra Madre oriental and occidental and the volcanism of the Trans-Mexican Volcanic Belt were taking place (Sedlock et al., 1993). This intense geologic activity culminating with the formation and expansion of highland forests might have played a crucial role on the diversification of several biotic components (see Morrone, 2010), perhaps including the tree squirrels.

The ancestors of the remaining South American genera (*Guerlinguetus*, "*Microsciurus*", *Hadrosciurus*) occupied areas east of the Andes with highest probability. This was the point (approximately 4.8 Mya) in the evolutionary history of Sciurini that tree squirrels crossed over the Andes and through a founder-event colonized large forested areas in the eastern portion of South America. *Guerlinguetus* showed the highest probability of ancestral occupancy in the Boreal Brazilian dominion, which includes the northern bank of the Amazon basin and the Guiana Shield. The ancestors of the genera "*Microsciurus*" and *Hadrosciurus* occupied an area including the Boreal Brazilian and South Brazilian dominions. Within *Guerlinguetus*, the ancestor of three species (*G. aestunas* "b", *G. aestunas* "c" and *G. brasiliensis*) colonized the South-eastern Amazonian dominion through a founder-event speciation. Through another founder event speciation, the ancestor of *G. aestuans* "c" + *G. brasiliensis* colonized the South-eastern Amazonian dominion. Subsequently, it expanded its range, reaching the Boreal Brazilian dominion and, for the first and only time in the tree squirrel diversification, the Atlantic Forest on the coast of Brazil (see **Supplementary Figure 6** for details). South-eastern Amazon and Atlantic Forest were the last two areas occupied by tree squirrels in South America.

The timing of Atlantic Forest colonization by tree squirrels (around 1 Mya) is comparable to the timing of the entrance of some species of sigmodontine rodents (such as species in the genera *Euryoryzomys*, *Hylaeamys* and *Oligoryzomys*; Steppan and Schenk, 2017; Maestri et al., 2019). At least two main distinct spatiotemporal routes have been proposed between the Amazon and the Atlantic Forest (Costa, 2003; Batalha-Filho et al., 2013; Ledo and Colli, 2017): older (middle to late Miocene) connections between southeastern Atlantic Forest and western Amazonia (the SE-NW bridge), and younger (Pliocene and Pleistocene) connections across northeastern Brazil (the NE bridge). As our results indicate a young invasion, squirrels might have used NE routes, possibly favored by interglacial periods of the Pleistocene, as the NE bridge hypothesis assumes.

Within the genera "*Microsciurus*" and *Hadrosciurus*, most species remained restricted to the Amazon basin and adjacent areas (along Boreal Brazilian and South Brazilian dominions). Only one species in each genus ("*Microsciurus*" *sabanillae* and *Hadrosciurus igniventris*) dispersed to the eastern slopes of the Andean cordillera and invaded the border of the Pacific dominion (**Figure 2**).

All South American genera originated from 4.4 to 3.2 Mya. This very short temporal window (about 1.2 My) overlaps with the South American Land Mammal Ages (SALMAs) of the Montehermosan (from about 6.8 to 4 Mya) and the Chapadmalalan (from about 4 to 3.4 Mya) (Flynn and Swisher, 1995). At this time, the Andean uplift was complete and the Amazon basin was in its final stages of formation with a large supply of Andean-derived sediments (Hoorn et al., 2010). These massive geological changes produced a great variety of new ecosystems in South America that favored the diversification of many groups of organisms.

South American genera usually do not overlap their distribution, except in a small area on the northern bank of the Amazon river, where one species of *Guerlinguetus* (*G. aestuans* "c") is sympatric with two species of *Hadrosciurus* (*H. igniventris* and *H. spadiceus*), and in the western Amazon basin, where species from "*Microsciurus*" and *Hadrosciurus* are mostly sympatric. It is important to mention that these last two genera are the most morphologically dissimilar among all South American forms. Species of "*Microsciurus*" are small squirrels with head and body length ranging from 125 to 180 mm, and species from *Hadrosciurus* are large, with head and body length ranging from 220 to 325 mm (Vivo and Carmignotto, 2015). It is also noteworthy that along lowland areas in the western Amazon basin, species of "*Microsciurus*" are more commonly found in non-flooded upland forests, while species of *Hadrosciurus* inhabit mainly seasonally inundated floodplain forests (Emmons and Feer, 1997). Besides tree squirrels, South America is the home of another remarkable radiation of Sciuridae, the Neotropical pygmy squirrel, *Sciurillus pusillus*. This singular radiation diverged early in the history of the family (about 35 Mya) and is included in its own subfamily, Sciurillinae (Mercer and Roth, 2003; Steppan et al., 2004; Roth and Mercer, 2008). The diversification and colonization of South America by the Neotropical pygmy squirrels are enigmatic pieces on the evolutionary history of this group.

Regarding speciation events generating the 43 extant species of Sciurini analyzed here, three species originated in the Miocene, 17 in the Pliocene, and 23 in the Pleistocene. The events in the Miocene were: the diversification of the Borneo endemic species *Rheithrosciurus macrotis*, the Eurasian *Sciurus anomalus*, and the North American *Neosciurus carolinensis*. During the Pliocene, the great majority of speciation events took place in South America, where ten species (from all genera) originated; in Central America five species originated (also from all genera) and in North America only one speciation event occurred (within the genus *Hesperosciurus*). In the Pleistocene, eight speciation events occurred in South America (within the genera *Leptosciurus*, *Guerlinguetus*, "*Microsciurus*", and *Hadrosciurus*), six in North America (within the genera *Tamiasciurus* and *Parasciurus*), and six in Central America (within the genera *Echinosciurus* and *Lepstosciurus*). In summary, most speciation events in North America occurred late in the Pleistocene, and in South America the majority occurred during the Pliocene or in the Pliocene-Pleistocene transition. Moreover, most cladogenetic events in South and Central America took place in the Pliocene. In North America and Eurasia, cladogenetic events occurred mainly early in the Miocene or late in the Pleistocene (**Figure 1**).

Pleistocene climatic fluctuations were probably a determining factor in the diversification process within North American lineages, for instance across the genera *Tamiasciurus* (see Arbogast et al., 2001; Chavez et al., 2011) and *Parasciurus*. In the Neotropics, Pleistocene fluctuations apparently played a secondary role, as dozens of speciation events occurred before the intensification of climatic cycles. It seems that the Central and South American lineages rapidly adapted to the new (and perhaps vacant) environments and experienced explosive diversification. This is quite different from the history of other rodent radiations in South America, rats and spiny rats, where most of the speciation events took place in the Pleistocene (Fabre et al., 2012; Steppan and Schenk, 2017).

Our analyses revealed a mostly constant (slightly decreasing) speciation rate through time for the tribe Sciurini. However, we observed a peak of lineage accumulation since the beginning of the Pliocene, which coincides with the Neotropical invasion, followed by a rapid diversification in this area. The constancy of the speciation rate (with no shifts during the Pliocene) might indicate that several extinctions may have occurred during the evolutionary history of tree squirrels. In this way, the number of species that went extinct possibly equalized the evident increase in species accumulation since the Pliocene and, therefore, the number of species maintained over time is similar to the number of extinct species. This could also strengthen the prevalence of founder-event speciation and overlook the presence of vicariant events in the ancestral range estimates of Sciurini, as extinctions might be expected to eliminate instances of vicariance and inflate instances of "jump" dispersal. Moreover, our analysis yielded a mean diversification rate around 0.29 for tree squirrels, which is slightly higher than the estimate of Zelditch et al. (2015) and similar to the diversification rate of muroid rodents in the same time frame (Steppan and Schenk, 2017). Finally, we conclude that the South American invasion was not as recent as previously inferred, but the speciation was indeed very fast, mostly restricted to a short period of time in the Pliocene, much earlier than for other groups of rodents.

DATA AVAILABILITY STATEMENT

The dataset generated for this study can be found in the Figshare Repository (https://doi.org/10.6084/m9.figshare.12288185.v1). GenBank accession numbers for all complete mitogenomes are provided in the **Supplementary Table S1**.

AUTHOR CONTRIBUTIONS

EA-J, and SP conceived the project. EA-J analyzed the data. EA-J, SP, AP, and JM interpreted and discussed the results. EA-J wrote the manuscript with contributions from SP, MT, DW, AP, JM. SP, AP, DW, and JM secured funding for this project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00230/full#supplementary-material

FIGURE S1 | Nucleotide substitution saturation plot. The number of transitions (s) and transversions (v) in the mitochondrial genome dataset is plotted against the F84 distances.

FIGURE S2 | Bayesian maximum clade credibility chronogram based on 13 mitochondrial protein-coding genes (11,278 bp) of 43 species of Sciurini and two species of Pteromyini used as outgroup. The mean heights along with the 95% highest posterior density intervals of dates are shown at each node.

FIGURE S3 | Chronogram employed on the range evolution estimation of the tribe Sciurini on a global scale, showing the numbers of the nodes.

FIGURE S4 | The most probable biogeographical scenario for Sciurini based on the set of ancestral ranges that received the highest likelihood in the global scale analysis, as inferred by DEC + J model. Colors in key boxes and map correspond to coloring of boxes within the tree (most probable ancestral ranges) and at terminals (current distribution of species).

FIGURE S5 | Chronogram employed on the range evolution estimation of Sciurini on a Neotropical scale, showing the numbers of the nodes.

FIGURE S6 | The most probable biogeographical scenario for Sciurini based on the set of ancestral ranges that received the highest likelihood in the Neotropical scale analysis, as inferred by BAYAREALIKE + J model. Colors in key boxes and map correspond to coloring of boxes within the tree (most probable ancestral ranges) and at terminals (current distribution of species).

TABLE S1 | List of specimens analyzed in this study, with geographic information and GenBank accession numbers for complete mitochondrial genomes. Taxonomic identifications follow Abreu-Jr et al. (2020). Voucher acronyms refer to the following scientific collections: American Museum of Natural History, United States (AMNH); Field Museum of Natural History, United States (FMNH); University of Kansas Natural History Museum, United States (KU); Museu Nacional da Universidade Federal do Rio de Janeiro, Brazil (MN); Museu Paraense Emilio Goeldi, Brazil (MPEG); Museum of Southwestern Biology, University of New Mexico, United States (MSB); Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Peru (MUSM); Museum of Vertebrate Zoology, University of California, United States (MVZ); Smithsonian National Museum of Natural History, United States (USNM).

TABLE S2 | Alternative states of ancestral range estimated by DEC + J model in the global scale analyses. Node numbers are the same depicted in the phylogeny of **Supplementary Figure S3**.

TABLE S3 | Alternative states of ancestral range estimated by BAYAREALIKE + J model in the Neotropical scale analyses. Node numbers are the same depicted in the phylogeny of **Supplementary Figure 5**. Areas acronyms are: Mexican transition zone (MEX), Mesoamerican dominion (MES), Pacific dominion (PAC), Boreal Brazilian dominion (BBR), South Brazilian dominion (SBR), South-eastern Amazonian dominion (SEA), and Parana dominion (PAR).

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Woody Cover Mediates Fox and Gray Squirrel Interactions

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Human activities are changing the environments that animal communities utilize, altering competition and co-existence dynamics as well as changing species distributions. A global consequence of human activity is increased woody cover, which changes environmental conditions on landscape, local, and fine spatial scales. These multi-scale processes are likely to change species interactions and distributions in unexpected ways. To elucidate these effects, we investigated the co-occurrence of two interacting species in a rapidly changing ecosystem. Eastern fox (*Sciurus niger*) and gray (*Sciurus carolinensis*) squirrels occur sympatrically throughout the southeastern United States. We used single-season, two-species occupancy modeling to understand what factors influence competition between the squirrels at three spatial scales. We found evidence that fox and gray squirrels compete at fine scales but not at local and landscape scales. The best model to explain fox and gray squirrel co-occurrence at fine scales was an additive fine-scale understory and tree canopy closure model. Increased fine-scale canopy closure correlated with increased fox and gray squirrel occupancy, while increased fine-scale understory cover correlated with decreased gray and fox squirrel occupancy. Fox squirrel occupancy probability declined in the presence of gray squirrels. The intensity of interaction was modified by fine-scale canopy cover and understory density. These findings support the hypothesis that species interactions can be an important factor in structuring biotic communities; however, the strength of the effect changes across spatial scales. Our results support the importance of considering species interactions at multiple scales when predicting and addressing changes in community composition in human modified and managed systems.

Keywords: gray squirrel, fox squirrel, competition, multispecies occupancy, woody encroachment

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INTRODUCTION

Human activities are changing the environments that animal communities utilize (Lewis and Maslin, 2015). These activities can alter species interactions and co-existence dynamics and change species distributions and community composition (Lyons et al., 2016). Changes in community composition are likely to be pronounced for ecologically similar species that often co-occur through specializations on multiple dimensions which result in multi-scale "trade-offs" (Kneitel and Chase, 2004). The benefits of ecological specialization at one scale often come with fitness costs at other scales (Fry, 2003; Bonsall et al., 2004). For example, local competitive advantage may be coupled with reductions in a species' ability to colonize new sites across a landscape (Cadotte, 2007). It is hypothesized that these multi-scale and dimensional competitive advantages allow similar species to co-occur (Kneitel and Chase, 2004). Nevertheless, the mechanism by which common, human-driven environmental changes alter how species interact at multiple scales is poorly understood (Tews et al., 2004).

Habitat loss and fragmentation, two major features of anthropogenic environmental change, are typically assessed at landscape spatial scales (Fischer and Lindenmayer, 2007). However, humans also change environments at more localized scales (Wilbanks and Kates, 1999), such as by suppressing fire or altering the number of trees within a forest patch through forest management. On a landscape, increased habitat edge from fragmentation may favor species that are poor competitors but can cross the matrix separating patches (Marvier et al., 2004). However, on the local level, fragmentation may favor species that are superior competitors, especially when resources are scarce (Ditchkoff et al., 2006). Understanding these patterns is critical to predicting how human activities will alter ecological communities.

One global environmental change that may be altering how species interact is woody plant encroachment hereinafter, woody encroachment (Eldridge et al., 2011; Stanton et al., 2018). Globally, grassy and savanna biomes are changing as woody species increase understory density and close canopies (Spector and Putz, 2006). Anthropogenic induced changes including fire suppression, increased atmospheric carbon, and unmanaged livestock grazing contribute to this environmental change (Roques et al., 2001; D'Odorico et al., 2012). Areas impacted by woody encroachment experience changes in species composition, such as open savanna specialists being replaced by generalists (Sirami and Monadjem, 2012). Woody encroachment can lead to increased homogeneity on landscape scales (Anthelme et al., 2007), while increasing structural complexity at fine scales (Sirami et al., 2009). Further, the physical characteristics of woody encroached areas may differ depending on successional conditions, with early stages exemplified by heightened understory cover and later stages defined by closed canopies with little to no understory (Van Auken and Bush, 2013).

To better understand how woody encroachment and habitat fragmentation alter species interactions on multiple scales, we investigated the co-occurrence of two sympatric species in a rapidly changing savanna ecosystem: the eastern fox (*Sciurus niger*) and gray (*Sciurus carolinensis*) squirrels. These two species have overlapping distributions throughout most of the southeastern United States. The historic pine savannas of this region are changing as hardwoods, especially oaks (*Quercus* spp.), have begun to dominate the understory and midstory, causing the once open canopy to close (Provencher et al., 2001; Freeman and Jose, 2009; Platt et al., 2015). Fox squirrels appear to be sensitive to competition and gray squirrels may be replacing fox squirrels in some parts of their range due to their competitive advantage in closed canopy systems (Brown and Batzli, 1985; Sexton, 1990; Sovie, 2019). Fox and gray squirrels partition resources but the scale, how they partition resources over time and space, and the intensity of interactions may change over ecological gradients (Sexton, 1990; Edwards et al., 1998; Derge and Yahner, 2000; Van Der Merwe et al., 2005; Sovie et al., 2019). Moreover, these ecological gradients and partitioning of resources are likely being altered by fragmented landscapes and woody encroachment.

Our objective was to identify if anthropogenically altered environmental conditions influence fox and gray squirrel co-occurrence across scales. Co-occurrence and conditional occupancy (i.e., the probability of a species occupying a site given the presence of another species) can be thought of as a measure of competition (Gotelli and McCabe, 2002; Ovaskainen et al., 2010; Richmond et al., 2010; Sebastian-Gonzalez et al., 2010). If a species is less likely to occur in the presence of another species, that may be a signal of competitive exclusion (Richmond et al., 2010).

We investigated squirrel co-occurrence at three biologically relevant spatial scales. In line with some ecological theories (Pearson and Dawson, 2004), we predict that species interactions are not an important driver of squirrel distributions at landscape scales. However, we predict that fox and gray squirrels interact in localized areas and their interactions may be influenced by landscape scale processes that regulate path connectivity and dispersal (Goheen et al., 2003). Finally, we predict that interactions between the squirrels may be moderated by fine-scale vegetation characteristics that increase predation risk (minimal understory and canopy cover) and decrease gray squirrel foraging advantage (Bowers et al., 1993; Van Der Merwe et al., 2005).

MATERIALS AND METHODS

Study Area

To investigate fox and gray squirrel co-occurrence, we conducted field surveys throughout North and Central Florida in 2012 and 2013. The region had a humid, sub-tropical climate, with warm wet summers (May–October: average high of 35°C and 121 cm of rain) and dry, mild winters (November–April: average high of 25°C and 25 cm of rain) (Hagemeyer et al., 2010). Vegetation communities across our study sites included open grasslands, pine-dominated forests, pine hardwoods, hardwood hammocks, bottomland hardwood forests, and pine clear cuts. Canopy trees commonly included longleaf (*Pinus palustris*), slash (*Pinus elliottii*), and loblolly (*Pinus taeda*) pines, and turkey (*Quercus laevis*), live (*Quercus virginiana*), laurel (*Quercus laurifolia*), and water (*Quercus nigra*) oaks.

Study Design

Squirrels select habitat on multiple scales, and variables that influence selection on one scale may not have predictive power at other scales (Johnson, 1980; Greene and McCleery, 2017a). Selection on smaller scales may be dependent on processes occurring at larger scales (McGregor et al., 2014). To assess the influence of environmental features on the co-occurrence of gray and fox squirrels, we surveyed using a nested (hierarchical) design at three spatial scales: fine (0.01 ha), local (5.3 ha), and landscape (765 ha) (**Figure 1**; Greene and McCleery, 2017a).

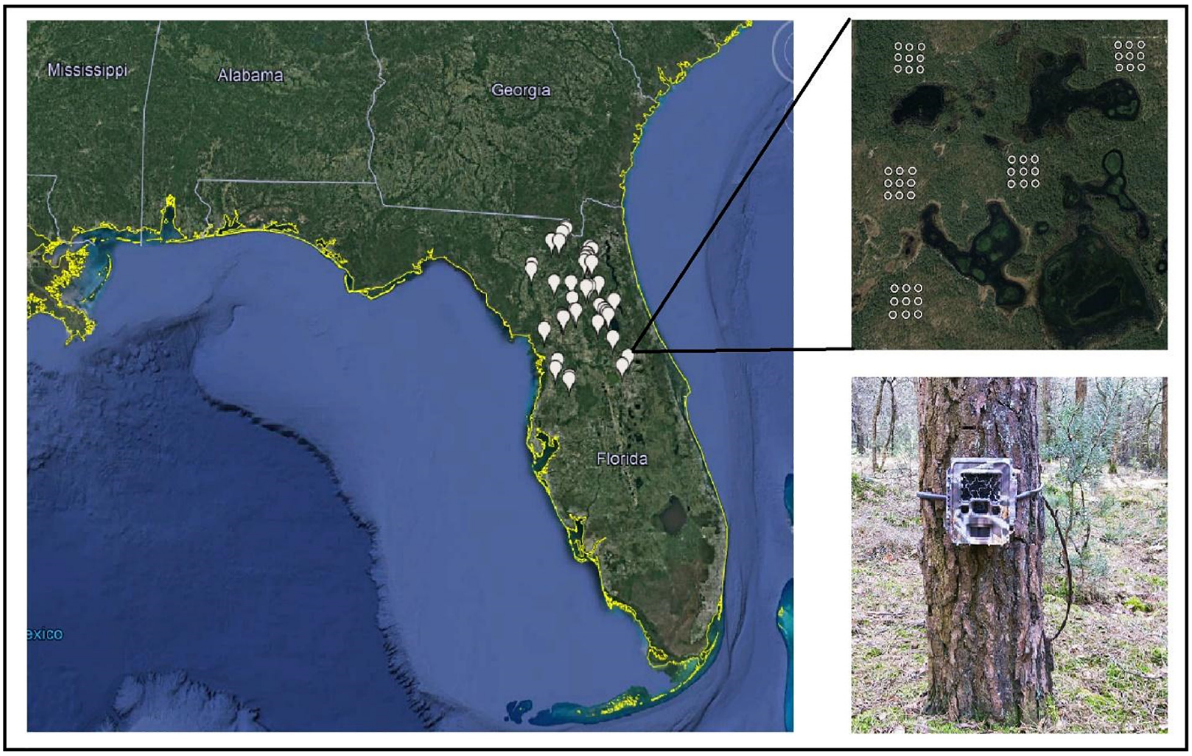

FIGURE 1 | The distribution of the 40 landscapes, local girds, and camera points (fine scale) surveyed in a multi-scaled study of habitat use and selection of fox squirrels and gray squirrels in North and Central Florida, United States, 2013–2014.

To represent the landscape scale, we placed 40 7.65 km^2 blocks throughout North and Central Florida using ArcGIS 10.1 (ESRI, Redlands, CA, United States). We selected 10 landscapes in upland pine or sandhills, 10 in mesic/shrubby flatwoods, and 20 landscapes randomly without regard to a land cover type. To represent the local scale, we randomly placed five 5.3 ha grids within each landscape. To maximize grid independence and to reduce spatial autocorrelation, we separated grids by ≥500 m; the pooled mean maximum distance moved from southeastern fox squirrel trapping and radio-telemetry studies (Greene and McCleery, 2017b). To study fine-scale occupancy, we placed nine camera traps within each grid as a 3 × 3 array with 115 m spacing between points.

We surveyed for each species using camera traps, a reliable method to survey squirrels (Greene et al., 2016; Greene and McCleery, 2017b). At each point, we placed a game camera (Bushnell Trophy Cam model 119436, Bushnell Outdoor Products, Overland Park, KS, United States) 50 cm above the ground and angled toward a bait pile of pecans and cracked corn. We set cameras to take three photos every time the camera was triggered using the camera's "normal" sensitivity setting. We placed camera traps within a landscape and grid concurrently and allowed cameras to collect data for <10 days. We conducted camera trapping from 01 January to 01 July in 2013 and 2014 and surveyed during this time because temperatures are mild and squirrels are more active (Moore, 1957; Weigl et al., 1989). See Greene and McCleery (2017a) for more details on the camera trapping protocol.

Environmental Measurements

We measured fine, local, and landscape scale variables hypothesized to affect squirrel occupancy, detection, and co-occurrence. Non-random co-occurrence may result from species-specific habitat preference, and thus, we controlled for species-specific habitat preference by measuring variables known to influence fox and gray squirrel occupancy. These variables included pine and oak density, understory cover, canopy closure, and canopy heterogeneity (Cottam and Curtis, 1956; Conner et al., 1999; Boone et al., 2017; Greene and McCleery, 2017a). We hypothesized that structural elements such as canopy cover, canopy cover heterogeneity, understory cover, and local and landscape fragmentation and connectivity mitigate squirrel interactions due to the ability of each species to move about the matrix differently (Goheen et al., 2003). Finally, we measured variables that we expected to influence the probability of detecting fox and gray squirrels. Specifically, these included visual obstruction that may interfere with the cameras field of vision and variation in camera trapping effort across the grid and landscape.

Fine scale

Interactions between fox and gray squirrels appear to be the most pronounced at fine scales, which includes interactions on individual trees (Conner et al., 1999). Fox squirrels will change their temporal behavior if they overlap with gray squirrels at individual cameras (Sovie et al., 2019). The competitive advantage of gray squirrels may be moderated by fine-scale vegetation characteristics that increase predation risk such as minimal understory and canopy cover (Caswell, 1978). Fox squirrels may use these risky areas and avoid antagonistic interactions with gray squirrels (Brown, 1999; Steele et al., 2015). Therefore, at each fine-scale site, we measured canopy closure using a concave spherical densiometer (Lemmon, 1956; Forestry Suppliers, Inc., Model-C). To capture variation in woody encroachment, we measured understory cover using the line-intercept method (Canfield, 1941; Greene and McCleery, 2017a). Finally, we measured visual obstruction that may interfere with the cameras field of vision (<45 cm in height) in five, 1/2 m × 1/2 m quadrats (Daubenmire, 1959; Greene and McCleery, 2017a).

Local scale

Fox and gray squirrels both respond to localized (∼1–4 ha) forest structure, specifically canopy and understory cover (Brown and Batzli, 1985; Greene and McCleery, 2017a). Gray squirrels occupy areas with elevated understory cover, closed canopy cover, or dense hardwoods (Brown and Batzli, 1985). Fox squirrels appear to avoid these areas but will enter them in the absence of gray squirrels (Sovie, 2019). Increased canopy and understory cover on landscape and local scales may deter squirrel predators (Potash et al., 2019), allowing gray squirrels to proliferate and out compete fox squirrels. How fox and gray squirrels interact in localized areas may also be influenced by the degree of canopy cover fragmentation or connectivity (Zollner, 2000; Goheen et al., 2003). To measure local environmental characteristics, we used the U.S. Forest Service Tree Canopy analytical layer (Jin et al., 2013) from the 2011 National Land Cover Database. We estimated the average percentage of canopy cover at each grid from 30 × 30 m pixels. Southeastern fox squirrels generally occupy areas with non-overlapping canopies typical of older, fire maintained pine stands (Perkins et al., 2008), and thus, we defined each 30 × 30 cell as either closed (>50% canopy cover) or open (<50% canopy cover) and calculated patch metrics for closed patches. We calculated mean patch size and patch density as measures of fragmentation and used the area-weighted mean patch radius of gyration as a measure of connectivity (McGarigal, 2014). We calculated patch metrics using the *calculate_lsm* function in the *landscapemetrics* package (Hesselbarth et al., 2019) in R version 3.6.1 (R Core Team, 2019). To estimate local measures of understory cover, we used the enhanced vegetation index (EVI) from March 22, 2013, from the MODIS satellite recording (Didan, 2015). EVI is remote-sensed measure of biomass that corrects for canopy background noise and is sensitive in areas with dense vegetation (Singh et al., 2015).

Landscape scale

We examined squirrel interactions at the landscape scale even though some ecological theories suggest that environmental gradients, not biotic interactions, should drive species distribution at larger spatial scales (Pearson and Dawson, 2004). However, it is possible that species compete across landscapes (Araújo and Luoto, 2007). Specifically, fragmentation generally favors species with higher dispersal ability (Cote et al., 2017). Fragmentation and heterogeneity may increase the fox squirrels' competitive advantage at the landscape scale, specifically when habitat characteristics mimic savanna-like conditions (Greene and McCleery, 2017a). Gray squirrels are generally found on homogeneous landscapes with large forest patches proximate to each other (Moore and Swihart, 2005). Landscape characteristics that act as barriers to gray squirrels, such as agricultural fields (Goheen et al., 2003), may diminish their competitive advantage and facilitate coexistence on large spatial scales. In contrast, processes like hardwood encroachment may increase landscape homogeneity and favor gray squirrels. Therefore, we calculated mean canopy cover, understory cover, mean patch size, patch density, and mean gyration for each 7.65 km^2 landscape using the methods described for local scale metrics.

Data Analysis

We elected to investigate fox and gray squirrel interactions using multi-species occupancy models (Rota et al., 2016). Multi-species occupancy models can reveal evidence of interactions between species that are imperfectly detected and from data that cannot reliably produce density estimates (Gotelli and McCabe, 2002; Ovaskainen et al., 2010; Richmond et al., 2010; Sebastian-Gonzalez et al., 2010). We converted raw camera trap data into encounter histories (detected or not) for each species and scale. We defined sites and surveys differently between scales. Starting with the fine scale, we considered each camera trap point ($N = 1,800$) as a site, and each 24-h period the camera was active a survey (≤10 days). For the local scale, we considered each grid as a site ($N = 200$) and each camera point as a survey [9 surveys (i.e., points per grid)]. For the landscape scale, we considered each 7.65 km^2 block as a site ($N = 40$) and the local scale grids within each landscape as a survey ($N = 5$).

We analyzed squirrel occupancy in R version 3.6.1 (R Core Team, 2019) using the package *unmarked* (Fiske et al., 2015). We used the Rota et al. (2016) single season, two-species occupancy model. In our parameterization, we estimated the probability of: (1) occupancy of fox squirrels (ΨA), (2) occupancy of gray squirrels (ΨB), (3) occupancy of fox squirrels given gray squirrels are present (ΨA:B), (4) occupancy of gray squirrels given fox squirrels are present (ΨB:A), (5) detecting fox squirrels (pA), and (6) detecting gray squirrels (pB). The Rota et al. (2016) parametrization allows for stable model behavior and the incorporation of covariates; however, it does not *a priori* assume one species is dominant over the other.

To identify if environmental variables influence how gray and fox squirrels interact on different scales, we were primarily interested in the conditional occupancy parameters (ΨA:B and ΨB:A). Nevertheless, we wished to control for detection and independent habitat associations [(pA), (pB), (ΨA) (ΨB)]. To determine the best models for (pA), (pB), and (ΨA) (ΨB), we undertook a sequential modeling process (**Supplementary Table S1**). Using the best predictors for (pA), (pB), and (ΨA) (ΨB), we then built models to test how woody encroachment, fragmentation, and habitat connectivity influence squirrel interaction ($\Psi A{:}B$ and $\Psi B{:}A$). Depending on the stage of succession, woody encroached sites may have elevated understory cover (as measured by line-intercept or EVI), or increased canopy closure (as measured by concave densiometer or canopy cover), or both (high understory and canopy closure) (**Table 1**; Van Auken and Bush, 2013). We measured fragmentation as the mean patch density of closed canopy forest at grid and landscape scales and connectivity as the mean gyration at grid and landscape scales. We normalized habitat variables using a log + 1 transformation to improve model convergence. For additive models, we examined the relationships between our covariates at each scale and dropped 1 of the pair if their Pearson's correlation coefficient was $>| 0.70|$. We allowed hypothesized drivers to influence squirrel occupancy across scales. For example, gray squirrels may use low canopy closure fine-scale areas and exclude fox squirrels if the broader environment has high canopy cover and supports gray squirrels (e.g., tree fall gaps) (Dill and Houtman, 1989; Van Der Merwe et al., 2005). Further, fox squirrels may use fine-scale areas with high canopy cover within highly heterogenous landscapes where gray squirrels do not usually occur (e.g., within pine savannas). Finally, we considered models that did not incorporate interaction (i.e., $\Psi A{:}B = 0$) or that treated species interaction as independent from environmental conditions (i.e., constant $\Psi A{:}B = 1$). We ranked models using AIC and model weight (Burnham and Anderson, 2002). We considered models within 2 AIC units of the top model competing models. We inspected the 95% CIs around the parameter estimates and β coefficients of competing models to see if the CIs crossed 0. If competing models differed from the best model by only one parameter and the CI of that parameter crossed 0, we based our inference off the simplest model (Burnham and Anderson, 2002).

RESULTS

At the fine scale, we successfully surveyed 1,737 sites. We detected gray squirrels at 270, fox squirrels at 217, and both at 17 points. At the local (grid) scale, gray squirrels were present at 86 of 200 sites, fox squirrels at 74 sites, and both at 23 sites. At the landscape scale, we detected gray squirrels on 38 of 40 landscapes, fox squirrels on 26 sites, and both on 20 sites. Our samples captured the environmental gradient in our system (**Supplementary Table S1**). We found that tree canopy and understory cover were weakly positively correlated at all scales (Fine = 0.27, $p < 0.01$; Grid = 0.28, $p < 0.01$; Landscape = 0.20, $p < 0.01$).

TABLE 1 | Measured variables.

Prediction	Fine	Local	Landscape
Canopy closure	Densiometer	Canopy cover	Canopy cover
Understory cover	Line-intercept	EVI	EVI
Fragmentation		Patch density	Patch density
		Mean patch area	Mean patch area
Connectivity		Mean gyrate	Mean gyrate

The best model to explain fox and gray squirrel fine-scale co-occurrence indicated that fox squirrels were less likely to occur at points occupied by gray squirrels. This interaction was moderated by fine-scale canopy cover and understory density ($\Psi A{:}B\ \beta_{\text{Fine canopycover}} = -6.25$, SE = 0.79; $\beta_{\text{Fine understorydensity}} = 3.19$, SE = 0.54). Fox squirrel occupancy increased with fine-scale canopy closure in the absence of gray squirrels and decreased with fine-scale canopy closure in the presence of gray squirrels (**Figure 2A**). Fox squirrel occupancy remained stable with increasing understory cover in the presence of gray squirrels and declined with increased fine-scale understory cover in the absence of gray squirrels (**Figure 2B**). We did not detect a change in gray squirrel conditional occupancy in the presence of fox squirrels (**Supplementary Figure S1**). Increased fine-scale canopy closure correlated with increased fox squirrel occupancy and increased fine-scale understory cover correlated with decreased fox squirrel occupancy ($\Psi A\ \beta_{\text{Fine canopycover}} = 0.60$, SE = 0.08; $\beta_{\text{Fine understorydensity}} = -3.30$, SE = 0.36). Increased fine-scale canopy closure correlated with increased gray squirrel occupancy and increased fine-scale understory cover correlated with decreased gray squirrel occupancy ($\Psi A\ \beta_{\text{Fine canopycover}} = 5.91$, SE = 0.76; $\beta_{\text{Fine understorydensity}} = -0.12$, SE = 0.36). Co-occurrence for all models at fine-scales included obstruction decreasing p for both species [pA $\beta_{\text{Obstruction}} = -0.29$, SE = 0.30; pB $\beta_{\text{Obstruction}} = -2.18$, SE = 0.31].

At the local scale, the best model did not include a parameter for co-occurrence, matching the no interaction prediction (**Table 2**). Increased landscape canopy cover correlated with decreased fox squirrel occupancy ($\Psi A\ \beta_{\text{Landscape canopycover}} = -4.59$, SE = 0.90), while local canopy cover correlated with increased gray squirrel occupancy ($\Psi B\ \beta_{\text{Local canopy}} = 1.16$, SE = 0.28). Survey effort influenced fox squirrel detection [pA $\beta_{\text{Effort}} = 0.39$, SE = 0.10], and gray squirrel detection was constant [pB $\beta_{\text{Intercept}} = -0.67$, SE = 0.08]. Models incorporating landscape understory cover, mean patch area, constant interaction, and mean patch gyration had similar AICc support to the top model (**Table 2**), but the 95% CIs for the parameter estimate for $\Psi A{:}B$ crossed 0.

At the landscape scale, the best model also did not include a parameter for co-occurrence, matching the no interaction prediction (**Table 2**). Fox squirrel occupancy correlated with decreased landscape canopy cover ($\Psi A\ \beta_{\text{Landscape canopycover}} = -5.21$, SE = 2.12), while gray squirrel occupancy was constant ($\Psi B = \beta_{\text{Intercept}} = 1.52$, SE = 0.44). The model included constant detection probability for both fox and gray squirrels [pA $\beta_{\text{Intercept}}$ 0.25, SE = 0.18; pB $\beta_{\text{Intercept}}$ 0.10, SE = 0.17]. The model representing constant species interaction had a similar AICc support (**Table 2**); however, the 95% CIs for the parameter estimate for $\Psi A{:}B$ crossed 0.

DISCUSSION

We found patterns of fox and gray squirrels co-occurrence that are indicative of potential competition at fine scales (0.01 ha) but not at local (5.3 ha) or landscape scales (765 ha). At fine scales, fox squirrels were less likely to

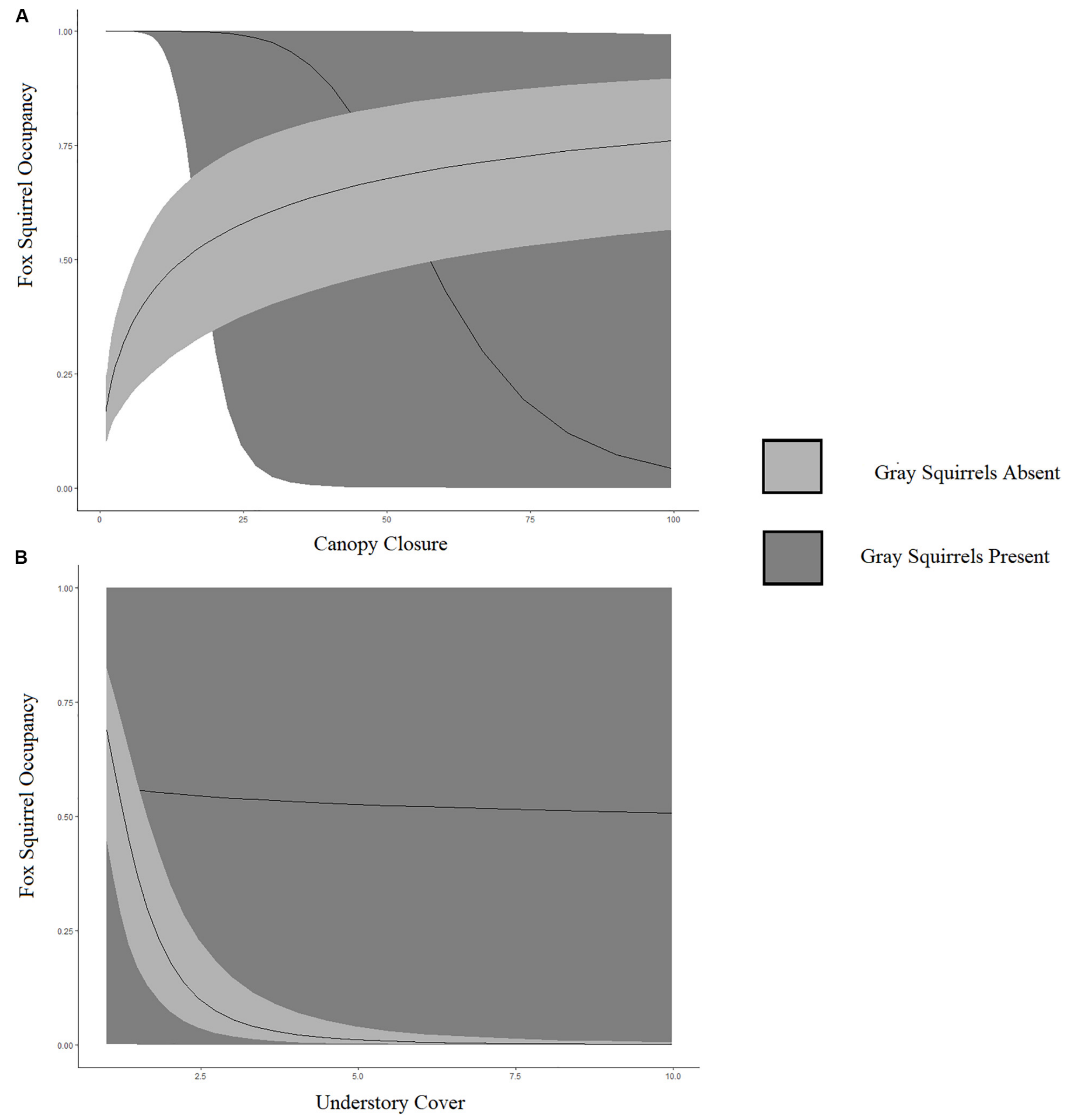

FIGURE 2 | Relationship between fox squirrel occupancy probability and fine-scale canopy closure **(A)** and undercover cover **(B)**, given gray squirrels are absent (light gray) or present (dark gray) in fine-scale sites located in North and Central Florida, United States, in 2013–2014. **(A)** Fox squirrel occupancy increased with fine-scale canopy closure in the absence of gray squirrels and decreased with fine-scale canopy closure in the presence of gray squirrels. **(B)** Fox squirrel occupancy remained stable with increasing understory cover in the presence of gray squirrels and declined with increased fine-scale understory cover in the absence of gray squirrels.

occur in areas occupied by gray squirrels, while gray squirrel occupancy was not influenced by the presence of fox squirrels. Further, physical site characteristics helped to explain the co-occurrence patterns of fox and gray squirrels. In the absence of gray squirrels, fox squirrels readily utilized closed canopy areas but did not utilize them in the presence of gray squirrels. These findings support the hypothesis that the strength of species interactions changes over spatial scales and vegetation gradients (Kelt et al., 1995; Pearson and Dawson, 2004).

In the few fine-scale environments where they co-occurred fox squirrels were limited by gray squirrel in areas of closed canopy. These fine-scale interactions may help us explain the broad habitat partitioning patterns we observed. Generalist gray squirrels were ubiquitous in human modified landscapes, while specialist fox squirrels were not. In the more open landscapes where fox squirrel still occur, they appear limited at the fine scale by gray squirrels in areas of closed vegetation which are spreading across the region. These fine-scale patterns suggest a competitive advantage for gray squirrels in conditions that are

TABLE 2 | Top four multispecies occupancy models for fox and gray squirrels in North and Central Florida, United States, for fine, local, and landscape scales based on AIC ranking.

Scale	Model name	*K*	AIC	ΔAIC	AIC*w*
Fine[1]	ΨA:B(Fine understory density + Fine canopy cover)	13	5982.40	0.00	1.00
	ΨA:B(Fine canopy cover)	12	6005.13	22.72	0.00
	ΨA:B(Fine understory cover)	12	6019.42	37.01	0.00
	ΨA:B(Grid EVI)	12	6019.62	37.22	0.00
Local[2]	ΨA:B(No interaction)	7	2159.85	0.00	0.16
	ΨA:B(Grid canopy cover)	9	2160.14	0.29	0.14
	ΨA:B(Constant)	8	2160.41	0.56	0.12
	ΨA:B(Grid patch density)	9	2160.71	0.86	0.10
Landscape[3]	ΨA:B(No interaction)	5	485.89	0	0.39
	ΨA:B(Constant)	6	487.45	1.56	0.18
	ΨA:B(Landscape canopy cover)	7	487.78	1.89	0.15
	ΨA:B(Landscape canopy cover + Landscape EVI)	8	488.91	3.02	0.08

Scale multispecies occupancy models included: [1]Fine: ΨA(Fine understory density + Fine canopy cover), ΨB(Fine understory density + Fine canopy cover), pA(Obstruction), pB(Obstruction); [2]Local: ΨA(Landscape canopy cover) ΨB(.) pA(.) pB(.); [3]Landscape: ΨA(Landscape canopy cover) ΨB(Grid canopy cover) pA(.) pB(Effort).

increasingly common and also suggest a potential mechanism for broader scale patterns.

Fox and gray squirrels both responded positively to increases in canopy closure and negatively to increases in understory cover; however, they differed in the intensity of their response to each factor. Fox squirrels responded strongly (>25× more than gray squirrels) to understory cover and gray squirrels responded strongly to canopy closure (∼10× more than fox squirrels). Our results suggest that gray squirrels are better at utilizing closed canopy areas and outcompete fox squirrels in these areas. In the absence of gray squirrels, fox squirrels appear to readily utilize sites with closed canopies. Our results support the idea that interaction between these species may also be mediated by predation (Caswell, 1978; Van Der Merwe et al., 2005). Increases in understory density limit sightlines to detect predators for fox squirrels, thus reducing foraging efficiency (Potash et al., 2019). Further, increases in canopy closure reduce the foraging efficiency of gray and fox squirrel avian predators (Bowers et al., 1993; Van Der Merwe et al., 2005). Increases in canopy and understory cover are often due to human-induced factors including increased atmospheric CO^2 and fire suppression (Roques et al., 2001; D'Odorico et al., 2012). Historically, fire shaped southeastern forests by maintaining broken canopies and low understory growth (Glitzenstein et al., 1995). Fire suppression on a local scale results in woody succession, first by increasing understory density (discouraging fox squirrels) and eventually leading to closed canopies (favoring the invasion of gray squirrels) (Keane et al., 2002; Swihart et al., 2007). Gray squirrels are efficient foragers and can persist in highly modified systems (Williamson, 1983; Kenward and Holm, 1989). Their ability to utilize resources in a small area may compensate for their poor dispersal ability across fragmented landscapes (Williamson, 1983; Goheen et al., 2003). Fine-scale interaction with fox and gray squirrels and increased canopy cover at the landscape scale may result in the loss of fox squirrels across woody encroached systems (Conner et al., 1999; Van Der Merwe et al., 2005; Greene and McCleery, 2017a).

As woody encroachment alters the interactions between fox and gray squirrels, there may be cascading effects throughout the ecosystem. Gray squirrels tolerate higher densities and overlap than fox squirrels (Koprowski, 1994), increasing both inter and intraspecific competition in invaded ecosystems. Fox and gray squirrels are important seed predators and scatter hoarders. Competition alters the probability that a seed is cached and where animals place those seeds (Muñoz and Bonal, 2011). Changes in scatter hoarding behavior alter the dispersal and reproductive success of many tree species (Whittaker and Jones, 1994; Terborgh et al., 2001; Silman et al., 2003). In the presence of competitors, squirrels are more likely to cache seeds in high-risk areas, such as broken canopy savannas (Heinrich and Pepper, 1998; Murray et al., 2006). Placing hard mast in risky, open areas may accelerate hardwood encroachment in these areas and act as a positive feedback mechanism. Thus, the invasion of pine savannas by gray squirrels due to woody encroachment may have long-term consequences for forest structure. Future investigations of the interactions between fox and gray squirrels may benefit from considering density dependent interactions and temporal changes in occupancy rates. Competition is a density dependent process and the relationship between fox squirrels, gray squirrels, and their environment may be highly dependent on their relative densities (Rosenzweig, 1991; van Beest et al., 2014). Further, we investigated a specific point in time for our analysis and compared different habitat types as a "space-for-time" substitution (Pickett, 1989). Longer term monitoring of fox and gray squirrel co-occupancy may reveal more nuanced relationships or expose competition at larger scales.

Our results suggest that human modification of environmental conditions favor generalist species that can utilize fine-scale resources. In our system, canopies are closing on fine, local, and landscape scales due to human activities. Generalists, like gray squirrels, have greater tolerances to the broad landscape changes occurring in our system (Sirami and Monadjem, 2012). Fox squirrels, like many longleaf pine savanna specialists, did not occur in areas with extensive canopy cover indicative of homogenizing human landscapes. In our system, generalists like gray squirrels, Southern flying squirrels (*Glaucomys volans*), and Virginia opossums (*Didelphis virginiana*) with good fine-scale competitive advantages are likely to replace specialists like fox squirrels, red cockaded woodpeckers (*Leuconotopicus borealis*), and gopher tortoises (*Gopherus polyphemus*), ultimately simplifying species diversity in this imperiled ecosystem. Our results support the importance of considering species interactions at multiple scales in predicting and addressing changes in community composition in human modified systems (Araújo and Luoto, 2007).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and review of the manuscript. AS conducted the data analysis. DG and RM conceived the study design. DG conducted the data collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00239/full#supplementary-material

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REVIEW
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Squirrel-Seed Interactions: The Evolutionary Strategies and Impact of Squirrels as Both Seed Predators and Seed Dispersers

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For many squirrel species, their intense – arguably coevolutionary – interactions with seed and nut producing trees have significantly shaped their biology and diversity. Here we provide an overview of this relationship in a range of forest types worldwide. We first review the evidence for how forest composition (conifer, hardwood, mixed hardwood and overall diversity of tree species) influences interactions between squirrels and seed trees and, ultimately, the role of squirrels as either seed predators or seed dispersers. We review, for example, the intense selective pressure squirrels exert on conifer trees as seed predators and the diversity of morphological traits and behavioral strategies that allow them to efficiently exploit this critical resource. In contrast, we show how the squirrel's role shifts to one of seed disperser in hardwood forests and how the specifics of this interaction varies further with forest structure, forest composition and climatic conditions. We then review the growing evidence for the tight ecological and evolutionary dance between the squirrels and the oaks that has shaped the biology of both across the globe. We show how a suite of seed (acorn) characteristics (e.g., chemical gradients, germination schedules, seedling morphology and tolerance-resistance strategies) are all intimately tied to the scatter-hoarding decisions of several squirrel species. And, based on studies in oak forests in Central America, Mexico, North America, and Eurasia, we also highlight the behavioral strategy of embryo excision now reported for six species across at least four genera of squirrels. This behavior, glaringly absent in other scatter-hoarding rodents worldwide, is now known be an innate trait in at least two species, one in Asia and another in North America. We review extensive recent research on one species of squirrel, the Siberian chipmunk (*Tamias sibiricus*), which exhibits a suite of behavioral strategies unique to that of other squirrels that independently contributes to seed dispersal and establishment. Finally, we outline numerous remaining questions concerning plants and other taxa of squirrels still open to investigation.

Keywords: sciuridae, seed predation, seed dispersal, scatterhoarding, coevolution, seed chemistry

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INTRODUCTION

Among the squirrels, the intensive use of tree seeds has clearly shaped both the biology of trees and their diaspores, which in turn have significantly influenced evolution of the squirrels' behavior, ecology and even some aspects of their morphology. In most, but not all cases, the evidence for tight coevolutionary interactions are not evident, yet the effects and outcomes of the interactions between squirrels and some tree species are nevertheless quite compelling. Steele et al. (2007) and Steele (2008) first reviewed interactions between tree squirrels and trees emphasizing numerous such interactions. Since this earlier research, a significant number of additional studies have further clarified our understanding of these relationships.

Our goal here is to highlight these recent findings and to further focus our understanding of the ecological and evolutionary interaction between squirrels and trees. We are especially interested in distinguishing direct ecological and evolutionary interactions between squirrels and seeds as well as those in which squirrels are more likely a member of a broader array of seed consumers that exhibit a diffuse suite of evolutionary interactions with seed trees and their seeds. We also review recent intensive studies of the Siberian chipmunk (*Tamias sibiricus*) a resident of conifer, mixed, and hardwood forests across northern Asia, which suggest a relatively tight evolutionary relationship with the oaks, somewhat distinct from that of other squirrel species.

SQUIRRELS AND CONIFER FORESTS

Tree squirrels are significant seed predators in conifer and mixed forests throughout both temperate and boreal biomes (Steele and Koprowski, 2001; Steele, 2008; Steele et al., 2008; Steele, in press) but under some limited conditions can act as agents of dispersal (Wauters and Casale, 1996; Zong et al., 2010, 2014). In these forests, squirrels consume large quantities of seeds, often approaching entire seed crops, usually in a highly efficient manner which places strong selective pressure on conifers (Smith, 1970, 1998; Elliot, 1974, 1988; Steele and Weigl, 1992; Benkman, 1995, 1999; Benkman et al., 2001; Talluto and Benkman, 2013). But, as several studies have shown, the conifers appear to also exhibit characteristics in direct or indirect response to squirrels. And, in other cases, there is evidence that behavioral and even morphological traits of squirrels follow from conifer and cone traits (see Smith, 1970, 1998).

Seed predation of conifers by squirrels usually results from (1) removal of large quantities of cones just prior to cone opening for the purposes of larder-hoarding for upcoming periods of food scarcity [e.g., *Tamiasciurus hudsonicus* and *T. douglasii* in boreal forests of North America (Smith, 1965, 1970, 1981; Steele, 1998, 1999; Boutin et al., 2000, 2006; Siepielski and Benkman, 2007, 2008a,b, 2010)], or (2) consumption of immature, green cones during the ripening of seeds long before cone opening. Examples of the latter include predation of longleaf pine (*Pinus palustris*) cones by the southeastern fox squirrel, *S. niger* (Steele, 1988, in press; Steele and Weigl, 1992; Steele and Koprowski, 2001), predation of cones of Ponderosa pine (*Pinus ponderosa*) by Abert's squirrel (*S. aberti*), and similar harvesting of immature cones by Eurasian red squirrels (*Sciurus vulgaris*) in stands of Scots (*P. sylvestris*) and Corsican pines (*P. nigra*) in Belgium, and of Norway spruce (*Picea abies*) and Arolla pine (*P. cembra*) in mixed conifer forests of the Italian Alps (Wauters and Dhondt, 1987; Molinari et al., 2006; Zong et al., 2010; Di Pierro et al., 2011). As reviewed by Steele (2008) another key manner in which squirrels can influence cone production and seed dispersal in conifers is by the indirect effects of bark stripping not reviewed here.

Direct evolutionary interactions between *Tamiasciurus* and conifers has been argued independently by both Smith (1970, 1998) and Benkman and colleagues (e.g., Mezquida and Benkman, 2005, 2014), with both suggesting strong evidence for the direct selection between conifers and cones (Steele, in press). Smith (1998) argues that east of the Cascade Range in western North America, the dry climate, due to the rainshadow, results in frequent forest fires. These conditions led to cones of Ponderosa pine that have a hard cone surface, strong, close attachment to the branch, asymmetrical shape at the base and fewer seeds per cone – all characteristics that appear to challenge seed predation by the red squirrel found on the eastern slope of the Cascades. There the American red squirrel exhibits larger body mass, stronger jaw muscles and a more robust lower jaw, necessary for harvesting and opening these serotinous cones. West of the Cascades, where the closely related Douglas squirrel (*T. douglasii*) is found, there is a wet maritime climate and no forest fires. Cones are non-serotinous and exhibit a soft surface, weak point of attachment to the stem and significantly more seeds per cone. The Douglas squirrel exhibits a smaller body size, weaker jaw muscles and a weaker lower jaw, all of which Smith (1998) suggest follows from the cone's characteristics.

Smith has also argued that across conifer species selection by *Tamiasciurus* spp. is likely to have differing selective pressures depending on cone and seed size (1970; 1998). Smith suggests that strong selection by *Tamiasciurus* on small cones is likely to result in cones with smaller and fewer seeds per cone, characteristics that contribute to high feeding costs for squirrels. In contrast, conifers species that produce larger seeds are likely to evolve cones that shed their seeds earlier to reduce predation by larder-hoarding squirrels.

Research by Craig Benkman and colleagues (e.g., Benkman, 1995; Parchman and Benkman, 2008; Siepielski and Benkman, 2008a,b) paints a similar but equally compelling picture about the evolutionary interactions between *Tamiasciurus* spp. and at least six conifer species in western North America. One such example is limber pine (*P. flexilis*), which the squirrels regularly feed on when still immature and attached to the tree (Siepielski and Benkman, 2008a). By comparing sites within the range of limber pine where red squirrels are present today (Rock Mountains) and sites where these squirrels have been absent for more than 10,000 years (Great Basin), the authors found significantly different cone characteristics, with trees investing only half as much energy in seeds per cone where squirrels were present. Where red squirrels were present, cones were found to have fewer seeds per cone, smaller seeds, larger cones, more resin per cones, and greater seed-coat thickness, all traits that likely defend against seed predation.

Selection of conifer seed and cone traits by these squirrels also significantly impedes avian dispersal by these pines. Conifers that depend on avian dispersal (by jays and nutcrackers [family Corvidae]) generally exhibit specific seed and cone characteristics (large seed size usually >90 mg, a greater seed-wing ratio, longer periods of seed retention, and specific cone orientation), that favor dispersal by these corvids (Lanner, 1999; Siepielski and Benkman, 2007). However, where pine squirrels are found, resulting seed and cone characteristics generally prevent dispersal by corvids (Siepielski and Benkman, 2007, 2010; Parchman et al., 2011). Where pine squirrels are found in both limber and whitebark pine (*P. albicaulis*) forests, for example, lower seed densities per cone prevent dispersal by Clark's nutcracker (*Nucifraga columbiana*) which in turn results in lower tree recruitment and as much as 50% lower stand densities (Siepielski and Benkman, 2008b).

Benkman and associates have also demonstrated how squirrels influence the close evolutionary relationship between the crossbill (*Loxia curvirostra*) and conifers. Crossbills appears to have coevolved with lodgepole pine in western North America (Benkman, 1999, 2003; Benkman et al., 2001, 2013), black spruce (*Picea mariana*) in Newfoundland (Parchman and Benkman, 2002) and in stands of the Allepo pine (*P. halepensis*) in forests of the Iberian Peninsula (Mezquida and Benkman, 2005). However, this close ecological and evolutionary relationship which significantly benefits seed dispersal is disrupted by pine squirrels in North America and Eurasian red squirrels (*S. vulgaris*) in the latter region. In the Italian Alps, the Eurasian red squirrel is an intensive seed predator of the Arolla pine during periods of low and moderate seed production but potentially shifts to a conditional mutualist during high seed years, possibly as a result of heavy mast production (Zong et al., 2010). The Arolla pine, which produces large, wingless seeds depends on nutcrackers and Eurasian red squirrels for dispersal. The squirrels are highly dependent on both seeds, directly eaten from the green immature cones and those scatterhoarded for use through the winter and the following spring. Further examination of caching behavior in different habitat types (Norway spruce vs. Arolla pine), show that seed densities per cache were lower and patterns of cache recovery were higher in spruce-dominated forests than in the Arolla pine forests (Zong et al., 2014). Also shown in Alpine forests of Europe, the Eurasian red squirrel avoids seeds of silver fir (*Abies alba*) over those of Norway spruce, on which they readily consume, most likely as a result of high levels of terpenes (i.e., limonene) in the scales and seeds of the former species (Rubino et al., 2012).

Yet another set of squirrel traits that appear to follow directly from their dependence on conifers for food are both their social system and key aspects of their reproductive strategies. In boreal forests of North America, the social structure of the red squirrel has been attributed to their dependence on conifer seeds, their behavior of larder-hoarding and the need to defend the larders (Smith, 1998). Other reproductive traits that likely follow for this intense dependence on cones is the bequeathal of territories from mothers to young (Larsen and Boutin, 1994), anticipatory parental care by larder-hoarding prior to conception and the ability to anticipate cone masting and increase reproduction well in advance of cone production (Boutin et al., 2006). Subtle differences in social structure, reproductive strategies and home range between Mount Graham red squirrels (*T. fremonti grahamensis*) in Arizona, United States, and Eurasian red squirrels in the Alps of Italy, both of which occur in conifer forests, appear to be tied closely to availability of cone resources (Mazzamuto et al., 2020).

THE SQUIRRELS IN DECIDUOUS FORESTS

In deciduous forests, the tree squirrels shift from almost exclusive seed predators to important agents of seed dispersal (Steele et al., 2005; Steele, 2008). This interaction, however, is always accompanied by both negative impact of both seed predation and their contributions to dispersal. It is also complicated by the greater number of seed types, as the diversity of seed trees increases significantly in most hardwood forests, as do the numerous characteristics of hardwood diaspores (Sundaram et al., 2015; Sundaram, 2016). Their positive impact on seeds and nuts follows from their behavior of moving these diaspores from their source trees, storing them in microsites that are often optimal for both germination and storage (e.g., Yi et al., 2013b). This behavior of directed dispersal in which a seed disperser moves seeds to sites also ideal for establishment, in the case of many tree squirrels (*Sciurus* spp.), occurs because cache microsites optimal for cache storage eventually facilitate seedling establishment if the seed remains in the cache.

Gray Squirrel Responses to the Oaks

Early studies focused on the food preferences of eastern gray squirrels for the many species of oaks (*Quercus* spp.) and the primary differences in acorns of red oak (RO, section *Lobatae*) and those of white oak (WO, section *Quercus*). Acorns of ROs exhibit higher levels of both lipid (∼20%) and tannin (5–15%) than those of the WO (lipid ∼10% and tannin <2%). In addition, RO species exhibit a delayed germination requiring a period of cold stratification before germination is possible. WO species, in contrast, germinate immediately upon seed fall pending water availability. Smallwood and Peters (1986), first demonstrated that these differences in seed chemistry resulted in a food preference for WO acorns with lower tannin, regardless of energy level, early in the autumn. Preferences then shifted to acorns high in energy, regardless of tannin levels later in the winter when energy requirements peaked.

Gray squirrels are highly sensitive to germination schedules, selectively dispersing and caching red oaks over those of WOs due to their differences in perishability that follow directly from these germination patterns (Hadj-Chikh et al., 1996). This sensitivity of gray squirrels to seed perishability extends to acorns infested with weevils (*Curculio* spp.) and other insect larvae, even when there is no external evidence of infestation, suggesting a particularly keen ability to detect acorn suitability for storage (Steele et al., 1996). Several studies (Steele et al., 2001a; Sundaram, 2016; Sundaram et al., 2020) now collectively show that gray squirrels rely on chemical cues from the shell or cotyledon to determine if acorns are no longer dormant. These cues, however, are masked by waxes during dormancy and are only detectable when the waxes degrade during the acorn's emergence from dormancy. Although acorns that are no longer dormant are still suitable for consumption, they are far less likely to be cached by squirrels.

When WOs are cached in early autumn, however, gray squirrels will consistently excise the embryo with a few scrapes of the incisors, removing the embryo and thereby killing the acorn (Fox, 1982), allowing the acorn to remain intact in the cache for up to 6 months (Steele et al., 2001b). Experiments with naive squirrels demonstrate that selective caching of RO acorns over those of WO and the behavior of embryo excision are both innate strategies among eastern gray squirrels (Steele et al., 2006), although the latter is hypothesized to be perfected by trial and error.

The field experiments by Hadj-Chikh et al. (1996), in NE Pennsylvania, showed that squirrels based caching decisions on acorn perishability over handling time (acorn size), an important determinant of caching decisions of gray squirrels in some conditions. Comparable experiments to that of Hadj-Chikh et al. (1996) in central Mexico, on caching decision of the Mexican gray squirrel (*S. aureogaster*), a closely related congener of the eastern gray squirrel, however, revealed markedly different results. In these temperate, but milder environments of Mexico, the Mexican gray squirrels selectively cached the larger acorns regardless of germination schedules and corresponding perishability. And, across all trials, there was a significant correlation between acorn size (handling time) and caching frequency (Steele, in press). However, most interestingly, when caching acorns of WO species, many of which are larger than those of RO species in Mexico, *S. aureogaster* consistently excised the embryo of these acorns. Thus, this squirrel is clearly aware of the perishability but likely caching larger acorns for the greater reward. Milder climate in central Mexico may mean this species scatter-hoards acorns for shorter periods and thus focuses on short term rewards rather than long-term storage.

Dormancy and nut size influence not only caching decisions but also the distance seeds are dispersed. For many tree species, dispersal away from the parent tree significantly reduces density-dependent mortality of seed and seedlings by seed predators and pathogens, which is highest near maternal sources (Janzen, 1970; Connell, 1971). Several studies have indicated that RO species are consistently dispersed farther and cached more than WO species (Steele et al., 2001b, 2007, 2011, 2014; Moore et al., 2007; Lichti et al., 2014; Steele, in press). And of course when WOs were dispersed embryos of the acorns were often removed, killing the fruit.

Acorn size, which varies tremendously within and across oak species and influences both dispersal and caching decisions (Steele et al., 2011, 2014). When other acorn traits are controlled for, larger acorns are cached more frequently and dispersed farther from the source (Steele et al., 2007, 2011). In addition, gray squirrels respond to these larger rewards (larger acorns) by dispersing acorns into open areas, some distance from canopy cover (Steele et al., 2011) where, even in the face of a high density of conspecifics, these more preferred acorns are far less susceptible to pilferage (Steele et al., 2014). Based on estimates of predation risks (Steele et al., 2015), we concluded that these sites are relatively immune to pilferage because they are placed where conspecifics are less likely to pilfer caches. Further, this series of studies open up a new line of investigation that suggests that scatter-hoarding squirrels that risk predation for cache sites with lower rates of pilferage may contribute significantly to seed dispersal when they are actually preyed upon when caching (Lichti et al., 2017; Lichti et al., in review).

Taken together, many of these studies suggest that gray squirrels, when faced with multiple choices among different oaks and even other hardwoods, are regularly making a diversity of decisions when scatter-hoarding. Lichti et al. (2014), for example, demonstrated that when presented with paired options of red oak (*Q. rubra*, RO), white oak (*Q. alba*, WO) and American chestnut, (*Castanea dentata*, AC), gray squirrels selectively dispersed RO over WO, RO over AC, but also AC over WO.

The Close Evolutionary Relationship Between Squirrels and Oaks Based on Germination Schedules and Cache Perishability

Embryo removal or embryo excision by tree squirrels to arrest germination in early germinating acorns (e.g., white oak species [section *Quercus*]) was first documented by Fox (1982) in eastern gray squirrels and is now known to occur in other species of *Sciurus* and at least three other genera of *Sciuridae* that frequently depend on acorns for food storage. To perform this behavior, squirrels simply flip the acorn and with a few quick scrapes of the incisors remove the embryo (Fox, 1982), thereby arresting germination and allowing the otherwise perishable acorn to remain intact during the food storing period (Steele et al., 2001b). Since previous reviews on this interaction (e.g., Steele et al., 2005; Steele, 2008), studies across the globe now show that this behavior occurs under a variety of circumstances, for at least six species of squirrels. Moreover at least two of these species exhibit a definitive innate tendency to perform the behavior (Steele et al., 2006; Xiao and Zhang, 2012).

Experiments on free-ranging gray squirrels Hadj-Chikh et al. (1996) clearly documented that this species is highly sensitive to acorn perishability when making caching decisions, selectively dispersing and caching dormant RO acorns (section *Lobatae*) over those WO (section *Quercus*) regardless of acorn size (handling time). And, when even limited numbers of white oak acorns were cached, the embryos of these acorns are removed.

In an extensive series of 18 experimental trials, similar to the six trials conducted by Hadj-Chikh et al. (1996), in which free-ranging Mexican gray squirrels (*S. aureogaster*) were presented with pairs of six species of native RO acorns and five species of WO acorns, Steele (in press) reports unexpected responses by this species. Squirrels consistently dispersed acorns short distances and stored larger acorns with greater handling time, regardless of oak type (section) and germination schedule.

Because many of these WO species are larger than those of RO, early germinating WOs were consistently cached more frequently than those of RO. Across these 11 oaks species, Steele (in press) reports a significant correlation between acorn mass and caching frequency. Steele (in press) suggests that this nearly opposite response to that of eastern gray squirrels, likely follows from the milder climate and the shorter period over which acorns are likely stored. Opposite of that reported by Hadj-Chikh et al. (1996), this study suggests that seed size was a more immediate determinant of caching decisions. However, when these WO acorns were cached, squirrels consistently excised embryos, thus indicating a sensitivity and ability to respond to acorn dormancy (Steele et al., 2001b; Steele, in press). These observations suggest that, although immediate priorities for caching shifted in this environment, the squirrels were still secondarily responding to early germination with embryo excision.

Several recent studies in Asia indicate that at least three additional squirrel species including the Pallas' squirrel (*Callosciurus erythraeus*) (Xiao et al., 2009), Pére David's Rock squirrel (*Sciurotamias davidianus*) (Xiao et al., 2010) and the Asian red-cheeked squirrel (*Dremomys rufigenis*) (Xiao and Zhang, 2012) all perform embryo excision on early germinating, non-dormant acorns, as well as a few other seed types. Yet the specific circumstances under which this occurs varies with seed types, squirrel species and masting conditions.

Xiao et al. (2009) reported the responses of free-ranging Pallas's squirrel to early germinating acorns of *Quercus variabilis* (QV) and nuts of *Castanea henryi* (CH), which exhibit a delayed germination period. In contrast, to most WO species in North America and Mexico, acorns of QV exhibit high tannin levels. When presented with these contrasting traits, Pallas's squirrels selectively cached QV acorns over nuts of CH, but excised the embryos of the latter at a much higher frequency. Squirrels also performed embryo excision on QV acorns as they recovered and re-cached these acorns through the hoarding season. Thus, Pallas's squirrels prioritize lower tannin levels over germination schedule when presented with these conflicting demands, but still depend on embryo excision for management of early germinating nuts.

Also reported by Xiao et al. (2010) in Central China, the endemic Pére David's Rock squirrel performs embryo excision on WO species (*Quercus aliena* var. *acutesevata*, *Q. serrata* var. *breviptiolata*, *Q. variabilis*) and two other species that produce dormant seeds: the Qinggang oak (*Cyclobalanopsis multinervis*) and the Chinese chestnut (*Castanea mollissima*). However, the frequency of embryo removal is much higher in the WOs and was significantly more evident in higher mast years (Xiao et al., 2010). The authors concluded that the interaction between seed germination and seed masting significantly influenced the squirrel's hoarding decisions. Xiao et al. (2013) also explored how acorn size and dormancy both interacted to influence the behavior of dispersal and embryo excision Pére David's Rock squirrel. As predicted, larger acorns were selectively dispersed father but for those that were early germinating species were more likely to have their embryos removed.

Xiao et al. (2010) presented only acorns of two WO species (*Quercus aliena* var. *acutesevata, Q. serrata* var. *breviptiolata*) to Pére David's Rock squirrel, both of which varied in the frequency of early germination during the hoarding period. These authors found that frequency of germination phenotypes accounted for as much as 40.5 and 21.4% of the dispersal success in these two species, respectively. This suggested that the relative selection of these phenotypes by this squirrel, combined with their behavior of embryo excision, resulted in frequency-dependent selection that likely helps maintain variation in the germination phenotypes in these two species. This suggests a strong evolutionary impact of the squirrel on the acorn germination phenotype.

Collectively, the studies reviewed above show how the behavior of embryo excision is an important behavioral strategy that enables several squirrel species across the globe to manipulate acorn scatter-hoards under a range of environmental situations. Further evidence for the adaptive significance of this behavior follows from two isolated studies, one in North America and another in Southwestern China, that demonstrate the innate basis of this behavior in two squirrel species. In the first, Steele et al. (2006) raised Eastern gray squirrels without any previous experience with acorns and then compared their responses to acorns of the northern red oak (*Q. rubra*) and the white oak (*Q. alba*) to those of captive wild-caught squirrels. Both naive and wild squirrels selectively cached RO acorns over those of WO. In addition, wild-caught squirrels consistently excised embryos of early germinating WO acorns. However, naive squirrels also attempted embryo excision of white oak acorns but often failed to successfully excise the embryo. Naive squirrels often attempted to excise embryos from the wrong (basal) end of the acorn or failed to insert the incisors deep enough to successfuly remove the acorn, indicating clearly that the behavior was innate but also in need of additional experience to better optimize outcomes (Steele et al., 2006).

Similarly, Xiao and Zhang (2012) presented free-ranging Asian red-cheeked squirrels (*D. rufigenis*) with acorns of an early germinating WO (*Q. variabilis*) and two dormant oaks (*Cyclobalanopsis stewardiana* and *C. glaucoides*) in an ancient forest where all WOs species had been extirpated hundreds of years earlier. These squirrels excised embryos of WOs when storing them and on occasion removed embryos of the dormant acorns as well. After eliminating the possibility that these squirrels likely emigrated from distant stands of WOs or learned from other squirrels (e.g., Pallas's squirrel), these authors concluded that the behavior was innate in this species as well.

Evidence of the presence of this behavior in six species of squirrels across four genera worldwide, along with evidence for the innate basis of the behavior in two species, one in North American and the other in Southeast Asia, strongly suggests convergence of this trait in squirrels. This is particularly evident when one considers the broad distribution of this behavior across the phylogeny of the Sciuridae (Steele, in press), coupled with evidence that the behavior appears absent in other squirrels that regularly handle acorns in some parts of their range (e.g., *Tamiasciurus* spp., *S. vulgaris, Tamias* spp.).

The behavior of embryo excision by squirrels appears to have also been met with an evolutionary response of WOs. One such example appears to have occurred in acorns of *Q. mongolica* which produces embryonic tissue (seed) set deeper in the acorn farther from the apical tip of the fruit (Zhang et al., 2014). Simulation of embryo excision in this species, compared with several other species with embryos closer to apical end of the fruit (*Quercus variabilis*, *Q. acutissima*, *Q. aliena*, *Q. aliena var. acutiserrata*, *Q. serrata. var. brevipetiolata*), suggests a clear advantage to this trait. In all other species, except *Q. mongolica*, artificial embryo excision resulted in a significant drop in germination and seedling performance. Acorns of *Q. mongolica* were consistently able to sustain attempts at embryo excision and still successfully germinate. Moreover, Zhang et al. (2014) reported that free-ranging squirrels of several species were far less likely to perform embryo excision on *Q. mongolica*, compared with acorns of these other oaks.

Partial Acorn Consumption and Diffuse Evolutionary Responses to Seed Size and Chemical Gradients in Acorns

Although a limited number of squirrel species (six, **Table 1**) worldwide are now reported to perform embryo excision on early germinating acorns, adaptations of these early germinating oaks present a significant challenge to numerous other seed predators that disperse or feed on these acorns, including several rodents, corvids and insect larvae (e.g., *Curculio*). Among the rodents, for example, Steele (in press) reports that in eastern deciduous forests of North America, the eastern chipmunk (*T. striatus*), deer mouse (*Peromsucus maniculatus*), white-footed mouse (*P. leucopus*) and the southern flying squirrel (*Glaucomys volans*) all selectively cache ROs over WOs but show no sign of embryo excision. The first two species are also known from field experiments to selectively disperse RO acorns over those of early germinating WOs. Despite the challenge these species face with early germinating WOs, there is yet another diffuse co-evolutionary relationship that all of these species appear to collectively engage in with the acorns of oak.

Several rodents, including squirrels, as well as blue jays (*Caynocitta cristata*) and even *Curculio* larvae, selectively feed on the basal end of these acorns well away from the embryo, consistently abandoning the seed with much of the cotyledon still intact (Steele et al., 1993, 1998; Perea et al., 2011; Yang and Yi, 2012; Steele, in press). Recent findings (Steele, in press) indicate that in addition to tannins–which are highest in the apical end of the fruit (Steele et al., 1993), near the embryo–lipids, Na and potentially other nutrients show an opposite gradient, with levels highest in the basal end of the seed (**Figure 1**, Steele, in press). Moreover, germination and seedling studies indicate that a notable portion of partially eaten acorns can still germinate and establish as seedlings (Steele, in press), despite the intensity of damage (Bartlow et al., 2018). Numerous early germinating oak species can even sustain complete acorn removal once the radicle is established (Yi et al., 2012b, 2013a, 2015, 2019a). Together these observations suggest a diffuse co-evolutionary relationship between multiple seed predators, including the squirrels, and the oaks that quite likely results in significant dispersal of oaks under conditions of high seed abundance.

BEHAVIORAL ADAPTATION OF SIBERIAN CHIPMUNKS TO EARLY GERMINATING OAKS

One species of squirrel that does not perform embryo excision of early germinating acorns appears to have overcome this problem with a suite of other behavioral strategies that allow it to manage the challenge of early germination (**Table 2**). Although this species is known to larderhoard food for winter hibernation, the relatively solitary species also regularly engages in scatterhoarding and, as a result, contributes significantly to seed dispersal (Wang et al., 2018; Yi et al., 2019b). In China, Siberian chipmunks (*T. sibiricus*) exhibit the unique behavior of pericarp removal of acorns of *Q. mongolica*, *Q. variabilis*, *Q. aliena* and *Q. serrata* var. *brevipetiolata* (Yi et al., 2012a, 2014; Yang et al., 2018). Similar observations in North America have been attributed to eastern chipmunks (*T. striatus*, Yi, personal

TABLE 1 | Summary of squirrels that perform embryo excision on early germinating white oaks.

Species	Location	Oak species/section	Comments	Literature cited
Eastern gray squirrel *Sciurus carolinensis*	NE, S. U.S., mid-western United States	*Quercus alba*, *Q. montanaQ. Macrocarpa*	1,2	Fox, 1982; Steele et al., 2001b, 2006
Western fox squirrel *Sciurus niger*	mid-western United States	*Q. macrocarpa*	2,3	Steele, in press
Mexican gray squirrel *Sciurus aureogaster*	Central Mexico, southward	*Q. glaucoides*, *Q. laeta*, Q. obtusata, Q. microfila Q. rugosa		Steele et al., 2001b; Steele, in press
Pallas' squirrel *Callosciurus erythraeus*	Southeast Asia, Southern China	*Q. vaiabilis*, *Castanea henryi*	2	Xiao et al., 2009
Pére David's Rock *Sciurotamias davidianus*	Endemic to East and Central China	Q. *aliena Q. serrata, Q. variabilisCyclobalanopsis multinervis* Castanea mollissima	2	Xiao et al., 2010
Asian red-cheeked squirrel Dremomys rufgenis	Southeast Asia, China	*Q. variabilis, Cyclobalanopsis stewardiana, and C. glaucoides*	1,2	Xiao and Zhang, 2012

1. Evidence of innate basis of the behavior. 2. Perform embryo excision on acorns or nuts with early germination. 3. No direct data reported; observed by collaborating technician.

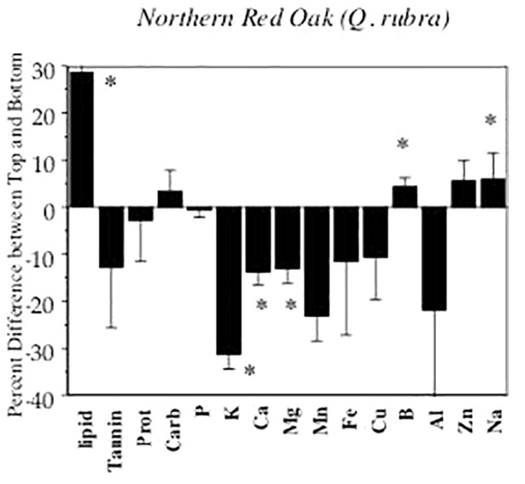

FIGURE 1 | From Steele, Michael A., *Oak Seed Dispersal: A Study in Plant Animal Interactions.* **Figure 6**.8b. @ 2021 Johns Hopkins University Press. Modified and reprinted with permission of Johns Hopkins University Press. (A) Distinction of the cotyledon of basal (top) and apical (bottom) halves of northern red oak acorns based on levels of lipid, tannin, carbohydrates, protein and 8 minerals. Results are based on composite samples of top and bottom halves of acorns from each of 20 trees (Steele, in press). Although tannin alone is not significantly higher in apical halves in this analysis (but see Steele et al., 1993), lipid and Na is higher in the basal half, suggesting that the top is more palatable but also higher in energy. Throughout the literature, it is now known that a suite of acorn seed predators (e.g., squirrels and other rodents, birds, and insect larvae) selectively consume the basal half of acorns and discard the apical halves, along with the embryo (Steele et al., 1993; Perea et al., 2011; Yang and Yi, 2012; Steele, in press), and that these damaged acorns can still germinate and establish (Steele, in press). * indicates significant difference in top and bottom halves at $P < 0.05$.

observation). By removing the pericarps of early germinating oaks, Siberian chipmunks are able to selectively discard nearly all insect-infested acorns, and, in turn, maximize the quality of their caches. However, unlike the behavior of embryo excision, pericarp removal does not prevent acorn germination. On the contrary, this behavior has a tendency to enhance seed germination rates of several oak species (Yi et al., 2014; Yang et al., 2018).

Yang et al. (2018) also found that pericarp removal by Siberian chipmunks may prevent cache pilferage by other rodents, especially conspecifics, possibly due to reduced seed odors associated with the pericarps (but see Sundaram et al., 2020). Moreover, field evidence showed that whole acorns cached by wood mice, *Apodemus peninsulae,* were more likely to be pilfered by Siberian chipmunks than acorns that had pericarps removed. Pilferage rates of whole intact acorns were estimated at 28% during both periods of both high and low seed abundance (Yi et al., 2019b).

Another behavioral strategy employed by Siberian chipmunks to deal with rapid germination of WOs, is radicle/acorn pruning. Early germinating acorns often develop a substantial radicle early in the autumn. Siberian chipmunks, as well as several other rodents (Yang et al., 2012; Zhang et al., 2017, 2018b), often cut and remove the acorn from the established radicle. This behavior has the potential to slow or stop re-sprouting of radicles and re-germination of acorns. However, it has also been shown that, under many circumstances, radicles over a minimum size are capable of producing new seedlings (Yi et al., 2012b, 2013a).

Yi et al. (2016c) tested the effects of seed odor on the scatter-hoarding and pilfering behavior of Siberian chipmunks. These chipmunks scatter-hoarded more seeds with weaker odors, supporting the hypothesis that olfaction likely plays an important role in the scatter-hoarding process and that chipmunks manage caches based on olfactory cues. This is further supported by experiments by Yi et al. (2016a) in which simulated nitrogen deposition significantly altered seed odor and other seed traits and, in turn, patterns of scatter-hoarding and pilfering by Siberian chipmunks (Yi et al., 2016a).

Acorn tannins and Siberian Chipmunks

Despite the close interaction between acorns and Siberian chipmunks, tannins in acorns appear to be an important chemical defense for the oaks. Yang and Yi (2012) showed that Siberian chipmunks and other sympatric seed-eating animals tend to selectively consume the basal end of WO (Section *Quercus*)

TABLE 2 | Summary of a suite of behavioral strategies employed by Siberian chipmunks (*Eutamias sibiricus*) to manage acorn scatterhoards.

Chipmunk characteristic(s)	Oak species	Oak/seed characteristic(s)	Literature cited
Pericarp removal prior to scatterhoarding	*Quercus mongolica, Q. variabilis, Q. aliena and Q. serrata var. brevipetiolata*	Early, rapid germination pericarp removal may enhance germination and reduce cache pilferage1	Yi et al., 2012a, 2014; Yang et al., 2018, 2016
Extensive acorn/radicle pruning which may slow germination	*Q. mongolica*, and other species	Rapid radicle production and ability to sustain acorn removal	Yang et al., 2012; Zhang et al., 2014, 2017, 2018a; Yi et al., 2019a
Partial acorn consumption	*Q. mongolica*	Ability to sustain damage and still germinate and establish	Yang and Yi, 2012; Zhang et al., 2014; Yi et al., 2015
Avoidance of acorns high in tannin; evidence of tannindegrading bacteria	*Q. mongolica*	Tannin causes significant weight loss/mortality	Zhang et al., 2018b
Extensive pilfering of other species' caches	*Q. mongolica*	N/A	Yi et al., 2019a
Selective predation of weevil (Curculio)-infested acorns and caching of only sound acorns	*Q. mongolica*		Yang et al., 2018

1. Also performed by eastern chipmunks in NA (Yi pers. observations) acorns, quite likely due to the chemical gradients described above in acorns of other oak species (Steele et al., 1993; Steele, in press). Acorns with this partial damage, given that embryos are avoided, have great potential to established either in the lab or field (Zhang et al., 2014; Yi et al., 2015).

Tannins in acorns also impose significant negative physiological effects on seed-eating animals. Although Siberian chipmunks employ a series of behavioral manipulations on acorns (e.g., pericarp removal and radicle pruning), tannins in acorns are typically not affected by these behaviors. Evidence from both enclosure and field studies showed that Siberian chipmunks tend to avoid artificial seeds with high tannins, but to eat and cache seeds with low tannins (Zhang et al., 2013, but see Xiao et al., 2009 for evidence of selective caching of high tannin acorns by other squirrels). Zhang et al. (2018a) provided further evidence that tannins in acorns results in loss of body mass and population declines of Siberian chipmunks, despite evidence of tannin degrading bacteria in the digestive tract of this squirrel.

Cache Site Selection of Siberian Chipmunks

The rapid sequestration hypothesis predicts that scatter-hoarding animals quickly store seeds near seed sources to reduce competition when seeds are abundant (e.g., during seed fall). Yi et al. (2019c), however, hypothesized that Siberian chipmunk, as multiple-prey loaders, may rely on an alternative hoarding strategy. By using multiple-compartment enclosures with different distances between the seed source and nest, they found chipmunks consistently placed their caches near nests but away from the seed source. Cache placement near nests may serve as an alternative strategy that not only guarantees a maximum harvest rate at the seed source but also ensures effective cache defense near nests. Moreover, scatter-hoarding seeds near nests may alternatively guarantee a food supply if larder-hoarded seeds are depleted or lost.

Another factor that may potentially influence both cache site selection by rodents is soil water content (SWC). For rodents, higher SWC increases olfactory detection and hence the probability of cache recovery by cache owners, although how it influences potential pilferers in unclear. Yi et al. (2013b) conducted a series of experiments with free-ranging chipmunks in a temperate forest of the Xiaoxing'anling Mountains of northeast China. They showed that Siberian chipmunks selectively cached acorns of *Q. mongolica* in soil of higher SWC, which may also facilitate later cache recovery. They argue that the selective scatter hoarding in soil of higher SWC represents a form of directed dispersal that may also enhance seedling establishment when seeds are not recovered.

Zhang et al. (2016) provided strong evidence that in large enclosures *T. sibiricus* preferred to establish their caches adjacent to visual landmarks, which they then later use for cache recovery. In the field Siberian chipmunks were also observed to established more caches in forest gaps rather than under understory (Yang et al., 2016), consistent with the observation of Steele et al. (2015) showing that gray squirrels prefer to establish caches in sites with higher predation risk to avoid cache pilferage.

Yang and Yi (2018) showed the importance of seed distribution patterns in affecting the hoarding behavior of scatter-hoarding rodents. Exposed seeds on the ground were more likely to be scatter-hoarded by *T. sibiricus*, while pilfered seeds from artificial caches were more likely to be larder-hoarded than those easily accessible on the ground. Recently, Deng et al. (2020) provided evidence that exclusion of interspecific competition by wood mice reduces seed dispersal and scatter-hoarding by Siberian chipmunks, despite an increase in per capita seed abundance. These studies collectively demonstrate that Siberian chipmunks show flexible behavioral strategies for hoarding in response to food availability.

Spatial Memory, Seed Odor and Scatter-Hoarding by Siberian Chipmunks

As the predominant scatterhoarder in coniferous and deciduous forests of northeastern China, Siberian chipmunks are expected to store more than several thousand acorns and other nuts annually. Therefore, spatial memory on these cached food items should play an important role in cache recovery by this scatter-hoarding animal. Li et al. (2018) found that *T. sibiricus* places more attention to, and greater memory of, their caches emitting weak odor for later recovery. This may represent the first study showing that scatter-hoarding animals trade off spatial memory on their caches emitting different intensity of seed odors. Wang et al. (2018) provided evidence that improved spatial memory by receiving oral addition of docosahexaenoic acid (DHA) and uridine-5-monophosphate (UMP) significantly increased the relative size of hippocampi of the chipmunks and increased concentration of hippocampal DHA and eicosapentaenoic acid (EPA). Consequently, chipmunks that received DHA and UMP scatterhoarded more seeds than control animals, indicating that scatter hoarding in this mammal is associated with improvement in spatial memory. Recently, Niu et al. (2019) reported that visual and/or auditory cues of conspecifics improve cache-pilfering and hoarding by Siberian chipmunks.

Although Vander Wall (1990; see also Dittel et al., 2017) proposed that scatter-hoarding animals show a cache recovery advantage over naive animals, Yi et al. (2016b) provided contrasting evidence that Siberian chipmunks consistently avoided its own caches while pilfering caches of others, suggesting that they are able to clearly distinguish caches they made from those made by naive conspecifics. The authors predict that spatial memory for cached seeds is necessary for scatter-hoarding animals to not only retrieve the food they buried but also for long-term management of caches (Yi et al., 2016b).

CONCLUSION

Squirrels exhibit close ecological and evolutionary interactions with the plants on which they feed. These interactions translate into a range of quite negative effects, such as heavy seed predation, herbivory and even bark stripping, as well as the critically positive (even mutualistic) effect on seed dispersal (Steele, 2008).

In mixed conifer and boreal forests, squirrels often exert strong selective pressure on trees directly as seed predators, indirectly by interfering with avian seed dispersal and only in limited situations contributing to conifer dispersal. In contrast, in deciduous forests across the globe, squirrels consistently contribute to dispersal and regeneration of numerous hardwood species. Moreover, a range of both hardwood seed traits and behavioral characteristics of squirrels point to a strong mutualism between the two.

Here, we reviewed recent studies that demonstrate a close co-evolutionary relationship between some squirrel species and the tree seeds they consume and disperse. These studies indicate both similarities in behavior and other traits across the globe, as well as differences in such traits that vary with climate and forest type. Some of these behavioral traits, such as embryo excision of early germinating WO species, are clearly unique to several squirrel species in North America, Mexico and Asia. Other interactions, in contrast, such as partial acorn consumption in response to chemical gradients in cotyledons, are shared with a community of other seed predators (insect, other rodents and some birds) and thus likely the result of diffuse co-evolutionary interactions. Still other species of squirrels, such at the Siberian chipmunk, exhibit a suite of behavioral responses to the oaks that appear independent of that of other squirrel species.

AUTHOR CONTRIBUTIONS

The review is based on a diversity of contributions by MS and XY. MS and XY contributed significantly to several revisions of the manuscript. Both authors contributed to the article and approved the submitted version.

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Evolution of Pelage Luminance in Squirrels (Sciuridae)

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Pelage luminance has been found in many mammalian systems to follow patterns predicted by Gloger's rule where darker colored animals are associated with environments that are warmer and more moist. Sciurids have one of the greatest diversities of color patterns and hues among mammalian families. We have used comparative methods to investigate whether the luminance of dorsal pelage in 137 species across Sciuridae conforms to prediction of Gloger's rule and other background matching expectations. We found using phylogenetic multiple regression, as well as univariate regression, that Sciurids generally conform to the expectations of Gloger's rule. Darker species are associated with environments with higher primary productivity, higher temperature, higher humidity, and lower solar radiation. Moreover, in support of the predictions of background matching, darker squirrel species were associated with environments with greater soil carbon content and higher fire frequency. Our macroevolutionary study sheds some light on selective pressures that are driving the evolution of coloration in Sciurids, but more comparative research is needed to fully understand other selective pressures that have led to the wide diversity of color patterns and hues.

Keywords: Gloger's rule, Sciuridae, multiple regression, pelage, phylogenetic comparative methods, ecogeographic rule

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INTRODUCTION

Gloger's rule is an ecogeographic rule describing the tendency of animals living in warm and wet environments to be darker (Gloger, 1833; Rensch, 1929). This rule has been supported by several quantitative tests from a wide variety of taxa including birds, mammals, reptiles, amphibians, and insects (Delhey, 2019; Delhey et al., 2019; Stanchak and Santana, 2019). There are multiple mechanisms that have been proposed to explain the color patterns that conform to Gloger's rule (Delhey, 2019) with the most common being that darker coloration is selected for in environments with lower light conditions to facilitate greater concealment from predators. Dark environments are often created by greater vegetative production, which is generally driven by higher temperatures and environmental moisture. Moreover, a high number of studies have found support for the UV protection hypothesis (Chaplin, 2004) by finding latitude, a strong correlate of solar radiation, to be significantly associated with pelage luminance (e.g., Stoner et al., 2003a,b, Caro, 2005; Santana et al., 2012). Thus, biologists that study Gloger's rule often investigate how well multiple environmental variables (i.e., temperature, environmental moisture, primary productivity, solar radiation, and their surrogates) predict the brightness of animal color. Another possible mechanism is selection for pelage color to match that of the background substrate (Nachman et al., 2003; Vignieri et al., 2010), the color of which may be predicted by the variables mentioned above.

The rodent family Sciuridae is a fascinating group to study color-pattern evolution because, unlike most other rodent families, squirrels have evolved extremely diverse pelages that include many types of patterns (e.g., stripes, spots, and patches) and hues (e.g., red, brown, tan, and black), and a range of luminances from black to very light (**Figure 1**) (Thorington et al., 2012). This phenotypic diversity is likely related to the evolution of squirrels in many different terrestrial biomes worldwide (e.g., desert, tundra, and rainforest), as well as their evolution of a diverse range of lifestyles (terrestrial, arboreal, and fossorial), life histories (diurnal, nocturnal, social, and solitary), and body sizes (16 g up to 1200 g) (Thorington et al., 2012). For example, many species of desert squirrels have pale-colored fur that is likely related to living in habitats with lightly colored soils that contain minimal amounts of dark organic content.

Patterns of Gloger's rule have been investigated in many mammal groups, but a large majority of these studies have been focused at the intraspecific level (Delhey, 2019). Studies of Gloger's rule in squirrels are limited, but are also focused on intraspecific variation. A study in fox squirrels (*Sciurus niger*) with melanistic polymorphisms has identified elevation and humidity as positively correlated with pelage luminance, but also showed that precipitation and temperature were negatively correlated with pelage luminance (Kiltie, 1989). They also found fire frequency was positively correlated with darker pelage. A similar study of the African giant squirrel (*Protoxerus stangeri*) found a negative relationship between pelage luminance and mean annual precipitation and a positive correlation between pelage luminance and mean annual temperature.

Studies of Gloger's rule in primates offer potentially intriguing insight to selective pressures that may drive color evolution in Sciurids because both mammalian groups have a wide diversity in color patterns and are similar in many other characteristics, such as possessing a mostly diurnal behavior, a range of both terrestrial and arboreal lifestyles, and a global distribution. A comparative study of Gloger's rule in primates showed that pelage luminance was negatively correlated with actual evapotranspiration (AET), which is a measure of the amount of water leaving an environment (Kamilar and Bradley, 2011). As AET is a composite measure of many environmental processes, this result implied a negative correlation between pelage luminance and precipitation, temperature, humidity, and primary productivity.

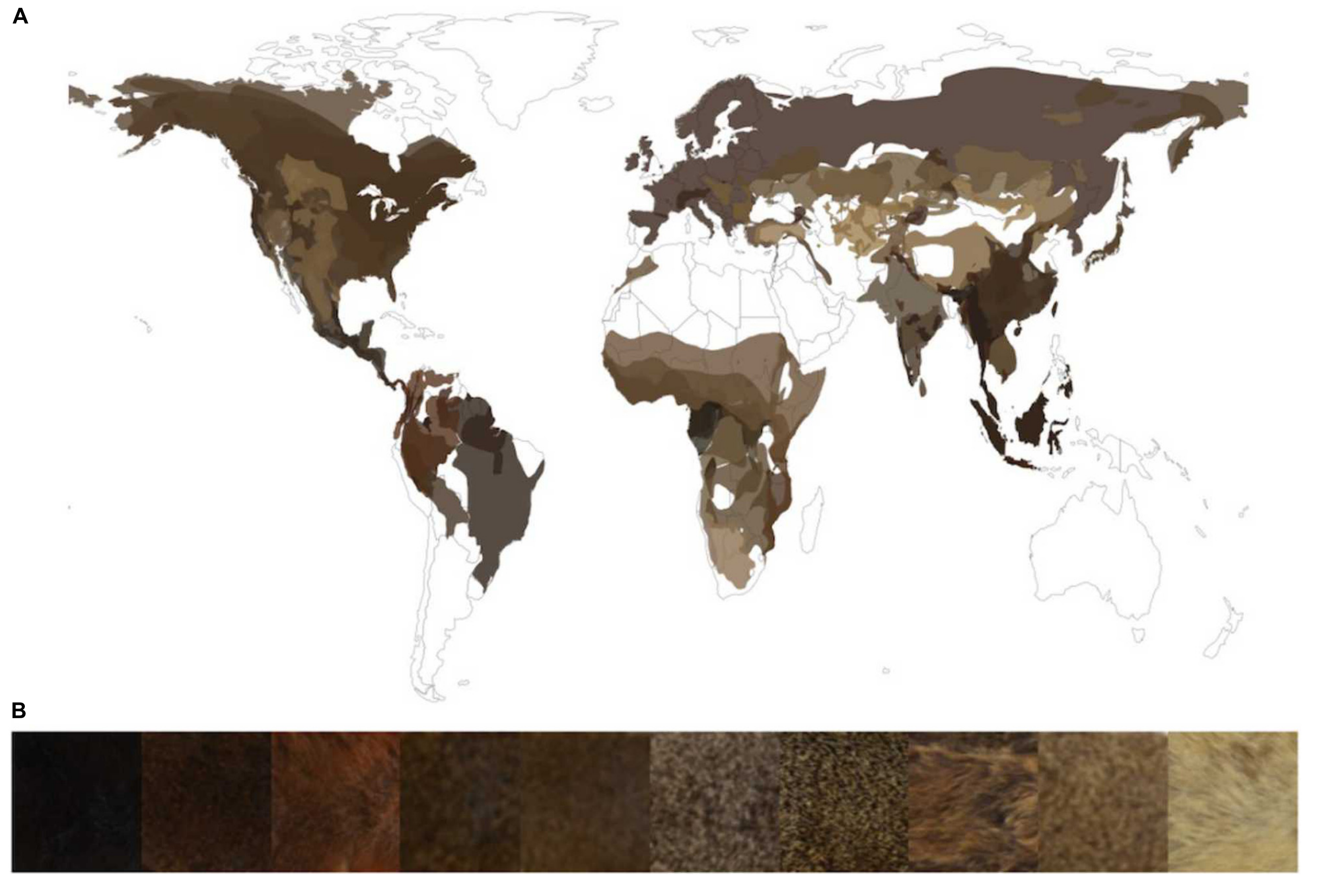

FIGURE 1 | (A) Average dorsal color of all species for which we have photo and distribution data superimposed onto that species range map. **(B)** Dorsal regions of select squirrel species showing the distribution of pelage luminance across the Sciurid family. The representative species from left to right are as follows: *Callosciurus finlaysonii, Sciurus aestuans, Sciurus vulgaris, Sundasciurus samarensis, Callosciurus nigrovittatus, Sciurus yucatanensis, Notocitellus adocetus, Petaurillus kinlochii, Urocitellus townsendii, Callosciurus finlaysonii.*

In this study, we used a comparative phylogenetic approach to investigate environmental predictors of dorsal-pelage luminance in Sciurids. We hypothesized that, in accordance with many other animal systems that conform to predictions by Gloger's rule, pelage luminance will be significantly associated with variables related to environmental moisture, temperature, and light conditions. We also hypothesized that pelage luminance will be significantly associated with other environmental variables that are more directly associated with background matching (soil carbon content, soil density, and frequency of wildfires).

MATERIALS AND METHODS

Phylogenetic Inference

To conduct phylogenetic comparative analyses, we constructed a phylogeny of 137 Sciuridae species across 51 genera. We downloaded all gene sequences from GenBank representing seven mitochondrial loci (12S, 16S, Cyt-b, COI, COII, COIII, mitochondrial control region) and four nuclear loci (C-myc, GHR, IRBP, RAG1). We then aligned all sequences using MUSCLE (Edgar, 2004) implemented in the EMBL web app (Madeira et al., 2019) and then manually edited egregious alignments. We then used Partition Finder (Lanfear et al., 2012) to find the best substitution model based on the Akaike Information Criterion. Finally, we inferred a phylogeny using Bayesian inference in MrBayes (Huelsenbeck and Ronquist, 2001) using Markov Chain Monte Carlo (MCMC) sampling. Two different runs (each with one cold and three heated chains) were analyzed for 3×10^7 generations (with trees sampled every 100 generations). Branch lengths were than calibrated according to the dates used by Zelditch et al. (2015).

Luminance Data From Museum Study Skins

To obtain color measurements of the 137 squirrels used in our phylogenetic analysis, we photographed the dorsal profile of museum study skins from the Division of Mammals at the Smithsonian National Museum of Natural History. We used a Sony Alpha 5000 to take digital photographs of specimens against a gray matte background. Two light-boxes containing ESDDI 85 W 5500K Day Light Florescent bulbs were positioned at opposing ends of the specimen stage in an interior, windowless room. All images were processed the MacOS image editing software Paintbrush 2.1 to exclude any region of the image depicting the specimen stage. We then processed masked images through a custom python script that converted the image data to CIE LAB colorspace. Average luminance was then calculated from all unmasked pixels of the image, thus capturing information from the entire dorsal region of the study skin. Higher luminance values indicate lighter pelage color.

Environmental Data

Our study largely used environmental variables that have been shown to be important predictors in Gloger's rule in other animal systems where studies have typically shown that animals are darker where environmental conditions are warmer, wetter, and darker (Delhey, 2019). The variables directly related to Gloger's rule that were used in our study can be grouped into three categories: (1) temperature (mean annual temperature), (2) environmental moisture (mean annual precipitation and humidity), and (3) light conditions (insolation, canopy closure, and net primary productivity). Given that more direct background matching with surrounding substrates can also be an important selective pressure on pelage color in rodents (Caro, 2005; Vignieri et al., 2010), we also included a fourth category of variables associated with the background environment (soil carbon content, soil density, and fire frequency). Soil density was included because sandy soils, which are generally lightly colored, have higher bulk densities than fine silts and clays because they have larger, but fewer, pore spaces (Ayres et al., 1973). Soil carbon content was included because soils with higher carbon content have higher rates of decomposition and thereby produce darker substrates (Schulze et al., 1993). Fire frequency was included because higher frequency of fires was shown in fox squirrels (Kiltie, 1989) to be an important predictor of darker pelage.

TABLE 1 | The 12 regression models tested by PGLS.

Model	AICc	Partial R^2	Alpha	Sigma^2	ΔAICc	*w*
Fire + Insolation + NPP + Temperature	347.34	0.29	0.53	0.73	0	0.72
Fire + Soil carbon + Humidity	349.88	0.26	0.53	0.76	2.54	0.2
Canopy closure + Fire + Insolation + Temperature	353.07	0.26	0.53	0.77	5.73	0.04
Fire + NPP + Soil carbon + Temperature	354.64	0.25	0.53	0.77	7.3	0.02
Fire + Insolation + Humidity	355.95	0.23	0.53	0.79	8.61	0.01
Canopy closure + Fire + Soil carbon + Temperature	357.21	0.23	0.53	0.79	9.87	0.01
Fire + NPP + Soil Density + Temperature	359.2	0.22	0.53	0.8	11.86	<0.01
Canopy closure + Fire + Soil density + Temperature	359.58	0.22	0.53	0.8	12.24	<0.01
Fire + Soil density + Humidity	361.68	0.19	0.53	0.83	14.34	<0.01
Fire + Precipitation + Soil carbon	377.28	0.1	0.53	0.93	29.94	<0.01
Fire + Insolation + Precipitation	379.01	0.09	0.53	0.94	31.67	<0.01
Fire + Precipitation + Soil density	381.13	0.07	0.53	0.96	33.79	<0.01

For brevity, fire frequency has been abbreviated to fire, solar radiation to insolation, soil carbon content to soil carbon, and net primary productivity to NPP.

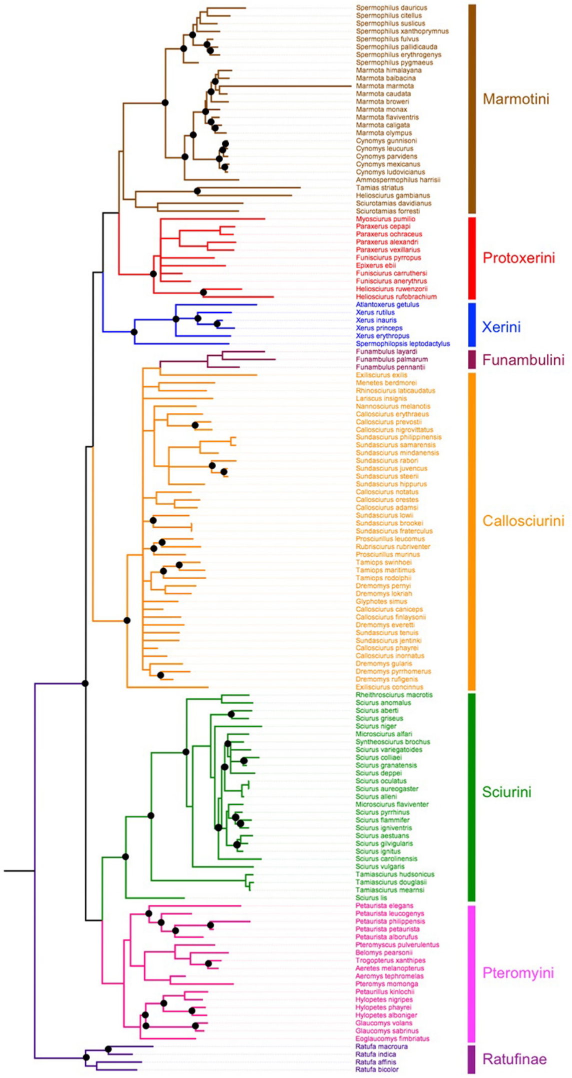

FIGURE 2 | Phylogeny representing 137 species within Sciuridae. Branch lengths are proportional to the expected number of changes per site. Dark circles represent nodes with >95% posterior probability.

TABLE 2 | Results of univariate regression analysis.

Variable	Slope 95% CI lower bound	Slope	Slope 95% CI upper bound	Partial R^2	*P*-value
Soil density	0.15	0.34	0.54	−0.33	<0.01
Soil carbon	−0.4	−0.21	−0.01	−0.4	0.04
Humidity	−0.88	−0.72	−0.57	0.1	<0.01
Insolation	−0.16	0.04	0.23	−0.45	0.72
NPP	−0.88	−0.71	−0.55	0.07	<0.01
Fire	−0.32	−0.11	0.09	−0.44	0.27
Canopy closure	−0.79	−0.62	−0.45	−0.06	<0.01
Temperature	−0.74	−0.57	−0.4	−0.11	<0.01
Precipitation	−0.84	−0.69	−0.52	0.05	<0.01

Some variable names have been abbreviated as described in the legend of ***Table 1****.*

The environmental data for the nine predictor variables (mean annual temperature, mean annual precipitation, humidity, insolation, canopy closure, net primary productivity, soil density, soil carbon, and fire frequency) for all 137 squirrel species were acquired from multiple databases. Both mean annual precipitation and mean annual temperature values were acquired across each species range from the PanTHERIA database, which provides point estimates at species level resolution for a range of environmental and life history characteristics of mammals (Jones et al., 2009) (PanTHERIA metadata available at http://esapubs.org/archive/ecol/E090/184/metadata.htm). Canopy closure raster data, which serves as a proxy measure for environmental light conditions, were acquired from high-resolution global maps of forest cover (Hansen et al., 2013). This dataset provides world-wide canopy closure data at 1 m by 1 m resolution. Due to memory constraints we reduced this dataset to a 5 m by 5 m resolution using the "aggregate" function in the *raster* package in R (R Core Team, 2013; Hijmans and van Etten, 2014). Both soil carbon content and soil density raster data at a 5-km resolution was acquired from the ISRIC Soil Grids web app (Batjes et al., 2019). Humidity (column water vapor amount), net primary productivity, fire frequency, and solar radiation were obtained from the NASA MODIS satellite hosted on NASA Earth Observations[1]. These data represent time averaged values for each month for approximately the past 20 years. We downloaded these data at an 11 km by 11 km resolution and averaged the monthly values into a single raster file. All raster data was clipped with extant range maps of all 137 species downloaded from the IUCN (2019) using the *raster* and *rgdal* packages in R (R Core Team, 2013; Hijmans and van Etten, 2014; Bivand et al., 2019) to obtain species level averages.

Model Selection

Following the recommendations of Cooper et al. (2016), we used an MCMC based approach to fit both a Brownian motion and Ornstein Uhlenbeck model of evolution to our data. This was implemented by the "fitContinuousMCMC" function in the R package *geiger* (Pennell et al., 2014). This was run for 1×10^6 generations with posterior likelihood values sampled every 100 generations.

Phylogenetic Generalized Least Squares

All data for our phylogenetic generalized least square (PGLS; Martins and Hansen, 1997) analyses were centered by subtracting the mean and scaled by dividing by the standard deviation. We then ran pairwise phylogenetic regressions using *phylolm* with the model specified as "OUfixedRoot" between each environmental variable and the phenotype with 1000 bootstrap replicates. Next, we ran 36 pairwise phylogenetic regressions using the same approach between each of the nine environmental variable and each of the eight other environmental variable to assess multicollinearity among the data. Variables were considered collinear if their correlation coefficient was greater than 0.7. Finally, we constructed 12 regression models representing all

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[1] https://neo.sci.gsfc.nasa.gov

possible combinations of non-collinear variables (**Table 1**). As above, this was implemented with the function "phylolm" from the R package *phylolm* (Ho and Ane, 2014). Model weights were then calculated according to Symonds and Moussalli (2011). Partial R^2 values were calculated using "R2.resid" function of the R package *rr2* (Ives and Li, 2018).

RESULTS

Phylogenetic Tree

Partition Finder selected the GTR + I + G substitution model for nearly all partitions, expecting the wobble positions of GHR and CMYC being better modeled by K80 + G and HKY + G, respectively. Our Bayesian phylogenetic inference of Sciuridae revealed 57 nodes with significant (>0.95) posterior probabilities (**Figure 2**).

Model Selection for Comparative Phylogenetic Analysis

Both runs of the MCMC converged as evidenced by the stationarity of the trace plots. The Ornstein Uhlenbeck model (Log likelihood −883) had a consistently higher likelihood than Brownian motion (Log likelihood −918) after burn-in and was therefore used for all comparative analyses.

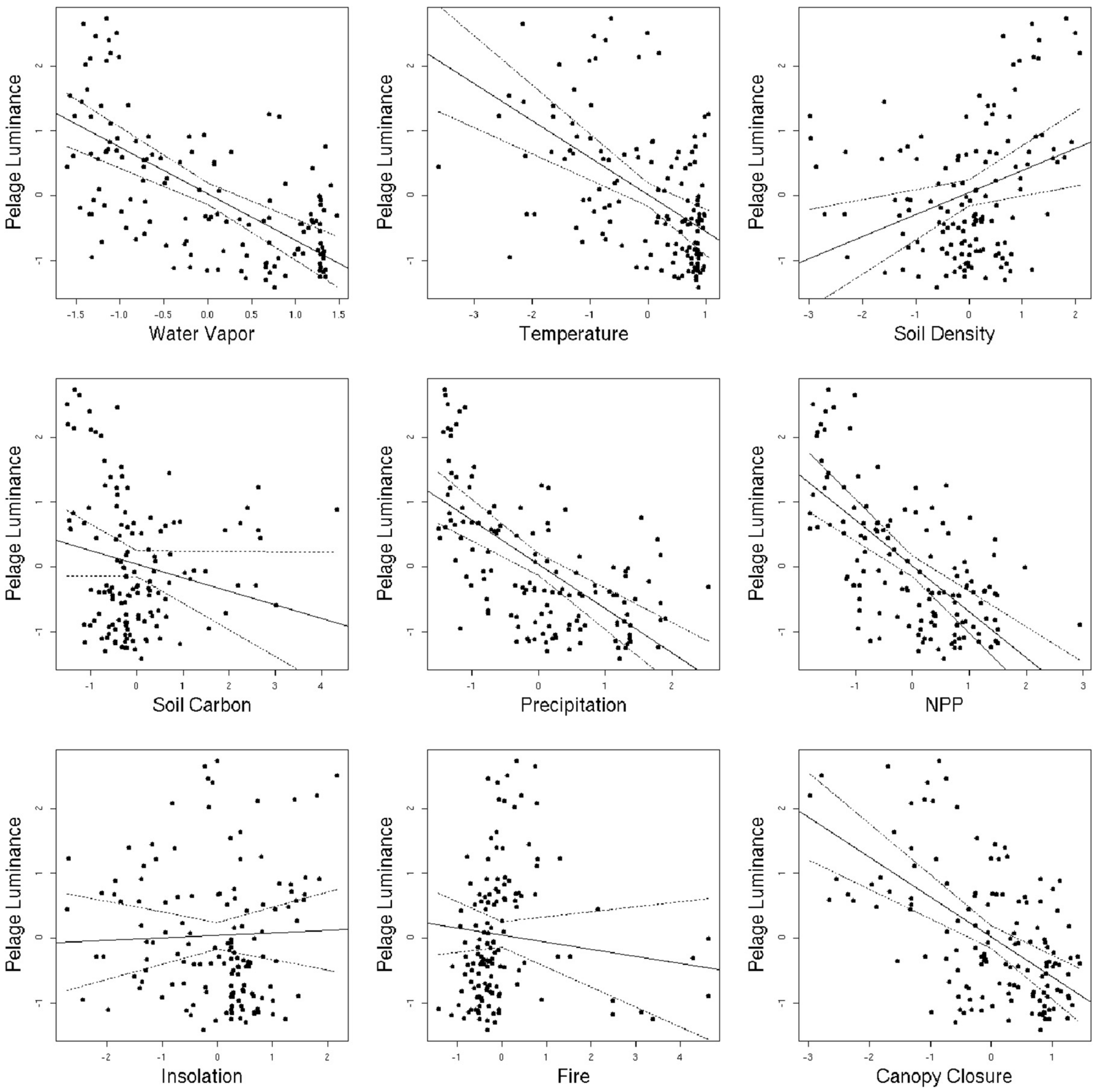

FIGURE 3 | Plots showing the relationship between pelage luminance and each of the nine environmental variables included in this analysis. The solid line represents the line of best fit, while the dashed lines represent 95% confidence intervals from 1000 bootstrap replicates.

Phylogenetic Generalized Least Squares

The univariate regression analysis revealed that seven out of nine environmental variables exhibit a significant correlation with the phenotype (**Table 2** and **Figure 3**). Pelage luminance is negatively correlated with soil carbon content, humidity, primary productivity, canopy closure, temperature, and precipitation. Soil density is the only variable to have a significantly positive correlation with pelage luminance. Solar radiation and fire frequency exhibit no significant correlation with pelage luminance.

The regressions among environmental variables revealed that soil density is collinear with soil carbon content and insolation. Soil carbon content is also collinear with insolation. Water vapor is collinear with primary productivity, canopy closure, temperature and precipitation. Primary productivity is collinear with canopy closure and precipitation. Canopy closure is also collinear with precipitation.

The top three multiple regression models form the credible set, the set whose cumulative model weight exceeds 0.95 (Symonds and Moussalli, 2011) and we reject the remaining models. Model one finds significant negative effects between pelage luminance and fire frequency, primary productivity, and mean annual temperature as well as a significant positive correlation between the phenotype and solar radiation. Model three differs from model one only in the replacement of primary productivity with canopy closure, where the correlation is likewise significantly negative. Model two includes significant negative correlations between pelage luminance and soil carbon content and humidity, while the correlation with fire frequency is non-significant (**Table 3**). It is worth noting that mean annual precipitation, commonly found to be a significant correlate of pelage luminance, does not appear in the credible set.

DISCUSSION

Our comparative study of dorsal pelage luminance across Sciuridae based on multiple regression found support for the predictions of Gloger's rule and other background matching expectations. The dorsal pelage of squirrel species become darker in environments with greater mean annual temperature, greater environmental moisture (humidity), lower light conditions (lower levels of solar radiation, greater primary productivity, and greater canopy closure), and darker background environments (greater soil carbon content and greater fire frequency). Our univariate regression analyses further supported these relationships. These findings are consistent with most predictions of Gloger's rule that have been observed in mammals, including carnivores (da Silva et al., 2016; Caro et al., 2017), artiodactyls (Stoner et al., 2003b), Palearctic shrews (Stanchak and Santana, 2019), primates (Kamilar and Bradley, 2011; Santana et al., 2012), rodents (Lai et al., 2008), and marsupials (Nigenda-Morales et al., 2018; Cerezer et al., 2020). These empirical studies have commonly used temperature, precipitation, latitude, and vegetation to explain patterns of color variation that are associated with climatic factors or surrogates of those factors. Our study extended the general predictions of Gloger's rule by also revealing that environmental variables related to background matching to surrounding substrates also contribute to pelage luminance.

TABLE 3 | Coefficients of top three models from **Table 1**.

Variable	**Model**					
	1		**2**		**3**	
	Coefficient	**P-value**	**Coefficient**	**P-value**	**Coefficient**	**P-value**
Fire frequency	−0.16	0.04	−0.02	7.60E−01	−0.22	<0.01
Insolation	0.61	<0.01	–	–	0.578	<0.01
Temperature	−0.76	<0.01	–	–	−0.9	<0.01
NPP	−0.34	<0.01	–	–	–	–
Soil carbon	–	–	−0.34	<0.01	–	–
Canopy closure	–	–	–	–	−0.2	0.04
Humidity	–	–	−0.81	<0.01	–	–

Some variable names have been abbreviated as described in the legend of ***Table 1****.*

Our results largely agreed with other quantitative investigations of Gloger's rule in other Sciurids that were focused on intraspecific variation in *Scirus niger* (Kiltie, 1989) and *Protoxerus stangeri* (Amtmann, 1965). The former investigated several populations within *S. niger* and found support for darker squirrels being associated with greater temperature, moisture, and fire frequency. The mechanism proposed by Kiltie (1989) for the role of fire frequency is that wildfires leave behind a dark charred substrate, which may provide greater concealment for darker squirrels. However, they also stated that the lasting effect of this mechanism may be tenuous because of the rapid regrowth of vegetation following fires usually obscures the dark background of charred wood. Perhaps the mechanism is not fire frequency, but other environmental characteristics that are associated with habitats that burn frequently. More research is needed to determine if there are other probable mechanistic explanations for the association of fire frequency with pelage luminance and whether this trend is generalizable beyond Sciuridae. Amtmann (1965) analyzed samples from across the range of *P. stangeri* and similarly found a negative relationship between mean annual precipitation and pelage luminance. However, contrary to our study, *P. stangeri* was found to exhibit a positive correlation between pelage luminance and mean annual temperature.

Primates and squirrels present an interesting opportunity to study parallel evolution in animal color patterns. Primates, like Sciurids, possess exceptional diversity in color patterns (Bradley and Mundy, 2008; Santana et al., 2012) and share many other attributes, including wide-spread diurnality, many species exhibiting an arboreal lifestyle, having similar predators (carnivores, raptors, and snakes), and having global distributions that span a range of biomes. Our results are consistent with prior work on primates (Kamilar and Bradley, 2011) that found pelage luminance across primates corresponded negatively with AET. AET measures the amount of water leaving an environment and is affected by temperature, precipitation, humidity and primary productivity. This result implies that primates are darker in areas with higher precipitation, higher temperature, higher humidity and higher primary productivity. The one major difference between primates and Sciurids with regards to color pattern evolution is that many primate species have evolved striking sexual dimorphisms in pelage coloration that are related to social signaling and sexual condition (Setchell, 2005; Dubuc et al., 2009; Marty et al., 2009). Squirrels do not exhibit sexual dimorphism in pelage coloration and thus the selective pressures on pelage coloration are likely to be strictly due to ecological and environmental factors.

The range of color patterns and hues in the family Sciuridae are incredibly broad and diverse (Thorington et al., 2012). This diversity includes species with patterns that are patchy (e.g., *Sciurus variegatoides*), striped (e.g., *Tamias striatus*), spotted (e.g., *Xerospermophilus spilosoma*), mottled (e.g., *Otospermophilus beecheyi*), and uniform (e.g., *Microsciurus mimulus*), as well as species with colors that are very light (e.g., *Callosciurus finlaysonii*), very dark (e.g., *Callosciurus prevostii)*, orangish-red (e.g., *Sciurus vulgaris*), reddish-maroon (e.g., *Ratufa indica*), reddish-brown (e.g., *Tamiasciurus douglasii*), and gray (e.g., *Sciurus griseus*). Furthermore, color patterns can vary tremendously just within a single animal (e.g., tail colors are often distinct from body colors). The evolutionary forces shaping this diversity of color patterns and hues across species and on different body parts are not well studied in Sciurids, but may include selection to enhance conspecific and heterospecific signaling, despite camouflage also likely being another important driver of pelage color evolution. Squirrels often vocalize and exhibit tail-flagging when they detect potential predators (Owings and Coss, 1977; McRae and Green, 2014). Thus, it is possible that striking color patterns on tails or even the main body may improve visual communication with predators during precarious encounters. We suspect that multiple types of selective pressures are shaping the evolution of fur color in squirrels and our study sheds some light that precipitation-related and background matching pressures may be shaping one dimension (pelage luminance) of these color patterns.

One of the general statistical challenges in studies of Gloger's rule is that many of the hypothesized explanatory variables are inherently correlated with each other and may be directly or indirectly related to animal-color patterns that conform to Gloger's rule. Ideally, studies can illuminate which variables are directly driving variation in pelage luminance and which variables may be mediated through others. Unfortunately, univariate or multiple regression approaches do not offer the ability to distinguish between direct and mediated effects when questions are ecologically complex. As an example, such approaches cannot distinguish between a model in which temperature and moisture drive primary productivity, which in turn drives pelage luminance; or one in which temperature and moisture drive primary productivity and pelage luminance simultaneously. One possible approach to differentiating between these two hypotheses is through statistical methods like path analysis, which has recently been extended for use with comparative phylogenetic data (von Hardenberg and Gonzalez-Voyer, 2013; van der Bijl, 2018). However, one limitation of path analysis is that it requires the hypotheses to be tested to explicitly specify all relationships among included variables. Given the complicated interconnectedness of variables typically included in Gloger's rule studies, this specificity is difficult to attain without further methodological development. We recommend that future research explores how path analysis can be effectively applied to questions concerning Gloger's rule.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Dryad repository doi: 10.5061/dryad.tqjq2bvv8.

AUTHOR CONTRIBUTIONS

AS and AC contributed to the conception and design of the study, and draft and revision of the manuscript. AS was responsible for the data acquisition, analysis, and data interpretation. Both authors approved the publication.

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A Study of Hybridization Between *Marmota baibacina* and *M. sibirica* in Their Secondary Contact Zone in Mongolian Altai

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The role of hybridization as one of the factors of speciation in mammals has been underestimated for a long time, but now there is a lot of data on its impact in mammalian evolution. Hybridization of species often occurs in their secondary contact zones, which is a natural model for testing factors that ensure species integrity. Studies of hybrid zones are increasingly revealing the essential role of ecological and behavioral features both in initiating crossbreeding and in maintaining interspecific barriers. We studied the hybridization of two species of marmots *Marmota baibacina* and *M. sibirica* in the zone of sympatry in Mongolian Altai Mountains. We used a bioacoustic approach to determine the localization of individuals of different species and their cohabitation sites. Genetic typing with two diploid nuclear markers and one marker each of paternal and maternal lines was used to identify hybrids. Habitat preferences of marmots were studied to understand the conditions for the formation of heterospecific pairs. We found a high proportion of hybrid individuals in boulder screes where conditions for the formation of heterospecific pairs probably exist. Our data indicate the viability and fertility of F1 hybrids and their descendants. We hypothesize that the environmental preferences and behavioral features of both species of marmots are important factors that both create conditions for hybridization and limit hybrid dispersal.

Keywords: *Marmota*, hybridization, secondary contact zone, molecular genetic markers, bioacoustics, behavior, ecology, Mongolian Altai

INTRODUCTION

The problem of defining species boundaries, their permeability, and estimating the impact of their disturbance for species delimitation is addressed according to the species concept that is a source of long-term discussions in evolutionary biology (Harrison and Larson, 2014; Zachos, 2016). Within the genetic species concept, maintaining genome integrity is one of the main species criteria (Wu, 2001). The genomic effects of interbreeding of heterospecific individuals are diverse and depend on many factors. On the one hand, interspecific hybridization and the breaking down of interspecific barriers can lead to the blurring of species boundaries and, in some cases, to the extinction of one of the species (Todesco et al., 2016). On the other hand, hybridization may enrich genomes with new variations, multiplies local adaptations, and in some cases, may initiate speciation (Abbott et al., 2013; Eberlein et al., 2019). But also hybridization may not have a significant impact on species genomes. The hybrid zones formed during secondary contact of previously spatially separated species may exist for quite a long time, appear and disappear periodically, and vary in their location depending on environmental changes (Wielstra, 2019). Formation of robust postzygotic barriers and gametic isolation during independent evolution requires long-term spatial isolation, disruption of which by secondary contacts under some conditions may lead to gene introgression (Abbott et al., 2013).

Advanced application of genetic approaches in studying gene flow and the consequences of species genome mixture makes it possible to detect population processes that do not appear on the phenotypic level or have occurred in the past (Chavez et al., 2011; Bono et al., 2018; Fadakar et al., 2020). At the same time, studies of phenotypic variability in hybrid zones allow us to understand the contribution of admixture to the adaptive capacity of a population (Fontsere et al., 2019).

Hybridization is more common in plant species (25% species involved) than in animals (10%) (Mallet, 2005). For mammals, hybridization has been previously recognized as extremely rare (Stebbins, 1959), but intensive researches using genetic approach have changed this view (Mallet et al., 2016). Current estimates suggest that at least 6% of mammal species are involved in interspecific hybridization to different extents (Mallet, 2005). This may vary across mammalian taxa, but numerous examples indicate that gene flow is a common phenomenon in mammals. The evolutionary role of hybridization in mammals has not yet been sufficiently understood. Most of the studied cases of hybridization in mammals are associated with secondary contacts of allopatric species that arose under the influence of cyclic glacial events (Colella et al., 2018). Revealing ancient hybridization in different taxa also broadens understanding of the contribution of hybridization to mammalian evolution (e.g., Marques et al., 2017). In the ground squirrels Marmotini up to a quarter of species are involved in hybridization events of varying intensity, from sporadic hybridization to the formation of hybrid zones and wide introgression according to our estimates of the literature data (Semenova, 1967; Nikol'skii et al., 1983; Smirin et al., 1985; Good et al., 2008; Stangl et al., 2012; Thompson et al., 2013; Ermakov et al., 2015; Frare et al., 2017; Ivanova et al., 2017; Leitner et al., 2017; Kapustina et al., 2018).

Marmots (*Marmota* Blumenbach, 1779) are large rodents distributed in North America and Eurasia, inhabiting open planes of mountain and lowland landscapes. Like most of the ground squirrels, they are burrowing animals. The distinctive features of marmots are the subdivision of the colony into family groups characterized by stability and territoriality, and the social behavior of most species (Armitage, 2003). Stable marmot family groups consist of a breeding couple and their offspring of different ages (juvenile and subadults) (Bibikow, 1996; Armitage, 2014). Hybridization was found only in Palearctic marmots. The crossing of species in all known cases occurs with the involvement of the gray marmot (*Marmota baibacina* Kastschenko, 1899) in zones of sympatry with the steppe marmot (*M. bobak* Müller, 1776), the long-tailed marmot (*M. caudata* Geoffroy, 1844), and the tarbagan (*M. sibirica* Radde, 1862; Semenova, 1967; Nikol'skii et al., 1983; Smirin et al., 1985).

To date, not a single hybrid zone of marmots has been studied comprehensively. One of the methods used in preliminary studies was bioacoustic. The advantage of acoustic signals as a model of variability is that they are amenable to visualization and quantification of their parameters using digital technology. Sound signals are widely used in the study of hybrid zones in birds and mammals (den Hartog et al., 2008; Wyman et al., 2011; Campbell et al., 2019). Most often, however, such studies focus on the role of specific signals in ensuring assortative mating by recognizing conspecific mates. Vocal traits can provide important information when studying hybridization in taxa with genetically determined acoustic signals not involved in mating behavior. Alarm calls typical for most ground squirrel species have exactly these properties. Species specificity and genetic determination of alarm calls of marmots and ground squirrels have been shown earlier (Nikol'skii, 1976, 1979; Blumstein, 2007). Studies of hybrid zones of four *Spermophilus* species revealed the formation of peculiar alarm calls in hybrids, which was confirmed by genetic data (Nikol'skii et al., 1984; Nikol'skii and Starikov, 1997; Kuzmin et al., 2011; Titov et al., 2020). Characters of alarm calls were used in *M. baibacina– M. bobak* secondary contact zone for hybrid identification (Nikol'skii et al., 1983). We initiated the study of the hybrid zone of *M. baibacina–M. sibirica* to confirm hybridization in marmots by genetic method in combination with bioacoustic and ecological approaches.

Gray or Altai marmot (*M. baibacina*) and Tarbagan or Mongolian marmot (*M. sibirica*) are closely related, relatively recent divergent species which have inherited significant morphological similarity (Kryštufek and Vohralík, 2013) despite appreciable genetic differences (Brandler et al., 2010a; Steppan et al., 2011). The chromosomal sets of both species have similar parameters (2n = 38, NF = 70) and minimal differences (Brandler et al., 2008). Both species are quite ecologically supple and inhabit a wide range of mountain and intermountain plains steppe outside the zone of sympatry. They have a social organization typical for marmots, based on family groups which consist of a breeding couple, juvenile and subadult individuals (Bibikow, 1996; Adiya, 2007). Pairs in these species form during the summer season long before reproduction time and are maintained for several years. In Mongolia, copulation in both species occurs in spring at the end of hibernation before the first exit to the surface from burrows (Adiya, 2007).

The secondary contact zone of *M. baibacina* and *M. sibirica* in the Mongolian Altai is an example of the limited sympatry of two widely distributed species of territorially conserved social mammals. Morphological hybrids were first discovered within this zone in the upper Ulagchin-Gol river valley during the study of the distribution boundaries of Mongolian marmots in the 1980s (Smirin et al., 1985). The first studies outlined the boundaries of a mixed population, described the peculiarities of fur coloration and craniometric characteristics of the supposed hybrids. Variability in the alarm call of marmots was studied

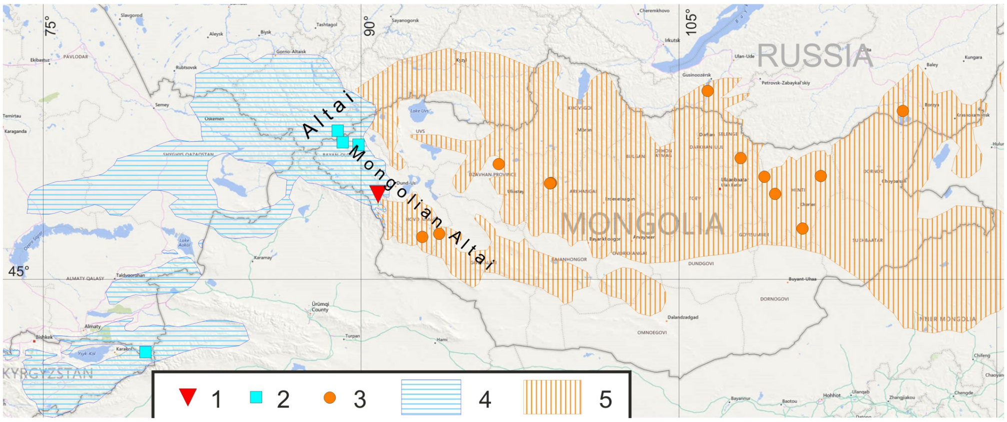

FIGURE 1 | Localities of studied marmot population and reference populations: (1)—population of Ulagchin-Gol valley; (2)—*M. baibacina* reference populations; (3)—*M. sibirica* reference populations. Distribution of marmots: (4)—*M. baibacina*; (5)—*M. sibirica*. Compiled from IUCN database, Brandler (2003); Kolesnikov et al. (2009), Kryštufek and Vohralík (2013).

as one of this species characteristics (Formozov and Nikol'skii, 1986). A single signal presumably belonging to a hybrid was identified among species-specific signals. At the same time, a difference in the habitat preferences of *M. baibacina* and *M. sibirica* was identified (Smirin et al., 1985; Rogovin, 1992). There is information about the existence of several more mixed settlements in the zone of overlapping areas of *M. baibacina* and *M. sibirica,* which to date remain unexplored.

The mixed population of *M. baibacina* and *M. sibirica* in the valley of the Ulagchin-Gol river is located within the limited area of a small mountain valley and is relatively isolated from the nearest marmot populations. It is a suitable model for studying the various factors affecting the hybridization process in the natural population. We aimed to study the genetic variability of this population in order to provide genetic evidence for hybridization and estimate the real ratio of parental species and hybrids. Since both species have species-specific vocalizations, we used recorded vocalizations as a potential marker of hybridization. Finally, both, genetic and acoustic data, were used to assess the spatial distribution and habitat preferences of each species in the study zone.

MATERIALS AND METHODS

The mixed population in the zone of sympatry of *M. sibirica* and *M. baibacina* was studied in Ulagchin-Gol river valley, which is a tributary of the Bulgan river in the central part of Mongolian Altai. The place of study is situated in Bayan-Ölgii Aimag in 180 km south-southeast from the Ölgii city with geographical coordinates of the central point at 47°27′N 90°53′E and absolute altitudes from 2500 to 3300 m above sea level (m a.s.l.) (**Figure 1**). The fieldwork was carried out by the team of the Joint Russian-Mongolian Complex Biological Expedition of the Russian Academy of Sciences and Mongolian Academy of Sciences during the summer seasons (June–July) 2007–2009. In our study, we used bioacoustic, genetic and ecological methods.

Bioacoustics Collecting and Analysis

An acoustic signal from marmots was used for remote species diagnosis of individuals in a mixed population. The main task of sound signal analysis was to study the spatial locations of marmots of different species in the population area and to identify places of immediate contact of heterospecific individuals. The alarm call of marmots has well-defined genetically inherited species-specific characteristics (Nikol'skii, 1976, 1984). It has been shown earlier (Brandler et al., 2010b) that the use of the alarm calls as a diagnostic feature is a convenient, non-invasive method to study the spatial distribution of marmots. During summer molting, which lasts from the end of May to mid-July on average, it is rather difficult to distinguish *M. sibirica* and *M. baibacina* in a mixed population by their color and structure of fur. Vocalizations of *M. sibirica* and *M. baibacina,* however, are well recognizable by hearing (**Supplementary Audios 1, 2**), which allows estimating the spatial distribution of individuals of these species during field surveys.

The marmots' alarm calls were recorded in the field in 2008–2009 using a digital recorder "Marantz-PMD 660" ("Marantz," Japan) and a condenser microphone "Audio-Technica AT897" ("Audio Technica," Japan) in uncompressed digital format Wave (WAV) with a discretization frequency of 48 kHz. The acoustic response was provoked by the researcher standing at some distance from the animal or moving slowly toward it. The coordinates of the calling animal locations were determined using a GPS navigator. GPS readings were obtained either directly on the burrow in which the calling marmot was hiding, or in case the burrow was located in a hard-to-reach place, by measuring the distance from the researcher to the animal using a laser rangefinder and azimuth counting by a compass, with the later calculation of coordinates by mapping these data on the space image in the program OziExplorer v3.95 (D&L Software Pty., Ltd.) using the researcher position GPS data. Sounds were also recorded during tissue sampling when marmots were in cages (SM1). In total, the records of 148 individuals from the contact zone of *M. baibacina* and

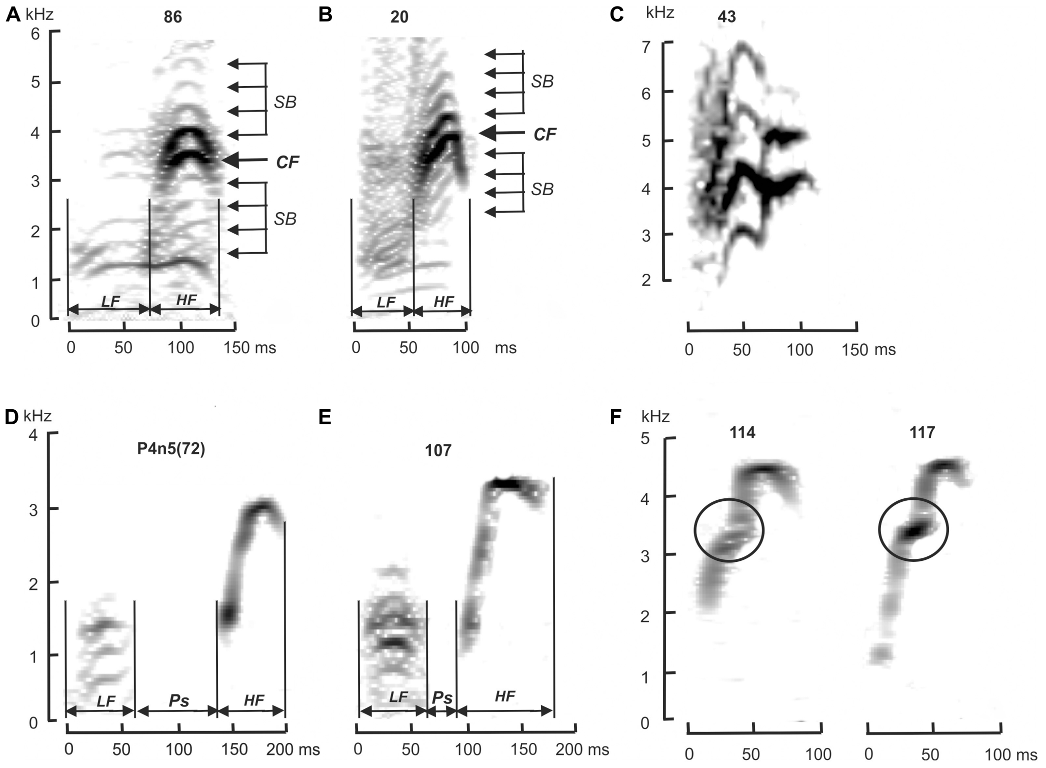

FIGURE 2 | Spectrograms of acoustic signals of *M. sibirica* **(A–C)** and *M. baibacina* **(D–F)**. **(A,D)**—reference populations; **(B,E)**—normal sound signal from Ulagchin-Gol population; **(C,F)** –high-frequency components of aberrant sound signals. *LF*, low frequency component; *HF*, high frequency component; *Ps*, pause between *LF* and *HF*; *CF*, fundamental frequency; SB, sidebands. Digits above—record numbers. KHz, kilohertz; ms, milliseconds.

M. sibirica in the Ulagchin-Gol River valley and its tributaries made in 2008 and 2009 were analyzed. The following records of alarm calls of marmots from populations located in the Mongolian Altai Mountains outside the zone of sympatry were used as species references: *M. baibacina;* 1 individual from the population located at a distance of 90 km NW from the Ulagchin-Gol valley (geographical coordinates 48°19′N 90°29′E), 2 individuals—180 km NW (49°9′N 89°55′E), 2 individuals—200 km NW (49°18′N 89°52′E), 2 individuals—280 km NW (49°40′N 89°05′E), and *M. sibirica;* 10 individuals—320 km SE (45°35′N 94°10′E).

The acoustic characteristics of the alarm calls were analyzed visually using graphical images of oscillograms and spectrograms in the SpectraLab 4.32.17 software ("Pioneer Hill Software LLC," United States) and Raven Pro 1.6.1 software (Center for Conservation Bioacoustics, 2019). The earlier defined temporal and frequency characteristics of *M. baibacina* and *M. sibirica* alarm calls were used as species-specific features (Nikol'skii, 1976, 1984). The alarm call of *M. baibacina* differs from *M. sibirica* by the presence of a pause between the low-frequency (*LF*) and high-frequency (*HF*) components (**Figure 2** and **Supplementary Figures 1**, **2**). The characteristic of the *HF* frequency modulation of *M. sibirica* alarm call is usually symmetrical, while in a *M. baibacina*, the final frequency of this element is significantly higher than the initial one. In addition, in most cases, the spectrum of an alarm call of a *M. sibirica* has sidebands above and below the fundamental frequency caused by the amplitude modulation of the base oscillation (Nikol'skii, 2007). Species identification of an alarm call of individual was identified based on the analysis of these features.

In addition to digitally recording alarm calls for acoustic analyses, we opportunistically recorded the location of individuals of both species when animals vocalized as researchers walked along the river in 2008 (**Figure 3**). In these cases, we assigned each call to a species based on species-specific differences (**Supplementary Audios 1**, **2**). The recognition distance of species alarm calls was about 300 m. Coordinates of the beginning and the end of this route were recorded on a GPS unit.

Tissues Sampling, DNA Extraction, Amplification, Sequencing, and Restriction Analyses

To estimate the genetic diversity of the mixed population of marmots and for genetical characterization of individuals tissue samples kept in the "Joint Wild Animal Tissue Collection for Basic, Applied and Conservation Research" of the Core Centrum of the Koltzov Institute of Developmental Biology RAS (state registration number 6868145) were used. Tissue samples were collected by us in 2007–2009 while catching marmots in the field. Methods of catching and describing of individuals, sampling,

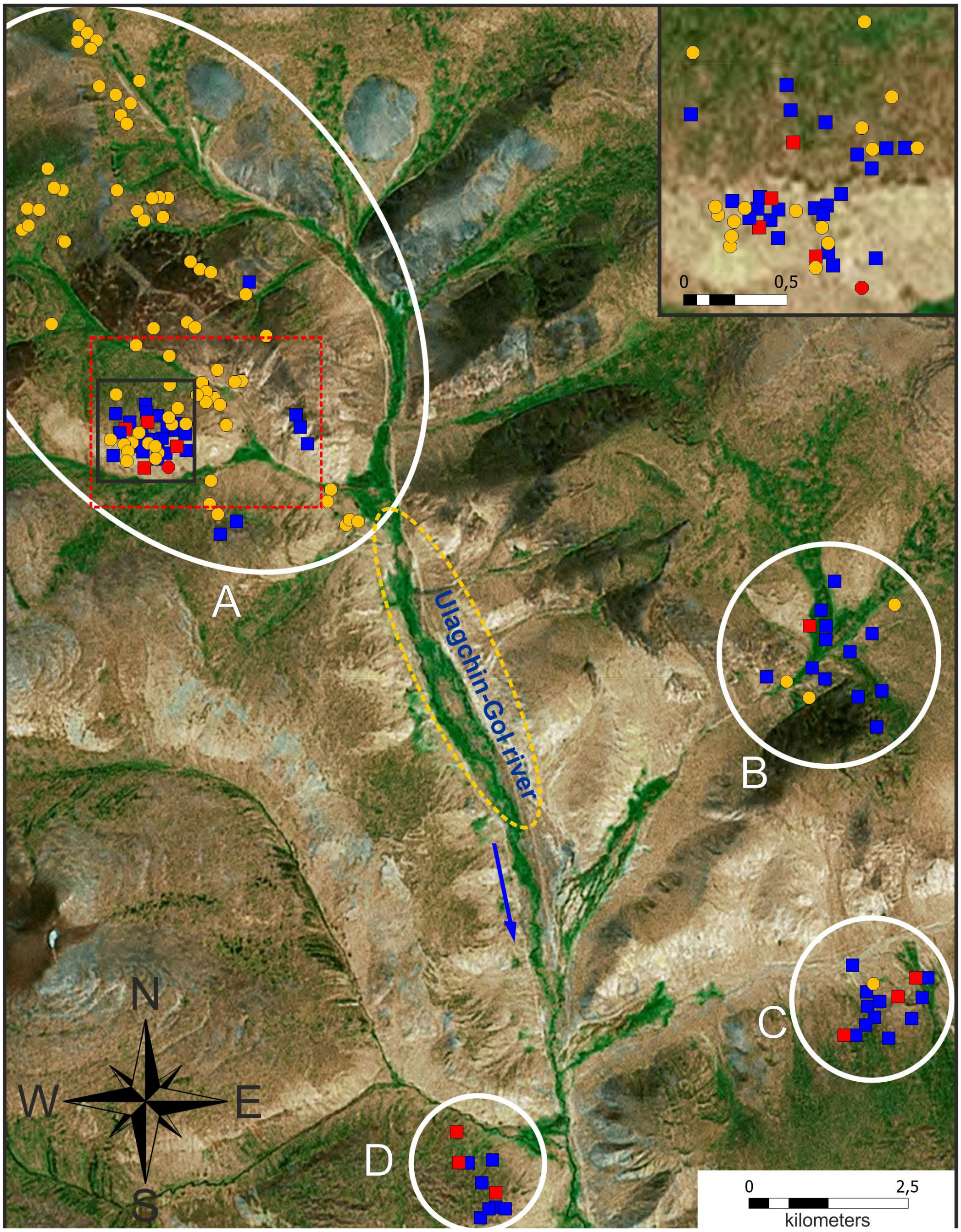

FIGURE 3 | Distribution of two marmot species in the Ulagchin-Gol valley based on analysis of acoustic signals. Blue squares indicate marmots with *baibacina* alarm calls, yellow circles indicate *sibirica*, red icons indicate individuals with aberrations of alarm calls (squares—*baibacina*, circle–*sibirica*). **(A–D)**—plots of surveying. The yellow dotted line indicates the area of the valley where the sound signals were identified as *sibirica* by the researcher by ear (explanation in the text). The blue arrow indicates the direction of the Ulagchin-Gol river flow. The red dotted frame indicates the area where marmots were genotyped (see **Figure 4**).

and transportation of samples are detailed in **Supplementary Material 1**.

Tissue samples of 58 marmots from the sympatry zone of *M. sibirica* and *M. baibacina* in the Ulagchin-Gol valley were investigated (**Table 1**). The information on the field species identification of animals by features of hair color, sex, age, GPS coordinates of the capture location was collected for each specimen. Due to difficulties in visual species identification during molting, species identification was made only for individuals with the least molting fur. The others were marked as *M.* sp. The tissues of 14 specimens of *M. sibirica* and 11 specimens of *M. baibacina* from "pure" (or "reference") populations located at distances from 150 to 1,300 km from the Ulagchin-Gol valley outside the zone of sympatry (**Supplementary Table 1**) kept in the same collection were examined to determine the species specificity of genetic markers.

DNA extraction from tissue samples was carried out using the standard salt method (Aljanabi and Martinez, 1997) or commercial kits for DNA extraction Diatom DNA Prep ("Isogen," Russia). The DNA precipitate was dissolved in deionized distilled water or a standard tris-EDTA buffer (TE) pH 8.0.

Genetic diagnosis of all specimens was carried out using nDNA markers: *i5HoxB*—5th intron of HoxB (homeobox containing gene); *i13BCR*—13th intron of BCR gene (breakpoint cluster region); *i8SmcY*–fragment of the 8th intron male-specific histocompatibility antigen, as well as mtDNA fragment–*cytb* (cytochrome b gene). Nucleotide sequences obtained from individuals originating from "pure" populations were used as references to identify the species specificity of each marker (**Supplementary Table 1**). Additionally, *cytb* sequences from GeneBank were used (*M. sibirica* AF143937, AF143938, and *M. baibacina* AF143915). Species-specific substitutions forming the target sites for specific endonucleases were found in three markers (*i5HoxB*, *i13BCR*, *cytb*). This allowed us to carry out genetic tests of the total mixed population sample using restriction analysis of PCR products of these fragments. The species specificity of *i8SmcY* was identified by nucleotide sequence analysis for all males.

Control region of mtDNA (CR) nucleotide sequences were studied in 51 individuals from a mixed population (**Table 1**) in order to evaluate a genetic variability in population samples of both species. Sequences of 3 *M. sibirica* and 3 *M. baibacina* individuals from "reference" populations were included in the analysis to identify the species specificity of CR haplotypes (**Supplementary Table 1**).

Specific primers (**Supplementary Table 2**) were used for the amplification of the investigated DNA fragments. Amplification reactions were carried out either in 25 μl of the reaction mixture using "DNA amplification" kit ("Silex M," Russia) covered with mineral oil in thermocycler "Tertsik" ("DNA-Technology," Russia) under the following conditions: initial denaturation 94°C 3 min; 30 cycles–denaturation 94°C 1 min, annealing (temperature in **Supplementary Table 2**) 1 min, extension 72°C 1 min; final extension 72°C 10 min, or in 20 μl reaction mixture Screen Mix ("Eurogen," Russia) without oil in Veriti Termo Cycler ("Applied Biosystems," United States) under the following conditions: initial denaturation 95°C 3 min; 30 cycles—95°C 20 s, annealing 40 s, 72°C 40 s (or 1 min for *cytb* and CR); final extension 72°C 7 min (or 10 min for *cytb* and CR).

Species identification of the *i5HoxB* alleles was carried out using *Msp*I*Hpa*II restriction enzyme ("Fermentas," United States). The *i13BCR* and *cytb* alleles were tested using *Pml*I restriction enzyme ("Fermentas," United States). Enzymatic reactions were carried out in 10 μl of reaction mixture containing 20–40 ng of the PCR product, reaction buffer and 2–3 units of the restriction enzyme (according to manufacturer's protocol). The samples were incubated at 37°C for either 1.5–3 h or overnight. The resulting DNA fragments were separated by electrophoresis in 2% agarose gel containing ethidium bromide with subsequent visualization using "Bio-Rad ChemiDoc MP System" ("Bio-Rad," United States).

Molecular Data Analyses

DNA sequences were aligned using ClustalW and edited manually where necessary in MEGA X software (Kumar et al., 2018). The detection of species-specific substitutions in the *i5HoxB*, *i13BCR*, *cytb* sequences, as well as the genetic typing of *i8SmcY* haplotypes, were carried out visually in aligned nucleotide sequences. The median-joining method (Bandelt et al., 1999) was used to construct a CR haplotype network using Network 10 software ("Fluxus Technology Ltd." at)[1].

Standard population genetic characteristics were calculated to estimate genetic diversity. Taking into account the high proportion of hybrids in the mixed population (see section "Results") reflecting the high potential of heterospecifics for interbreeding, we made the assumption that the population of two cohabiting marmot species of the Ulagchin-Gol valley is a single panmictic population. Frequencies of species-specific alleles (*sibirica* and *baibacina*) for each locus, heterozygosity expected (He) and observed (Ho) for each diploid locus and means for samples for all diploid loci, gene diversity, and genetic distances, Hardy–Weinberg equilibria tests were calculated in Arlequin 3.5.2.2 software (Excoffier and Lischer, 2010). The Wright fixation index (F_{is}) was calculated in the FSTAT 2.9.3.2 software (Goudet, 2001).

Spatial Analysis and Habitat Assessment

Spatial distribution of the studied marmots in the mixed population was analyzed using satellite images with a resolution of up to 30 m/pixel freely available by Google (United States) and digitized topographic maps at scales of 1:200,000 and 1:100,000, on which points of location of animals with identified genotypes, as well as vocalizing animals, were mapped in accordance with the recorded GPS coordinates in the OziExplorer v3.95 ("D&L Software Pty., Ltd.") and MapInfo 9.5 ("Pitney Bowes Software Inc.," United States) software.

To assess the habitat preferences of marmots with different genotypes, the locations of all genotyped individuals were classified as one of three major habitat types: (1) valley bottom and lower slope, (2) upper slope and watersheds, (3) large stone plots (boulder screes). An analysis of the population density of marmots in each of these habitats and the distribution of both species of marmots and hybrids was carried out. The significance of the association between the allocation of marmots with different genotypes and habitat types was examined using Fisher's exact test in STATISTICA 8.0 software ("StatSoft, Inc."). The *p*-values from the test were computed for 2 × 2 contingency tables with pairwise grouping of the evaluated factors. We applied the two-tailed Fisher's exact test for all tables except those that included cells with 0, for which the one-tailed version was used. Altitude above sea level was recorded for each animal that was genotyped to understand the impact of altitude on the spatial distribution of marmots.

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[1] http://www.Fluxus-engineering.com

TABLE 1 | Characteristics and results of genotyping of marmots from mixed population of the Ulagchin-Gol valley.

Collection ID	Field morphological species identification	Sex	Age[a]	Altitude, m a.s.l.	Genetic markers[b]					Diagnosis
					i5HoxB	*i13BCR*	*i8SmcY*	*cytb*	CR	
24825	*M. sibirica*	F	juv	2,897	ss	ss	–	s	s3	*M. sibirica*
24826	*M. sibirica*	M	juv	2,744	ss	ss	s	s	s3	*M. sibirica*
24827*	*M. baibacina*	M	ad	3,018	sb	sb	b	s	s5	hybrid (F1)
24828	*M. sibirica*	M	ad	2,818	ss	ss	s	s	s3	*M. sibirica*
24829*	*M. baibacina*	F	ad	3,057	ss	sb	–	s	s5	hybrid
24830	*M. sibirica*	M	ad	2,888	ss	ss	s	s	s3	*M. sibirica*
24831*	*M. baibacina* [55]c	M	ad	2,956	sb	bb	b	b	–	hybrid
24832*	*M. baibacina* [55]	M	ad	2,956	bb	bb	b	b	–	*M. baibacina*
24834	*M. sibirica*	M	ad	2,710	ss	Ss	s	s	s5	*M. sibirica*
24835	*M. sibirica*	F	sub	2,710	ss	ss	–	s	s3	*M. sibirica*
24836	*M. sibirica*	F	sub	2,718	ss	ss	–	b	–	hybrid
24837	*M. sibirica*	M	sub	2,810	ss	ss	s	s	s5	*M. sibirica*
24838*	*M. baibacina*	F	ad	3,024	ss	sb	–	s	s3	hybrid
25051	*M. sp.*	M	–	2,900	bb	bb	b	b	–	*M. baibacina*
25052*†	*M. baibacina*	F	ad	3,062	ss	ss	–	b	–	hybrid
25053*	*M. sp.*	F	ad	2,940	ss	sb	–	s	s3	hybrid
25054*†	*M. sibirica*	M	sub	2,897	sb	sb	s	b	b5	hybrid (F1)
25055*†	*M.* sp. [66]	M	sub	3,041	sb	sb	b	s	s4	hybrid (F1)
25057*†	*M.* sp. [66]	M	ad	2,897	ss	sb	s	s	s3	hybrid
25060	*M. sibirica* [79]	M	juv	2,873	ss	ss	s	s	s3	*M. sibirica*
25061	*M. sibirica* [79]	M	juv	2,873	ss	ss	s	s	s3	*M. sibirica*
25062†	*M. sibirica* [79]	F	sub	2,873	ss	ss	–	s	s3	*M. sibirica*
25063†	*M. sibirica* [79]	F	ad	2,873	ss	ss	–	s	s3	*M. sibirica*
25064†	*M. sibirica* [79]	M	ad	2,873	ss	ss	s	s	s3	*M. sibirica*
25065†	*M. sibirica* [79]	M	sub	2,873	ss	ss	s	s	s3	*M. sibirica*
25066	*M.* sp.	M	ad	2,882	ss	ss	s	s	s5	*M. sibirica*
25069*†	*M.* sp.	M	ad	3,023	ss	ss	s	b	b4	hybrid
25070*†	*M.* sp.	M	ad	3,057	ss	ss	s	s	s3	*M. sibirica*
25071†	*M.* sp.	F	ad	2,880	ss	ss	–	s	s3	*M. sibirica*
25072†	*M.* sp.	M	ad	3,044	sb	bb	b	b	b9	hybrid
25075*	*M.* sp.	M	sub	3,044	sb	bb	b	b	b8	hybrid
25080*	*M.* sp.	F	ad	2,980	bb	bb	–	b	b3	*M. baibacina*
25084†	*M.* sp.	F	ad	2,859	ss	ss	–	s	s3	*M. sibirica*
25085†	*M.* sp.	F	ad	2,877	ss	sb	–	s	s3	hybrid
25086	*M.* sp.	F	ad	2,878	ss	ss	–	s	s3	*M. sibirica*
25098*†	*M.* sp.	F	ad	2,910	ss	ss	–	s	s3	*M. sibirica*
25099*	*M.* sp.	M	ad	2,895	ss	ss	s	b	b7	hybrid
25100	*M.* sp.	F	ad	2,887	ss	ss	–	s	s3	*M. sibirica*
25106*†	*M.* sp.	F	sub	2,940	sb	ss	–	b	b5	hybrid
25107*†	*M.* sp.	F	ad	2,983	ss	ss	–	s	s3	*M. sibirica*
25108	*M.* sp.	F	ad	3,003	sb	sb	–	s	s3	hybrid (F1)
25109	*M.* sp.	M	ad	3,058	ss	ss	s	s	s5	*M. sibirica*
25113	*M.* sp.	F	ad	3,010	ss	ss	–	b	b7	hybrid
25124*	*M.* sp.	M	–	2,816	ss	ss	s	s	s3	*M. sibirica*
25125*†	*M.* sp. [86]	F	ad	2,816	ss	ss	–	s	s3	*M. sibirica*
25126*†	*M.* sp. [86]	M	ad	2,816	ss	ss	s	s	–	*M. sibirica*
25127†	*M.* sp. [144]	M	sub	2,773	ss	ss	s	s	s6	*M. sibirica*
25128†	*M.* sp. [144]	F	ad	2,773	ss	ss	–	s	s5	*M. sibirica*
25129†	*M.* sp. [144]	M	ad	2,773	bb	bb	b	b	–	*M. baibacina*
25130*†	*M.* sp.	F	ad	2,776	ss	ss	–	s	s3	*M. sibirica*
25131*	*M.* sp.	M	ad	2,802	bb	bb	b	b	b6	*M. baibacina*

(Continued)

TABLE 1 | Continued

Collection ID	Field morphological species identification	Sex	Age[a]	Altitude, m a.s.l.	Genetic markers[b]					Diagnosis
					i5HoxB	*i13BCR*	*i8SmcY*	*cytb*	CR	
25132*	*M.* sp.	M	ad	2,788	ss	ss	s	s	s3	*M. sibirica*
25133*	*M.* sp.	M	ad	2,841	sb	sb	s	b	b3	hybrid (F1)
25134	*M.* sp.	F	ad	3,084	ss	ss	–	s	s3	*M. sibirica*
25135*	*M.* sp.	F	ad	2,827	sb	sb	–	b	b5	hybrid (F1)
25260	*M. baibacina*	F	ad	2,680	bb	bb	–	b	b3	*M. baibacina*
25262*	*M. baibacina* [2]	F	ad	2,800	ss	sb	–	b	b3	hybrid
25263*	*M. baibacina* [2]	M	ad	2,800	ss	ss	s	b	b3	hybrid

[a] *juv–juvenile; sub–subadult; ad–adult.*

[b] *i5HoxB, i13BCR—diploid nDNA markers; i8SmcY—haploid nDNA Y-chromosome marker, identified in males; cytb, CR–haploid mtDNA markers. Alleles: s–sibirica; b–baibacina. Numbers indicate the allelic types of CR.*

[c] *Digits in square brackets denote numbers of family groups including more than one genotyped individual.*

* *Individuals inhabiting boulder screes.*

† *Tagged individuals.*

Alarm calls of individuals marked in bold were recorded.

M. sp.—individuals had not species identified in field by colors and structure of fur due to molting.

It is known that marmot population densities are highest in high-quality habitats where environmental needs of individuals are met (Bibikow, 1996). Based on this, we used population density by the number of family groups per unit area (family/km^2) to estimate the suitability of the above three major habitat types for marmots. The family group sites were determined based on the relative location of permanent and/or winter burrows following the common approach (Mashkin and Chelintsev, 1987). The specific placement of visually different types of burrows (permanent, winter, temporary) allowed an accurate identification of the family sites' area frontiers. The family sites were accounted totally within the surveyed area in the upper part of the Ulagchin-Gol valley. The area of the surveyed territory and areas with different habitat types was calculated by mapping the GPS data to a digitized physical map of 1:100,000 scale.

All experimental protocols were approved *post hoc* by the recently formed Ethics Committee for Animal Research of the Koltzov Institute of Developmental Biology RAS (Approval No. 36, 05\03\2020) in accordance with the Recommendations for Laboratory Practice in Russian Federation. Animals were treated according to established international protocols, such as Guidelines for Humane Endpoints for Animals Used in Biomedical Research and Guide for the Care and Use of Laboratory Animals. The study is classified as one of moderate severity and the number of the animals used in this study does not exceed the number needed to meet statistical significance.

RESULTS

Bioacoustics Data

Our data suggested that frequency-temporal features of *M. sibirica* and *M. baibacina* alarm calls from reference populations outside the zone of sympatry correspond to the specific features described earlier for these species (Nikol'skii, 1976, 1984). All signals of *M. sibirica* had no pause between the low and high-frequency components and had a symmetrical high-frequency component with sidebands induced by amplitude modulation (**Figure 2A** and **Supplementary Figures 1A–D**). All signals of *M. baibacina* from all reference populations had a pause between *LF* and *HF* and an asymmetrical *HF* frequency modulation (**Figure 2D** and **Supplementary Figures 2A–D**). No amplitude modulation was found in the last signals. Oscillograms and spectrograms of these alarm calls were used as reference standards for species-specific diagnostics of acoustic signals of marmots from mixed populations.

In total, alarm calls of 148 individuals of marmots were analyzed in the study area; 77 of them were identified as *M. sibirica* and 71 as *M. baibacina*. Among the analyzed signals, 5 were recorded from animals in cages. Three of them were *M. sibirica* and two were *M. baibacina* based on a genetic test. Sound signals of other captured and genotyped marmots could not be recorded due to the short time of keeping in cages and the absence of their vocalization at this time. All the analyzed individuals had clear corresponding species-specific features of the acoustic signal described above (**Figure 2** and **Supplementary Figures 1**, **2**). The spatial distribution of these individuals is shown in **Figure 3**. The records of *M. sibirica* alarm calls were collected mostly in the northern upper part of the valley right up to the headwaters of the Ulagchin-Gol river, where there are more gentle slopes with rich alpine meadow grass (**Figure 3A**). In contrast, the marmots producing calls typical for *M. baibacina* prevailed in the southern lower part of the valley (**Figures 3B–D**). During the survey of the middle part of the valley by walking along the Ulagchin-Gol riverbed, we heard only *M. sibirica* alarm calls (21 notices) on the valley bottom (yellow dotted line in **Figure 3**). We have identified in the upper part of the valley the territory with predominance of boulder screes where both types of acoustic signals were equally common (framed in **Figure 3**). 17 *sibirica* and 25 *baibacina* phonotypes were recorded in this place.

An aberration of *HF* modulation was found in 12 individuals (17%) with *baibacina* phonotype (**Figure 2F**). In contrast to the normal form, the frequency of *HF* of the aberrant signal initially increased with normal rate, then the frequency rose sharply in a short time interval, followed by a weakly modulated fragment of the signal, including the maximum of the fundamental frequency. The signal ended with a weak frequency decrease similar to the normal alarm call. Only one individual had an alarm call that was different from the normal ones among all *M. sibirica* signals (**Figure 2C**), which is a rare single aberration that has not been previously described in this species. Despite the differences between aberrant signals and normal ones, they could be assigned to one of the species based on the above-mentioned species–specific features.

Thus, the acoustic characteristics described here showed that the species specificity of the alarm call of each specimen from the Ulagchin-Gol valley was clearly diagnosed, and the intra-population variability of the acoustic signal through aberrations of its frequency-temporal properties was detected. Differentiated spatial distribution of individuals with different species alarm calls was detected along the valley.

Molecular Genetic Data

In order to be used as references, nucleotide sequences of *i5HoxB*, *i13BCR*, *cytb*, and *i8SmcY* were sequenced from individuals of *M. sibirica* and *M. baibacina* from reference populations outside the zone of sympatry (**Supplementary Table 1**). Nucleotide variabilities of all molecular markers were presented in **Supplementary Materials** (**Supplementary Figures 3–6**). No intraspecific variations in autosomal markers were detected. Species-specific substitutions in *i5HoxB*, *i13BCR*, and *cytb* formed recognition sites for some restrictases. The *Pml*I restrictase cut sequences of *i13BCR* of *M. sibirica* and *cytb* of *M. baibacina* at positions 395 and 292, respectively, into two fragments of unequal length. The *Msp*I*Hpa*II restrictase cut the *i5HoxB* sequence of *M. baibacina* at position 305. This sequence was also cut by this enzyme at positions 392, 456, and 579, forming a set of fragments identical for both species (**Supplementary Figure 7**).

The obtained data made it possible to use restriction analysis for molecular testing of the total sample of marmots (58 individuals) from a mixed population for genetic typing by *i5HoxB*, *i13BCR,* and *cytb* loci. Individuals from reference populations of both species are included additionally as a control of test results. Genotyping of 31 males from the mixed population was carried out using *i8SmcY* based on two species-specific replacements (**Supplementary Figure 5**) by sequencing.

A species test of the total sample of marmots from the mixed population was carried out on the basis of the analysis of allele composition. The alleles of both species of each genetic marker were found in the studied sample. An individual was associated with one species of marmots if it had alleles of only one species for all markers. In other cases, the individual was found to be a hybrid. The results are presented in **Table 1**.

According to the results of genetic diagnostics, more than half of marmots (62%) of the examined sample may be classified as one of the species (51.72% *M. sibirica*, 10.34% *M. baibacina*). The others (38%) were classified as hybrid marmots and had alleles of both species in different combinations (**Table 1**). In the total sample of marmots, the frequency of *sibirica* nDNA alleles was three times higher on average, and the frequency of *sibirica* mtDNA alleles almost twice higher than that of *baibacina* (**Table 2**).

The prevalence of nDNA *sibirica* alleles was also observed in the sample of hybrids. At the same time, maternal lines (*cytb*) of *M. baibacina* prevailed in hybrids and paternal lines (*i8SmcY*) of both species and were represented almost equally (**Table 2**). Most of the hybrid males (8 of 11) had different species markers of mtDNA and Y-chromosomes. The total ratio of species-specific haplotypes of sex markers in hybrid males was close to equal (41% *sibirica* and 59% *baibacina*), but *baibacina* mitotypes prevailed in their mtDNA (73%). An approximately equal ratio of species-specific alleles was also found in their nuclear genome (59% *sibirica* and 41% *baibacina*).

Since the bioacoustic analysis had detected a place with a predominance of boulder screes where individuals with alarm calls of different species live together, a sample of marmots from this area was analyzed separately. The analyses showed a high prevalence of hybrids (62%) in this sample. The number of *M. sibirica* individuals found here was only twice as high as *M. baibacina* (25 and 13%, respectively). In this sample, the frequency of diploid marker alleles of *sibirica* was higher than that of *baibacina*, and their ratio was similar to that observed in the sample of hybrids (**Table 2**). At the same time, the ratio of *cytb* mitotypes was near to 1:1.

The observed heterozygosity (Ho) for each of the diploid markers of nDNA was lower than expected one (He), while the Wright fixation index (F_{is}) is > 0 in the total sample (**Table 3**). The same trend was observed in the sample from the boulder screes place. In this sample, the heterozygosity values in the latter were appreciably higher, and the F_{is} value was lower by half.

The spatial distribution of genotyped individuals is shown in **Figure 4**. "Pure" tarbagans inhabit the entire studied area but are more attracted to the banks of creeks and slopes. "Pure" gray marmots, which are much less numerous, are concentrated in or near large rocky places. One *M. baibacina* was also identified in the lower part of the valley where this species predominates according to bioacoustic analysis (**Figure 3D**). Hybrids, as well as tarbagans, were present throughout the area but concentrated more in boulder screes (**Figure 4** and **Supplementary Tables 3**, **4**).

TABLE 2 | Frequencies of species-specific haplotypes of molecular genetic markers in the mixed marmot population in the Ulagchin-Gol valley.

Genetic marker	Total sample (*n* = 58)		Hybrids (*n* = 22)		Boulder screes (*n* = 28)	
	Sibirica	***Baibacina***	***Sibirica***	***Baibacina***	***Sibirica***	***Baibacina***
i5HoxB	0.81	0.19	0.74	0.26	0.75	0.25
i13BCR	0.74	0.26	0.61	0.39	0.64	0.36
cytb	0.66	0.34	0.36	0.64	0.50	0.50
*i8SmcY**	0.71	0.29	0.55	0.45	0.62	0.38

**Only for males: in the total sample, n = 31; in hybrids, n = 11; in boulder screes, n = 16.*

TABLE 3 | Population genetic characteristics of the mixed marmot population of the Ulagchin-Gol valley.

Genetic marker	Heterozygosity		Mean heterozygosity (± SD)		F_{is} ($p < 0.05$)	
	He	Ho	He	Ho	Per locus	All
Total sample ($n = 58$)						
i5HoxB	0.3100	0.1724	0.3484 ± 0.0543	0.1897 ± 0.0244	0.446	0.458
i13BCR	0.3868	0.2069			0.467	
Boulder screes ($n = 28$)						
i5HoxB	0.3818	0.2857	0.4247 ± 0.0606	0.3214 ± 0.0505	0.255	0.247
i13BCR	0.4675	0.3571			0.239	

Of the six family groups in which all or part of the individuals were genetically tested, only two consisted of "pure" *M. sibirica* (**Table 1**); only hybrids were found in two family groups; one group contained a hybrid and *M. baibacina* and another contained both *M. baibacina* and *M. sibirica*.

In total, 57 complete nucleotide sequences of the mtDNA control region (998–1,001 bp) were sequenced to estimate the level of general intra-population genetic variability. Fifteen haplotypes of CR (**Table 1** and **Supplementary Table 1**) were found in the studied sample, including individuals from "pure" populations and from the contact zone. All haplotypes formed two clusters on the haplotype evolutionary network (**Figure 5**). One cluster combined two haplotypes of "pure" *M. sibirica* with four haplotypes from the mixed population, while two haplotypes of "pure" *M. baibacina* formed another cluster together with seven haplotypes from the mixed population. Interspecific differences consisted of 72 nucleotide substitutions. The genetic variability indices for this locus in the sample of marmots from the mixed population are presented in **Table 4**.

Of the 24 marmots for which specific identification was visually determined in the field, only in 15 individuals did the visual identification match by the results of genetic typing (**Table 1**). It should be noted that in 2 out of 9 cases of incorrect field identification, alleles of the species to which it was visually assigned prevailed in specimen genotypes. These results showed that the species identification of these marmots in the hybrid population was not reliable, based on external features.

All five marmots for which sound records were made in cages were not genetic hybrids (three *M. sibirica* and two *M. baibacina*).

In summary, the genetic analysis revealed a high proportion of hybrids in the population and their high concentration in boulder screes in studied part of the colony. All new DNA haplotype sequences were deposited in GenBank under accession numbers MT412445–MT412473.

Spatial Analysis and Habitat Assessment Data

Half of the total sample of genetically tested marmots inhabited boulder screes, 38% on the lower parts of mountain slopes and at the bottom of the valley, 12% on the upper parts of slopes and on watersheds (**Supplementary Table 3**). The majority of *M. sibirica* individuals (62%) were found in the lower parts of the mountain slopes and at the bottom of the valley. Equal numbers of *M. baibacina* were found in the latter habitat type and in the boulder screes. Most hybrid individuals (78%) originated from boulder screes. Fisher's exact test *p*-values (**Supplementary Table 4**) indicate no differences in the frequency of occurrence of pure individuals of both species in all habitats, and a non-random distribution of hybrids across habitats.

A survey of 103 family sites of marmots on the area of 5.71 km^2 was carried out to estimate a population density in different habitats in the upper part of the Ulagchin-Gol valley at the plot where genetic and acoustic surveys were conducted (**Figure 3A**). The population density was 48.6 families/km^2 in lower parts of mountain slopes and at bottom of the valleys, 2.4 families/km2 in upper parts of the slopes and watersheds, and 6.4 families/km2 in boulder screes.

Our data did not reveal the influence of altitude on the distribution of marmots with different genotypes. The average habitat altitude of *M. sibirica* was 2,862 ± 16.8 m a.s.l. in the range of 2,710 m a.s.l. minimum to 3,084 m a.s.l. maximum; *M. baibacina* was 2,849 ± 47.5 m a.s.l., 2,680–2,980 m a.s.l. (min–max); hybrids was 2,942 ± 21.3 m a.s.l., 2,718–3,062 m a.s.l. (min–max).

DISCUSSION

Population Genetic Features of *M. baibacina* and *M. sibirica* Hybridization

Our data on molecular genetic typing of marmots from the area cohabited by *M. baibacina* and *M. sibirica* indicated an active hybridization of these two species. The high frequency of hybrid individuals in the population (38% of the total sample) and the presence in their genomes of almost all possible combinations of parent species alleles of studied markers (**Table 1**) indicated viability and fertility of F1 hybrids and viability of their offspring.

The predominance of *sibirica* alleles in the nuclear genome in both the hybrids and the general sample most likely reflected the prevalence of *M. sibirica* individuals in the studied area that could lead to more frequent reciprocal crossbreeding with this form. The lack of *M. baibacina* in cohabitation sites may be the cause of the relatively low observed proportion of F1 hybrids. At the same time, the high proportion of hybrids in the population indicated intensive crossbreeding with both parent species and other hybrids in different combinations. Taking into account the presence of genotypes with almost all possible allele combinations

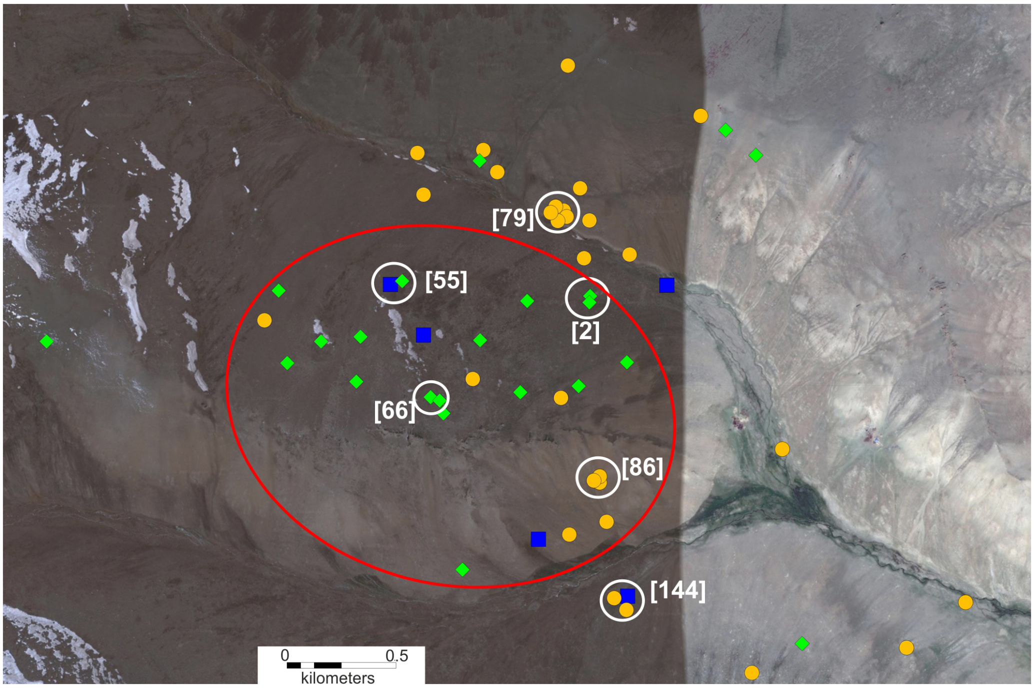

FIGURE 4 | Spatial distribution of genotyped individuals in the area of cohabitation of two species of marmots (it is marked by a red dotted frame in **Figure 3**). Symbols: orange circle—*M. sibirica*; blue square—*M. baibacina*; green rhombus—hybrid; white circle—single-family individuals; red oval indicates the area of boulder screes. Family identification numbers are in square brackets (see **Table 1**).

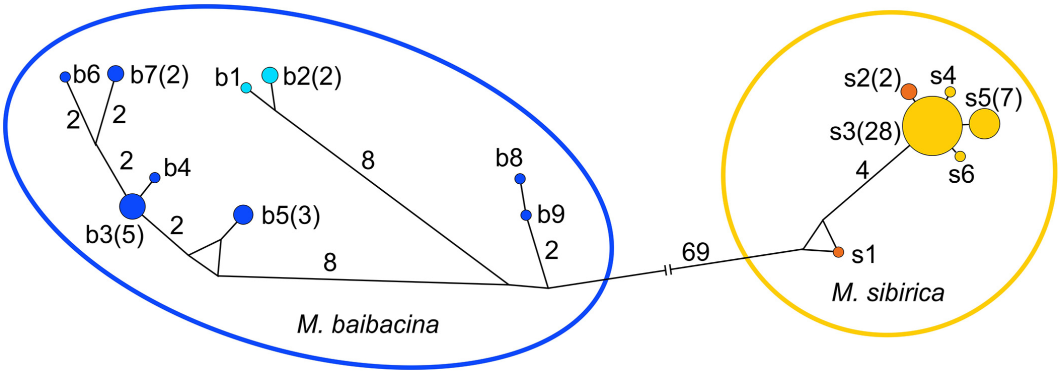

FIGURE 5 | The mtDNA control region haplotypes network. Haplotypes: light blue—"pure" *M. baibacina*; orange—"pure" *M. sibirica*; blue and yellow for *M. baibacina* and *M. sibirica*, respectively, from the mixed population. Haplotype designations as in **Table 1** and **Supplementary Figure 1**. Figures in brackets are the number of individuals.

TABLE 4 | Ratios of CR genetic diversity of marmots in the Ulagchin-Gol valley population.

Haplotypes	n	Number of haplotypes	Number of polymorphic loci	Gene diversity (± SD)	Average genetic distance over loci (± SD)
M. sibirica	37	4	3	0.401 ± 0.087	0.0004 ± 0.0004
M. baibacina	14	7	21	0.846 ± 0.074	0.0065 ± 0.0036

in the population, we supposed that crossbreeding of individuals with different genotypes occurred without significant limitations, and F1 or backcross sterility may be absent or very poorly expressed. However, the amount of our data was not sufficient to confirm the fertility of backcrosses. The presence of both species' alleles in hybrids in both the mitochondrial genome (maternal lines) and Y-chromosome (paternal lines) (**Table 2**) indicated the involvement of males and females of both species in the hybridization. In this case, a partial or total violation of Haldane's rule, i.e., sterility and non-viability of the heterogametic sex in hybrids, may be assumed, however, this requires more in-depth study. Apparently, the independent evolution of these species during about 2.5–3 million years in allopatric conditions (Steppan et al., 2011) did not lead to the development of effective postzygotic isolation.

However, there was a heterozygote deficiency in the mixed Ulagchin-Gol valley population (Ho $<$ He, $F_{is} > 0$ both for each diploid autosomal marker and on average in all samples (**Table 3**), indicating a lack of panmixia and population subdivision–the "Wahlund effect" (Wahlund, 1928). Although our data are insufficient to demonstrate the absence of fertility limitations in backcrossing with hybrids, they indicate a high genetic compatibility of these species. High potential for panmyxia in the mixed population is also supported by a smaller F_{is} value and low Ho and He differences in the sample from boulder screes, where the increase in heterozygosity can be prevented by a constant flow of "pure" individuals into this area. Taking this into account, it may be assumed that, besides the possible reduced viability of hybrids, one of the source of population subdivision may be pre-mating mechanisms such as behavioral or ecological features that limit free crossbreeding in a mixed population.

Phenotype and Genotype Intrapopulation Variability

The frequency-time structure of the alarm call is a good diagnostic characteristic for the studied pair of marmot species. The alarm call similar to the aberrant signals of *M. baibacina* that we found (**Figure 2F**) was considered a hybrid attribute in earlier studies of this population (Formozov and Nikol'skii, 1986). No genetic analysis has been carried out in this research. We cannot say that the found aberrations were indications of the hybrid origin of the individual, although this cannot excluded. Recently, aberrations of the sound signal that differ from those described by us were found in the Tien Shan population of *M. baibacina* in the contact zone with *M. caudata* (Nikol'skii and Vanisova, 2020). However, the authors believe that the formation of these aberrations in the result of hybridization is unlikely. Earlier, aberrations of the sound signal with frequency modulation characteristics similar to those we found in the Ulagchin-Gol valley were found in the closely related species *M. bobak* (Nikol'skii, 2008). They were variants of intraspecific variability, the frequency of which increases in marginal populations under isolation. The population of the Ulagchin-Gol valley is located near the boundaries of distribution of both contacting species, and the found aberrations of the sound signal may be elements of intraspecific variability that arise under the influence of the same factors as in *M. bobak*. The relationship between the sound signal aberrations and interspecies hybridization in the peripheral populations of *M. baibacina* requires additional genetic studies.

The high variability of *baibacina* phonotype structure corresponds to the variability of *baibacina* control region of mtDNA haplotypes in the investigated population (**Table 4** and **Figure 5**). According to a central-marginal hypothesis (Eckert et al., 2008), marginal populations are more characterized by a low level of genetic diversity formed by the founder effect and/or genetic drift. The variability of *sibirica* haplotypes, but not *baibacina*, corresponds to this hypothesis. Moreover, a high level of genetic variability in marginal populations is often associated with the areal oscillation (Vandermeer, 2006), such as in the greater long-tailed hamster *Tscherskia triton* under the influence of Pleistocene glacial fragmentation of their area of distribution (Sheremetyeva et al., 2017). We may hypothesize that the high level of phonotype and haplotype variability of baibacina either have been maintained during the reduction of the *M. baibacina* range accompanied by the loss of monogenic marginal populations and the transition of central populations to the species distribution boundary or formed by gene flows due to cyclic fragmentation-defragmentation of marginal parts of the range during Pleistocene climate changes. Although populations in hybrid zones are often characterized by a high level of variability, we do not have enough data to conclude that high levels of variability in *M. baibacina* acoustic signal and CR is associated with hybridization.

Individuals of hybrid origin were detected significantly more by molecular genetic typing than by comparing spectrograms. However, the predominance of individuals with the alarm call of *M. baibacina* (60%) in the sample from boulder screes (**Figure 3**) with the high proportion of hybrids did not correspond to the observed low frequency of *baibacina* alleles in this site (**Table 2**). Probably, intermediate phenotypic traits are not expressed or weakly expressed in *M. baibacina-M. sibirica* hybrids, in contrast to that found in *S. major-S. fulvus* and *S. major-S. suslicus* hybrids (Titov et al., 2005; Kuzmin et al., 2011). The lower incidence of intermediate phenotypic traits compared to the level of hybridization detected with genetic markers is also known in other mammals, for example, in wolf-dog hybrids (Lorenzini et al., 2014). An understanding of the phenotypic expression of mixed genotypes in marmots requires further investigation.

Spatial, Habitat, and Behavioral Background of Hybridization

The spatial analysis of locations of individuals with different species traits (**Figure 3**) indicates that two marmot species are distributed unequally in the Ulagchin-Gol valley space. *M. sibirica* prevails in the upper part and *M. baibacina* prevails in the lower part of the valley. The optimal habitat for both marmot species in the study area seemed to be the bottom of the valley and the lower part of the slopes. The density of family sites found in this habitat type was four times higher than the average population density (11 families/km^2) in other places of Mongolian marmot areas with similar environmental conditions (Kolesnikov et al., 2010). According to this and results of Fisher's exact test (**Supplementary Table 4**), marmots in the Ulagchin-Gol valley least preferred watersheds, and boulder screes, although more favorable, are also suboptimal. Predominant occupation of valley bottoms by *M. sibirica* may indicate that it displaced *M. baibacina* from these habitats. At the same time, it is known that stony habitats are unfavorable for *M. sibirica* (Badmaev, 2020), and it is reluctant to inhabit such habitats. On the other hand, *M. baibacina* is a more "mountainous" species (i.e., more adapted to living in composite topography) than *M. sibirica* (Bibikow, 1996), and therefore it is more tolerant of living in boulder screes. The observed ecological segregation of two species of marmots is consistent with the found "Wahlund effect" and may be one of the factors limiting panmyxia and, consequently, hybridization in a mixed population. Taking into account the fact that both species of marmots inhabit all three types of habitats outside the area of range overlap (Rogovin, 1992; Brandler et al., 2010b), the detected habitat separation of these species may indicate their biotopic reorientation (change in habitat preferences) as a result of competitive relationships in a hybrid population leading to a decrease in the frequency of interspecific contacts similar to that described for mixed settlements of ground squirrels *S. major* and *S. suslicus* (Titov et al., 2012).

The spatial distribution of genetically analyzed individuals (**Figure 4**) is generally consistent with sound signal analysis. The high incidence of hybrid individuals and their concentration in boulder screes (**Supplementary Tables 3**, **4**) apparently indicates that the most numerous contacts between heterospecific individuals and a mixed pair formation occur precisely in these places as well as subsequent crossbreeding of hybrids. In addition, the family group, which consists of a heterospecific couple of adults [144] (**Table 1** and **Figure 4**), inhabited close to a large rocky area. Nevertheless, hybrids were found not only in boulder screes but, apparently, they were able to penetrate adjacent areas and coexist with both parent species, however, insufficient data are currently available to quantify a dispersion of hybrids in this population. Previous studies using tagged animals have shown that the migration distance for *M. baibacina* is 1–4.5 km, but only 13.7% of migrants move more than 1 km (Bibikow, 1996). Based on these data, we assume that the dispersion of hybrids declines rapidly as they move away from their birthplace.

One of the possible hypothetical hybridization scenarios for this population is based on the ecological segregation of different species, high occupancy of habitats, and the species-specific structure of colonies, basis of which is formed by stable territorially-conservative family groups not allowing non-residents to enter their area (Bibikow, 1996; Adiya, 2007; Armitage, 2014). Young animals are also generally banished by their parents from the family site after reaching puberty and they become migrants. Considering the high level of intrapopulation aggression and the low number of interfamily regroupings found earlier in this population (Kolesnikov and Svininykh, 2010), we may assume that non-resident migrants are highly exposed to elimination in this area by predators, diseases and injuries inflicted by resident individuals during territorial conflicts as well as in other marmot species in similar conditions (Suntsov, 1981; Armitage, 2003; Mashkin et al., 2010). As a result, animals are more likely to succeed if they settle close to their native site in suboptimal habitats where mixed couples may be formed in conditions of limited partner choice. In this case, conspecific assortative mating is significantly reduced due to specific copulation time. Male and female of these marmot species choose mates and form pair bonds in the fall prior to hibernation, and copulation occurs in the burrow prior to emergence in the spring (Bibikow, 1996). Thus, conditions of limited mate choice may contribute to the formation of hybrids in suboptimal habitats. The high probability of mixed couples and the short distance of successful dispersion of migrants may explain the detection of higher concentrations of hybrid individuals in boulder screes places. Obviously, it is not the only factor determining the spatial features of these species hybridization. Further studies on the migratory behavior of hybrids may provide the necessary data to better understand this process.

Divergence times of the *bobak* group including *M. baibacina* and the *camtschatica* group including *M. sibirica* were estimated to be 3.5–4 Ma (Steppan et al., 2011), i.e., the event occurred relatively soon after an initial invasion of marmots into Asia (4.6 Ma). Allopatric speciation in these groups developed during the epi-platformal orogenesis of Eurasia (Nikol'skii and Rumiantsev, 2012) under the influence of glacial and interglacial events of Middle and Late Pliocene and Pleistocene (Erbaeva, 2003; Polly et al., 2015). Taking the above dates into account, we can say that the duration of *M. baibacina* and *M. sibirica* speciation corresponds to the average time of evolution of hybrid inviability in mammals (2–4 million years) (Fitzpatrick, 2004), which is consistent with relatively high genetic interspecific differences between them (Steppan et al., 2011). At the same time, as our study shows, intrinsic postzygotic reproductive barriers between these species have not yet been formed. It can be assumed that the mountain glaciations of the Altai formed an effective geographical barrier between these species at the end of the Pliocene and the beginning of the Pleistocene, which agrees with the model of reduction of marmots' ranges during cold glacial cycles (Nikol'skii et al., 1999; Polly et al., 2015). In any case, the recent overlapping of *M. baibacina* and *M. sibirica* areas might occur only as a result of their expansion after the last spatial isolation. We assumed that ranges of *M. baibacina* and *M. sibirica* were isolated during mountain glaciations. The last climatic cooling in the Mongolian Altai that may have a significant impact on mountain-steppe ecosystems and, consequently, on the distribution of marmots corresponds to the Sartan Glaciation and has occurred at about 25 Ka (Devyatkin, 1981). On the other hand, *M. baibacina* and *M. sibirica* may have been isolated during an expansion of forests in the mountains of Mongolia the last of which was dated to the Middle Holocene and ended 4,000 years ago (Vipper et al., 1989). In any case, it may be assumed that the overlapping zone of *M. baibacina* and *M. sibirica* areas followed the disappearance of isolation barriers and existed for a relatively long time, possibly with some interruptions related to short-term climatic changes.

Comparing the studied pair of marmot species with other hybridizing Marmotini, it can be noted that the contact between *M. baibacina* and *M. bobak* in Kazakh upland also has a relatively long duration resulting from the expansion of *M. bobak* 5–7 Ka (Nikol'skii et al., 1983). In most other known cases of intense hybridization, contact zones were formed relatively recently as a result of an expansion of one or several species under influence of anthropogenic factors that transform the natural environment. Examples of this scenario are the contact zones of *S. major* with the neighboring species *S. suslicus*, *S. fulvus*, and *S. pygmaeus* (Ermakov et al., 2002) and *I. parvidens*–*I. tridecemlineatus* (Stangl et al., 2012).

The level of hybridization we documented between *M. baibacina* and *M. sibirica* is lower than what has been reported in Palaearctic *Spermophilus*, both in the proportion of hybrids in populations and in spatial coverage (Ermakov et al., 2002, 2015; Titov et al., 2005; Spiridonova et al., 2006; Ivanova et al., 2017). But in general, hybridization occurs more frequently in secondary contact zones within Palaearctic *Marmota* and *Spermophilus* than Nearctic Marmotini (Hird et al., 2010; Stangl et al., 2012; Frare et al., 2017; Leitner et al., 2017). This may be due to the greater divergence of Nearctic species and, consequently, forming more effective interspecific barriers compared with Palearctic species (Herron et al., 2004; Brandler et al., 2010a). However, neither our study nor those addressing ground squirrel hybrid zones have identified adaptive effects of gene introgression as found in other mammalian hybrid zones [e.g., formation of a hybrid swarm in *Odocoileus* (Haines et al., 2019); introgressive replacement of the mitochondrial genome in *Lepus* (Marques et al., 2017)].

CONCLUSION

Our study is the first description and characterization of the hybrid zone in marmots using genetic, bioacoustic, and ecological approaches. We revealed active hybridization in the secondary contact zone of two marmot species *M. baibacina* and *M. sibirica*. Genetic data detected no limitations for heterospecific crossbreeding and indicated fertility of F1 and their descendants, but also indicated subdivision of the mixed population, the source of which requires more extensive genetic research. The differential spatial distribution found in the valley and the change in habitat preferences of the two marmot species may limit the free interbreeding of heterospecifics. We previously suppose that behavioral features of marmots associated with long-term territory maintenance and family structure of the colony combined with ecological specificity contribute to the appearance of hybrids. At the same time, aggressive elements of intrapopulation behavior in combination with landscape heterogeneity of surrounding area may limit the dispersion of hybrid individuals reduce the impact of gene introgression on species genomes. Further studies of hybrid migration and gene pools of surrounding marmot populations may provide required data for understanding the gene flow between these hybridizing species. The adaptive significance of hybridization remains unclear in the study population, and further studies might shed light on it.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank NCBI, accession numbers MT412445–MT412473.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Research of the Koltzov Institute of Developmental Biology RAS.

AUTHOR CONTRIBUTIONS

OB designed the study and prepared this manuscript. OB, SK, VK, and BB collected tissue samples. SK extracted DNA and performed PCRs and restriction. OB and SK performed molecular data analysis, mapped spatial data, recorded sound signals, and contributed equally to the study. AN and OB performed bioacoustic analysis. VK tagged marmots and performed population density assessment. YA provided guidance and field works in Mongolia. All authors contributed to the editing and finalizing of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.555341/full#supplementary-material

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Adaptation-Driven Evolution of Sirtuin 1 (SIRT1), a Key Regulator of Metabolism and Aging, in Marmot Species

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The sirtuin protein family plays a role in the lifespan of various species and is involved in numerous key metabolic processes. To understand the evolutionary role of sirtuins in marmots, a long-living rodent species group with remarkable metabolic shutdown during hibernation, we conducted a phylogeny-based substitution rate analysis of coding genes based on genetic information of seven marmot species. We show that sirtuin 1 (SIRT1) has evolved under positive selection in the marmot lineage. We pinpoint three amino acid changes in four different marmot species that underlie the signal of positive selection and that may favor increased longevity in marmots. Based on a computational structural analysis we can show that all three substitutions affect the secondary structure of the same region in human SIRT1. We propose that the identified region is close to the catalytic domain and that the potential structural changes may impact the catalytic activity of the enzyme and therefore might be playing a functional role in marmot's extended lifespan and metabolic shutdown.

Keywords: non-model species, dietery restriction, hibernation, positive selection, 3D structure

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1. INTRODUCTION

Maximum lifespans of mammals differ greatly between species (Kowalczyk et al., 2020). Although the rodent species evolved a tenfold variability in lifespan (Gorbunova et al., 2014), the underlying molecular and evolutionary mechanisms are still little understood. Studies on ageing processes have identified the highly conserved sirtuin family (Zhao et al., 2020), whose effect on lifespan was first demonstrated in yeasts and is suspected to have a similar influence in mammals (Haigis and Sinclair, 2010).

The mammalian sirtuins have seven members (SIRT1 to SIRT7) (Frye, 2000) and are a protein family of nicotinamide adenine dinucleotide (NAD+)-dependent deacylases (Haigis et al., 2006). While they have a conserved catalytic core domain in common (Frye, 2000), they differ by cellular localization, functions, and substrates (Maissan et al., 2021). The best studied protein of this family is SIRT1 which is predominately a nuclear protein and acts as a transcription regulator and deacetylates certain histones such as H3, H4, and H1 (Toiber et al., 2011), but also modifies more than 50 non-histone proteins (Nakagawa and Guarente, 2014), such as p53 (Luo et al., 2001). Generally, it influences energy metabolism, cell survival, DNA repair, and tissue regeneration as well as numerous other important cellular processes (Bonkowski and Sinclair, 2016). Like SIRT1, SIRT6, and SIRT7 are located in the nucleus and involved in histone modification. While SIRT6 controls the expression of multiple glycolytic genes and is a regulator of glucose homeostasis

(Zhong et al., 2010), SIRT7 is a positive regulator of RNA polymerase transcription and is linked to cell proliferation (Ford et al., 2006). In contrast, SIRT2 is primarily located in the cytoplasm where it regulates various metabolic enzymes. However, under stress and during mitosis, it also migrates into the cell nucleus where it regulates the cell cycle and cell differentiation (Vaquero et al., 2006). The other mammalian sirtuins SIRT3, SIRT4, and SIRT5 are mitochondrially localized. There, SIRT3 and SIRT5 influence a variety of metabolic enzymes (e.g., in fatty acid oxidation) (Verdin et al., 2010), while SIRT4 is active in amino acid metabolism by inhibiting glutamate dehydrogenase (Haigis et al., 2006).

In addition to the functions already mentioned, sirtuins, especially SIRT1, have been investigated for their role in caloric restriction (Finkel et al., 2009; Derous et al., 2021). It has long been known that calorie restriction significantly delays the aging process and extends life span in a variety of animal species, including rodents, and has been shown to reduce the incidence of age-related disorders (e.g., diabetes, cancer, and cardiovascular disease) in mammals (Bordone and Guarente, 2005). In humans, the association between SIRT1 expression and caloric restriction (Civitarese et al., 2007) as well as an age-related increase in SIRT1 expression intensity (Kilic et al., 2015) has been demonstrated. Furthermore show transgenic mice with brain-specific SIRT1 overexpression a significant delay of the aging process and an extension of life span (Satoh et al., 2013).

Since sirtuins play an important role in aging processes it has been postulated that the evolution of sirtuins may be associated with the heterogeneity of maximum lifespan between species (Tian et al., 2017). Despite the numerous analyses of sirtuin functions, the insights of evolutionary processes of this important protein family is fairly limited. Currently existing studies mainly focus on model organisms, such as mice (Vanhooren and Libert, 2013). Exceptionally, a recent study pointed out amino acid substitutions in beaver SIRT6 relative to mouse SIRT6 leading to lifespan extension (Tian et al., 2019). Based on these promising results we were intrigued whether positive selection might have been played a role in the evolution of sirtuin protein members in other rodent species. A sequence-based analysis of positive selection might reveal particular amino acid with potential impact on sirtuin catalytic activity and may explain an association with longevity. As the sirtuins are associated with dietary restricted longevity here we focused on a species group that experiences long periods of dietary restriction in form of deep hibernation and conducted a phylogeny-based substitution rate analysis based on genetic information of seven marmot species (Cardini and O'Higgins, 2004). We find that sirtuin 1 (SIRT1) has evolved under positive selection in the marmot lineage.

2. MATERIALS AND METHODS

2.1. Data Retrieval

For the analysis of the seven mammalian sirtuin protein family members SIRT1 to SIRT7 we obtained coding DNA sequences, including several isoforms or different prediction entries from NCBI Genbank (Clark et al., 2015). The sequences were retrieved for human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), American beaver (*Castor canadensis*), and guinea pig (*Cavia porcellus*). The coding DNA sequence for SIRT5 for rats could not be retrieved. Additionally, we obtained some sirtuin coding DNA sequences for marmots from Genbank, including sequences of SIRT1, SIRT3, SIRT5, and SIRT6 for yellow-bellied marmot (*Marmota flaviventris*) and the sequence of SIRT 5 for Alpine marmot (*Marmota marmota marmota*, Gossmann et al., 2019). A complete list of retrieved sirtuin sequences along with their respective identifiers can be seen in Supplementary Table 1 (see **Supplementary Material**). To conduct an analysis tailored to marmots, additional publically available coding sequences for SIRT1 to SIRT7 homologs were predicted. For this, coding sequences were predicted from genomic data based on sequence homology to the available Refseq sirtuin marmot sequences using Exonerate v2.2.0 (Slater and Birney, 2005). The genomes of the woodchuck (*Marmota monax*), the Himalayan marmot (*Marmota himalayana*) and the Vancouver Island marmot (*Marmota vancouverensis*) were retrieved from Genbank. Furthermore, coding sequences for the Mongolian marmot (*Marmota mongolia*), long-tailed marmot (*Marmota caudata*) and Altai marmot (*Marmota baibacina*) could be obtained by using re-sequencing information (Bai et al., 2019) (http://www.marmotdb.org/). Specifically, coding information were obtained by using the vcf's information obtained from mapping short read data onto the Himalayan reference genome by Bai et al. (2019). Each species for which sequence data was used was included in the phylogenetic tree that was provided obtained from Timetree (Kumar et al., 2017).

2.2. Sequence Preparation and Selection Analysis

To prepare the obtained sequences a customized python pipeline was designed based on available scripts from Afanasyeva et al. (2018). In brief, it contained general automated preparation steps, e.g., renaming data files, adjusting identifier information and storing the coding sequences for each sirtuin SIRT1 to SIRT7 from each species. Note, that this pipeline could be easily extended to handle large scale data, such as entire proteomes or across large trees (Afanasyeva et al., 2018; Yusuf et al., 2020). Then, each coding DNA sequence was translated into the corresponding protein sequence using the standard genetic code. All sequences containing a stop codon within the sequence were disregarded for further analysis. The translated sequences were then aligned using MUSCLE v3.8 using default parameter settings (Edgar, 2004). These alignments were used for a manual inspection and limited to the selection of one sequence per species and protein. This was necessary, because alignment artifacts may artificially create a signature of positive selection (Afanasyeva et al., 2018). Criterions for the exclusions of sequences included sequences with missing fragments in comparison to the human canonical sequence as well as sequences with potentially erroneous intron exon boundaries. The identifier of the selected sequences which were retrieved from Genbank are listed in **Supplementary Table 2**, while the selected sequences of the predicted sirtuin sequences for SIRT1 are shown in Supplementary Table 3 (**Supplementary Material**).

Alignments of the selected and translated sequences where further processed using PRANK v.170427 using default parameter settings (Loytynoja, 2013) and critical residues were masked with ZORRO (Wu et al., 2012) when the confidence score was smaller than nine. Combined with the previous coding DNA sequences the results were used as input for PAL2NAL v14 (Suyama et al., 2006) to create coding DNA sequence alignments. Thereby, output files with renamed identifier were created as input for PAML (Yang, 2007). Prepared codeml files and phylogenetic tree files pruned down to the number of available proteins were then used as input for codeml of the PAML package.

To conduct a selection analysis based on substitution rates, codeml from the PAML package version 4.9j was used. Two model types were considered. First, a branch model comparison was executed to determine heterogeneity in dN/dS between the marmot clade and the rest of the tree (two-ratio model) relative to a model where a single dN/dS ratio (one-ratio model) was assumed. Secondly, branch-site comparisons were used to test for the evidence of positive selection within the marmot clade. We compared the branch-site model A to a model where a potentially positively selected site category was restricted to drift (e.g., dN/dS = 1). Furthermore, the site models model 1 (M1a, NearlyNeutral, 2 categories) (Wong et al., 2004; Yang et al., 2005) and model 22 (M2a_rel, PositiveSelection, 3 categories) (Weadick and Chang, 2012), that allow the ω ratio to vary among sites (among codons or amino acids in the protein), were compared to a Clade model C (Bielawski and Yang, 2004).

2.3. Selection Model Comparison

The codeml-generated output files were further processed using a customized python script. For the branch test comparison dN/dS values were extracted from the one ratio and two ratio models and twice the log likelihood difference was assumed to be χ^2 distributed with one degree of freedom. Same accounts for the three different branch-site model comparisons. Here, the dN/dS values were extracted for model A, model A fixed, model=1 (M1a) and model=22 (M2a_rel), as well as for Clade model C. Again, twice the log likelihood difference of model A against model A fixed (with $\omega = 1$) was assumed to be χ^2 distributed with one degree of freedom. For model M1a against Clade model C d.f.=3 was used while one degree of freedom was used for model=22 (M2a_rel) against Clade model C. Furthermore, the NEB (Naïve Empirical Bayes) and BEB (Bayes Empirical Bayes) values were extracted from rst-files from the codeml output for data plotting.

2.4. Structural Prediction

A 3D model was calculated using the SWISS-MODEL (Guex et al., 2009) (with access on SWISS-MODEL Interactive Workspace, n.d.) a template for human SIRT1 protein structure from Protein Data Bank (4I5I) (Zhao et al., 2013) and the coding sequence of human SIRT1. With FirstGlance (FirstGlance in Jmol, n.d.) the model was visually processed. The influence of the substitution of selected amino acids regarding the secondary structure of the protein was analyzed using Jpred4 (Drozdetskiy et al., 2015) as well as Porter 5.0 (Torrisi et al., 2019) submitting the protein sequence of human SIRT1 in comparison to the same sequence with single amino acid substitution [with substitution of lysin (K) to threonine (T) in amino acid sites 499, serine (S) to leucin (L) in amino acid sites 508 and substitution of arginine (R) to isoleucine (I) in amino acid sites 613] deducted from the marmot sequences of *M. sibirica*, *M. himalayana* and *M. caudata*, and *M. flaviventris*, respectively.

3. RESULTS

3.1. Branch Test: Differences in Evolutionary Rates in Marmot Clade

Differences in the evolutionary rates, recovered from between-species comparisons, may reveal molecular footprints of selective pressures. Here we investigated heterogeneity in the rate of molecular evolution of sirtuins between the marmots and other mammalian species (**Figure 1**).

The ratio of non-synonymous to synonymous substitutions ω = dN/dS was used as a measure for the rate of molecular evolution at the protein level. The dN/dS ratio indicates the driving selective forces in the evolutionary process, as elevated dN/dS ratios above one suggest the influence of positive selection ($\omega > 1$) while a low ratio ($\omega < 1$) reveals a process driven mainly by negative selection (Gossmann and Schmid, 2011). The comparison of the estimated evolutionary rate between the one ratio (single dN/dS ratio for all considered species) and two ratio model (different dN/dS ratio for marmot clade) revealed higher dN/dS ratios in the marmot clade relative to the compared species for six of the seven sirtuin proteins (SIRT1, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) (**Table 1**).

Indeed, we find a significant difference of the d_N/d_S ratios between the marmot clade and the remaining tree for SIRT1. This shows that the marmot clade has a different evolutionary rate regarding SIRT1 than the remaining branches of the phylogenetic tree. The explanation for the higher dN/dS ratios could be relaxed purifying selection, but it could also suggest a possible influence of positive selection specific to marmots.

3.2. Branch-Site Test: SIRT1 Repeatedly Evolved Under Positive Selection

Since the branch test revealed an increased rate of molecular evolution in the marmot clade relative to other mammalian species, a possible role of positive selection events was further analyzed. To investigate positive selection in the evolution of SIRT1 in the marmot clade three branch-site model comparisons were executed. The branch-site model A was compared to the model A fixed where a potentially positively selected site category was restricted to drift (with dN/dS = 1). Furthermore, Clade C was compared to model 1 (M1a, NearlyNeutral) and also to model 22 (M2a_rel, PositiveSelection). All three comparisons revealed a significant difference (**Figure 2**), indicating the occurrence of positive selection events within the marmot clade.

A significant difference for the three different likelihood comparisons is assumed if twice the log likelihood difference

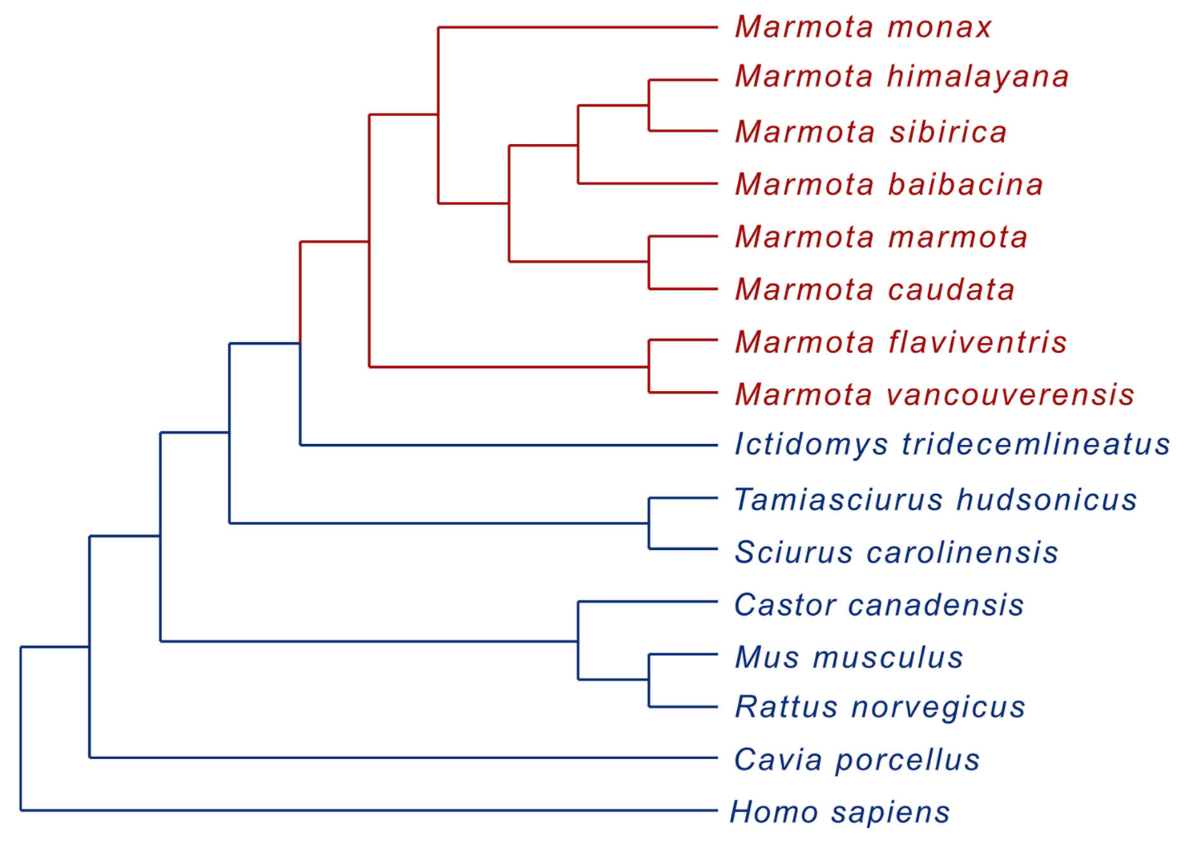


FIGURE 1 | A cladogram of the phylogenetic relationship of the species used for substitution rate analyses, divided into A: marmot clade (red) and B: other mammalian species (blue).

TABLE 1 | Results of the branch model comparison for SIRT protein coding genes.

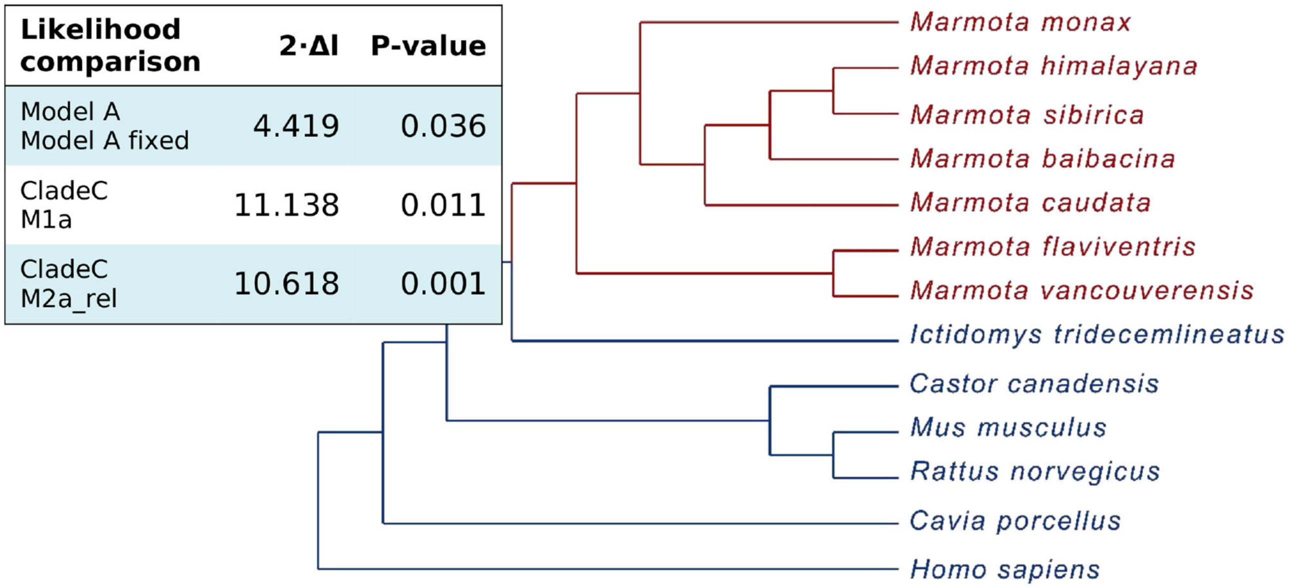
Sirtuin	d_N/d_S (1ratio)	d_N/d_S_A (2ratio)	d_N/d_S_B (2ratio)	2 Δl	*P*-value
SIRT1	0.149	0.700	0.144	3.973	0.046
SIRT2	0.085	0.066	0.086	0.202	0.653
SIRT3	0.150	0.175	0.149	0.048	0.826
SIRT4	0.078	0.152	0.076	1.093	0.296
SIRT5	0.179	0.300	0.173	1.377	0.241
SIRT6	0.041	0.104	0.039	1.953	0.162
SIRT7	0.049	0.065	0.046	0.768	0.381

For the comparison of the estimated evolutionary rate between the one ratio (single dN/dS ratio for all considered species) and two ratio model (different dN/dS ratio for A: marmot clade and B: other mammalian species) a significant difference is assumed if twice the log likelihood difference (2Δl = 2 · (l1 – l2) with l1 and l2 as the log of the maximum likelihood (ML) estimated of the two models) is χ^2 distributed with d.f. = 1.

(2Δl = 2 · (l1 − l2) with l1 and l2 as the log of the maximum likelihood (ML) estimated of two models) is χ^2 distributed. A likelihood comparisons between the branch-site model A and model A fixed, where a potentially positively selected site category was restricted to drift (e.g., dN/dS = 1) was performed to test for the evidence of positive selection within the marmot clade (χ^2 distribution with d.f. = 1). Furthermore, results of a likelihood comparisons between Clade model C (Bielawski and Yang, 2004) and site models model 1 (M1a, NearlyNeutral, 2 categories) (Wong et al., 2004; Yang et al., 2005) (χ^2 distribution with d.f. = 3) and a comparison between Clade model C and model 22 (M2a_rel, PositiveSelection (3 categories)) (Weadick and Chang, 2012) (χ^2 distribution with d.f. = 1) differed significantly.

To identify individual amino acid substitutions that were likely promoted by positive selection, we determined every amino acid's probability to belong to a class with either low (dN/dS < 1), neutral (dN/dS = 1) or a high dN/dS ratio (dN/dS > 1). As the different models revealed similar results, we focused on model A results. The gained NEB (Naive Emperical Bayes) probabilities show three amino acid sites with high posterior probabilities (> 75%) for site class 2 with ω = dN/dS = 1.68 (**Figure 3**), indicating that the footprints of positive selection can be pinpointed to particular amino-acid replacements. Similar results have been obtained for posterior probabilities based on BEB (Bayes empirical Bayes, **Figure 3**) probabilities. This approach accounts for sampling error (Yang et al., 2005) illustrating that the amino acids sites underlying the signal of positive selection can be robustly identified.

The three outlined amino acid sites presumably have undergone positive selection. The alignment of the analyzed



Likelihood comparison	2·Δl	P-value
Model A Model A fixed	4.419	0.036
CladeC M1a	11.138	0.011
CladeC M2a_rel	10.618	0.001

FIGURE 2 | Results of branch-site model comparisons for SIRT1 protein coding genes with a cladogram of the phylogenetic relationship of the species used for analyses, divided into A: marmot clade (red) and B: other mammalian species (blue).

sequences for SIRT1 shows the amino acid sites and the corresponding substitutions within the marmot clade (**Figure 4**). In respective to coding sequence of human SIRT1 the amino acid sites are 499 with substitution of lysin (K) to threonine (T) in *M. sibirica*, 508 with substitution of serine (S) to leucin (L) in *M. himalayana* and *M. caudata* and 613 with substitution of arginine (R) to isoleucine (I) in *M. flaviventris*.

3.3. Secondary Structure Prediction

Since we identified substitutions that presumably evolved under positive selection, we were interested whether we could identify a possible impact of these residues on the structural level. To investigate this effect further, a secondary structure prediction of human SIRT1 protein was performed along with *in-silico* mutated variants of the human protein, substituting the three residues we identified from the marmot species. Intriguingly, we find that a differently predicted substructure within the amino acid sites 518 to 523 of the human SIRT1 sequence is observed for each of the 3 residues (**Figure 5**). Interestingly the secondary structure of this 6 amino acid long sequence stretch can be predicted only with low confidence, but it is embedded in a coiled coiled region that can be confidently predicted. The observed effect on the secondary structure is also remarkable, because one of the mutations is 270 bp (90 aa) away from the affected amino acid stretch. While the human sequence is predicted with a helix structure for the mentioned amino acid sites, the sequences with the single substitutions each are predicted with a predominantly coiled structure instead. We conducted control mutations where we randomly substituted amino acid residues nearby the identified positions and did not find such an effect on the structure prediction for serine and arginine (**Supplementary Figures 3E–G**). We find that changes of nearby lysin residues to the observed mutations had a similar impact that lead to a coiled-coiled prediction in the target region (**Supplementary Figures 3H–J**).

This structural difference may alter the enzyme function. As Zhao et al. (2013) reported the catalytic domain of SIRT1 reaches up to the residue 516. Their estimated structure (shown in **Supplementary Figure 1**) which ends with the amino acid E 512 suggests a very close position of the subsequence QKELAY with amino acid sites 518 to 523 to the substrate bounded in the catalytic domain. Assuming a coiled structure for the subsequence (as predicted in **Figure 5**) instead of the shown strand in **Supplementary Figure 1** it is conceivable that the subsequence QKELAY might have an influence on the substrate affinity.

4. DISCUSSION

The association of sirtuins and dietary restricted longevity has been at the center of ageing research for more than a decade (Dali-Youcef et al., 2007; Guarente, 2007; Imai, 2007). Nevertheless, little is known about the role of molecular adaptations of sirtuins especially in non-model animals experiencing long periods of dietary restriction under natural conditions. These studies hardly deviate from the usual models, such as yeast, mouse and human, leading to a limited amount of insights (Vanhooren and Libert, 2013; Mitchell et al., 2015). Our analyses, as well as work of Tian et al. (2019) with highlighted results found in beaver SIRT6 protein, underline the importance of considering animals outside the common model organisms. To understand the influence of sirtuins on dietary restricted longevity, it is important to compare the different species with their different phenotypic characteristics regarding dietary

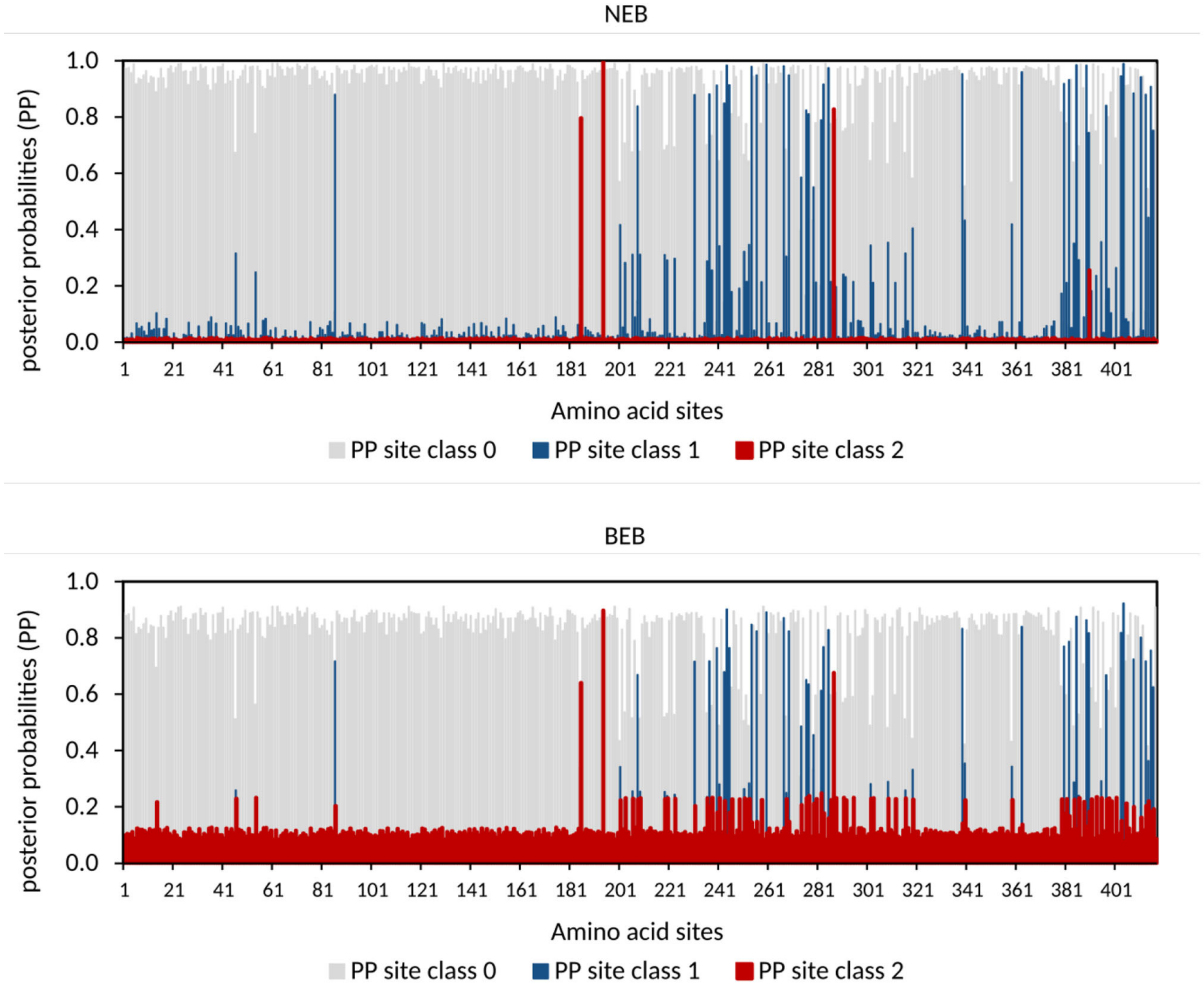
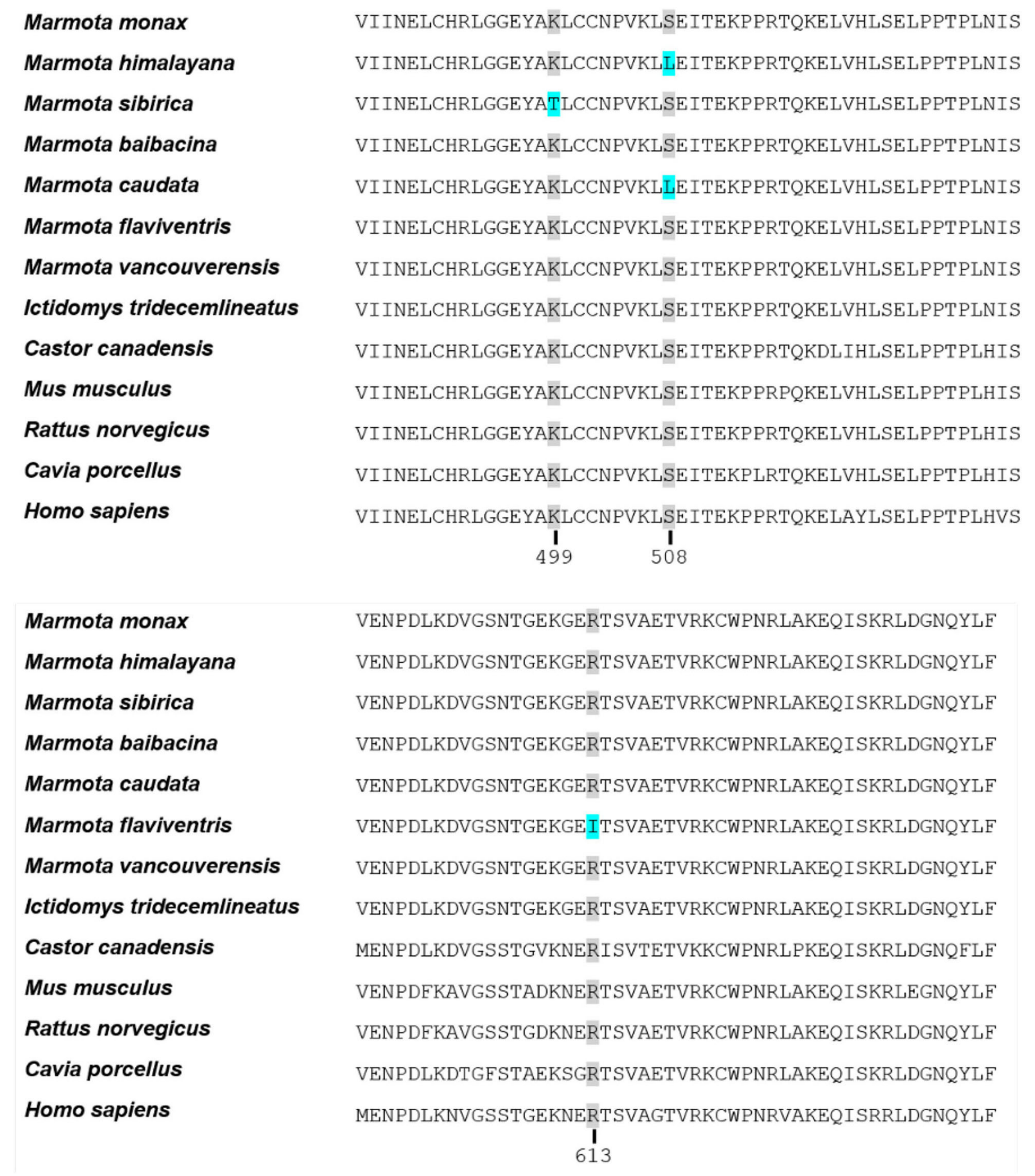


FIGURE 3 | Quantitative representation of the Naive Empirical Bayesian (NEB) and Bayes Emperical Bayesian (BEB) class probabilities derived from the branch-site model comparison as a test for positive selection. The amino acid sites respective to human SIRT1 protein sequence fit into the classes with certain dN/dS ratios with the given posterior probabilities. Posterior probabilities for site class 2 indicate the probability for positive selection and are shown in red.

restriction and longevity. Learning about the correlation of the genomic differences and phenotypic characteristics helps discovering the genetic basis for a long life span. In order to gain such important insights, species with extreme characteristics in the respective area, such as in this case animals with extreme dietary restriction, should be especially considered.

Here, we report differences in sirtuin evolution in the marmot clade relative to other mammalian species. We find a significantly increased rate of molecular evolution for one of the sirtuin family members, SIRT1. Increased rates of d_N/d_S might stem from relaxed purifying selection, or reflect an overall lower effective population size in the marmot lineage (Gossmann et al., 2019). We tested a third hypothesis that could drive this pattern: positive selection. Indeed, We identify three amino acid substitutions occurring in four specific marmot species but not in humans or the common model organism mouse. Interestingly, for two species, *M. himalayana* and *M. caudata* the same amino acid substitution may have appeared independently or may stem from common ancient genetic variation, illustrating the possibility of parallel evolution. The fact that significant differences were observed for three branch-site model comparisons underlines the robustness of the analysis and demonstrates the independence of the result from the model assumptions.

With the huge difference in dietary restriction between these species the results outline the importance of an even broader analysis. Our phylogeny-based substitution rate analysis presented here can easily be extended to sequences of many other species to learn more about sirtuins and their evolution. The investigation of other deep hibernators such as dormice, chipmunks, hamsters, fat-tailed lemurs, bats and hedgehogs (Geiser, 2011) could provide further findings. For this gaining

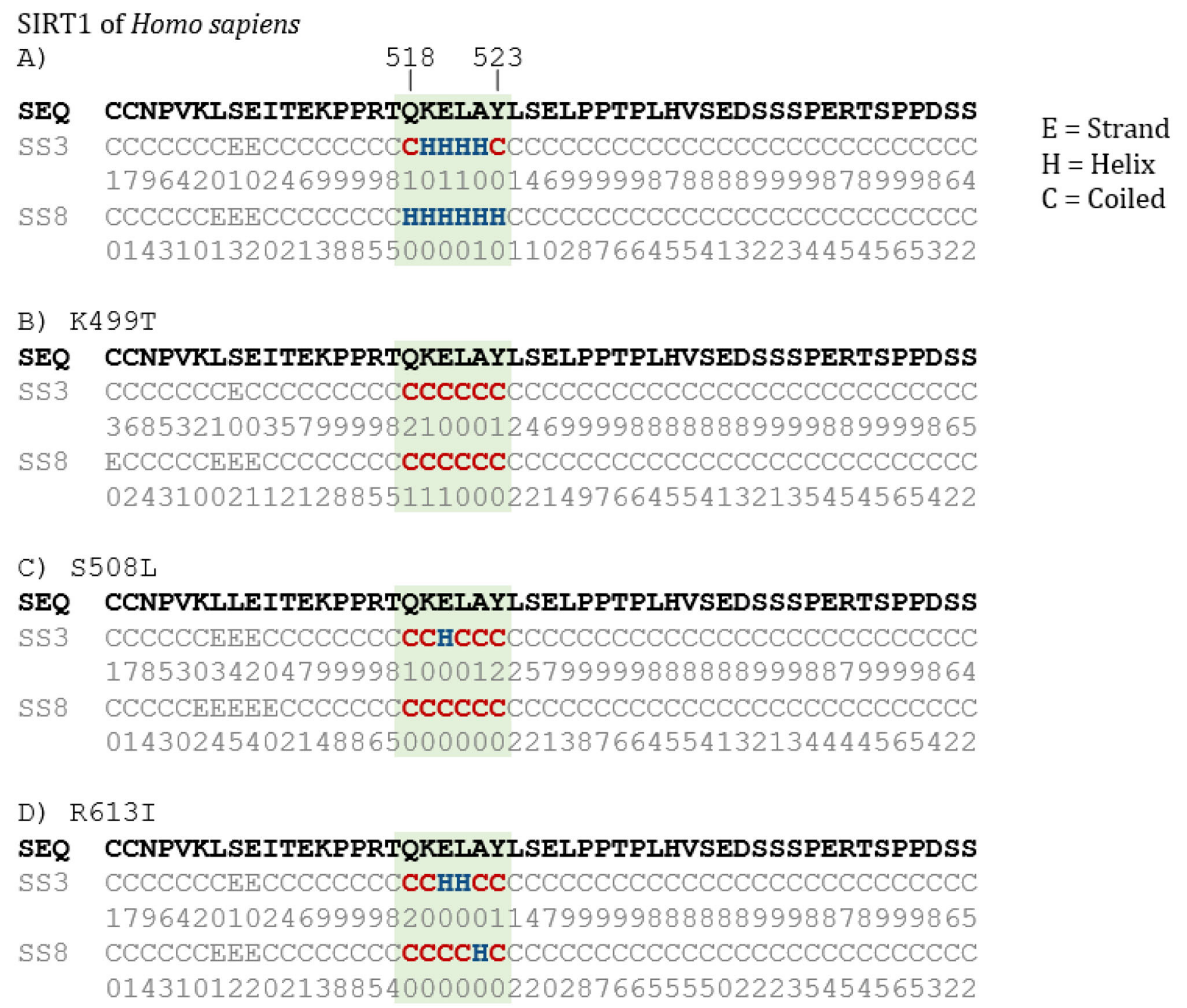


Marmota monax	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Marmota himalayana	VIINELCHRLGGEYAKLCCNPVKLLEITEKPPRTQKELVHLSELPPTPLNIS
Marmota sibirica	VIINELCHRLGGEYATLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Marmota baibacina	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Marmota caudata	VIINELCHRLGGEYAKLCCNPVKLLEITEKPPRTQKELVHLSELPPTPLNIS
Marmota flaviventris	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Marmota vancouverensis	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Ictidomys tridecemlineatus	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLNIS
Castor canadensis	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKDLIHLSELPPTPLHIS
Mus musculus	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRPQKELVHLSELPPTPLHIS
Rattus norvegicus	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELVHLSELPPTPLHIS
Cavia porcellus	VIINELCHRLGGEYAKLCCNPVKLSEITEKPLRTQKELVHLSELPPTPLHIS
Homo sapiens	VIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVS
	499 508

Marmota monax	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota himalayana	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota sibirica	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota baibacina	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota caudata	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota flaviventris	VENPDLKDVGSNTGEKGEITSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Marmota vancouverensis	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Ictidomys tridecemlineatus	VENPDLKDVGSNTGEKGERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Castor canadensis	MENPDLKDVGSSTGVKNERISVTETVKKCWPNRLPKEQISKRLDGNQFLF
Mus musculus	VENPDFKAVGSSTADKNERTSVAETVRKCWPNRLAKEQISKRLEGNQYLF
Rattus norvegicus	VENPDFKAVGSSTGDKNERTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Cavia porcellus	VENPDLKDTGFSTAEKSGRTSVAETVRKCWPNRLAKEQISKRLDGNQYLF
Homo sapiens	MENPDLKNVGSSTGEKNERTSVAGTVRKCWPNRVAKEQISRRLDGNQYLF
	613

FIGURE 4 | Relevant sections of the alignment of SIRT1 proteins from 13 mammals including seven different marmots. Substitutions with the signature of positive selection (analyzed by branch site model comparison with codeml of the PAML package) are highlighted in blue (amino acid sites 499, 508, and 613). The Alignment was performed with MUSCLE.

further insights depend on the genome sequences of additional species. Large scale genome projects such as the Bat1K (Teeling et al., 2018) will allow for more phylogenetic analyses in the future. Furthermore, population genetic analyses can be performed to better understand the recent evolutionary mechanisms underlying lineage specific mutations.



SIRT1 of *Homo sapiens*

E = Strand
H = Helix
C = Coiled

```
A)                           518  523
                              |    |
SEQ  CCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERTSPPDSS
SS3  CCCCCCCEECCCCCCCCCHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     17964201024699998101100146999998788889999878999864
SS8  CCCCCCEEECCCCCCCCHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCC
     01431013202138855000010110287664554132234454565322

B) K499T
SEQ  CCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERTSPPDSS
SS3  CCCCCCCECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     36853210035799998210001246999988888889999889999865
SS8  ECCCCCEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     02431002112128855111000221497664554132135454565422

C) S508L
SEQ  CCNPVKLLEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERTSPPDSS
SS3  CCCCCCEEECCCCCCCCCCHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     17853034204799998100012257999998888889998879999864
SS8  CCCCCEEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     01430245402148865000000221387664554132134444565422

D) R613I
SEQ  CCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERTSPPDSS
SS3  CCCCCCCEECCCCCCCCCCHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     17964201024699998200001147999998888889998878999865
SS8  CCCCCCEEECCCCCCCCCCCCHCCCCCCCCCCCCCCCCCCCCCCCCCCCC
     01431012202138854000000220287665555022235454565322
```

FIGURE 5 | Relevant section of secondary structure prediction. Protein sequences for secondary structure prediction are **(A)** human SIRT1 protein sequence, and human SIRT1 protein sequences with substitution of **(B)** lysin (K) to threonine (T) in amino acid site 499, **(C)** serine (S) to leucin (L) in amino acid site 508, **(D)** arginine (R) to isoleucine (I) in amino acid site 613. Predictions were performed with Porter 5.0 (Torrisi et al., 2019). Secondary structures are divided into three classes: E, Strand; H, Helix; C, Coil; SS3, 3 class secondary structure; SS8, 8 class secondary structure, each followed by the line of confidence values for 3 and 8 class predictions, respectively (with 9, maximal confidence; 0, very little confidence).

As experimentally shown in the work of Tian et al. (2019), the discovered amino acid substitutions have an influence on lifespan, demonstrated by swapping the critical amino acids of mouse and beaver SIRT6. Similar experiments using mouse models and inserting the amino acid substitutions highlighted in this work could verify the effect on molecular mechanisms and life span. The protein changes observed are in regions of human Sirt1 involved in NAD binding and a potential phosphorylation site at residue 530 (Shan et al., 2017; Ling et al., 2018). It would be intriguing to investigate further whether this could indicate the regulation of the Sirt1 interaction networks (Elibol and Kilic, 2018) being under selection. Due to lack of quality sequencing data of the annotated marmot genomes that were used in this study, we could not analyse the region that is homologous to the N-terminus of human/mouse Sirt1. Since the SIRT1 N-terminal region that has been shown to interact with histone proteins (Vaquero et al., 2004) future investigation could shed further light on the functional implications of evolutionary transitions in SIRT1.

In conclusion, this work demonstrates the ability of phylogenetic studies to determine the signature of molecular adaptation, especially when looking at a range of species with specific life-history features, which is enabled by large quantities of genome information and the use of computational approaches. However we note, that studies on a comparable scale involving other species clades will be crucial to shed light on the evolutionary signatures of extreme life styles to deduct the mechanistic underpinnings of metabolic adaptation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NJ and TG contributed to conception and design of the study. NJ performed the analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.666564/full#supplementary-material

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