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Misinterpretation of Genomic Data Matters for Endangered Species Listing: The Sub-specific Status of the Peñasco Least Chipmunk (*Neotamias minimus atristriatus*)

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We provide a response to a recently published evaluation of the subspecies status of the Peñasco least chipmunk (*Neotamias minimus atristriatus*). The work we discuss used exon capture genomic approaches and concluded that their results did not support the distinction of this taxon as a subspecies, with recommendation that it be synonymized with *N. m. operarius*. We refute the interpretations, conclusions, and taxonomic recommendations of this study, and explain in clearer terms how to interpret genomic analyses for applied management. We identify four broad conceptual issues that led to errant recommendations: (1) interpretation of subspecies and diagnosability, (2) inappropriate use of reciprocal monophyly as a criterion for subspecies, (3) importance of geographic isolation, and (4) error in hypothesis testing and misinterpretation of results. We conclude that the data from this genomic appraisal add to information from prior studies providing strong support for recognition of *N. m. atristriatus* as a subspecies. Our conclusions have important and immediate implications for the proposed listing of *N. m. atristriatus* as an endangered species under the U.S. Endangered Species Act.

Keywords: conservation genomics, Distinct Population Segment, Endangered Species Act, independent evolutionary trajectory, integrative taxonomy, reciprocal monophyly

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Recent decades have seen a surge in technological development of genomic sequencing methods for non-model organisms, along with associated bioinformatic data processing, and downstream evolutionary analyses. In parallel with these advances, genomic data are increasingly being applied to questions of taxonomic validity among wildlife, and toward systematic rearrangements within and among species of conservation concern. These applications may have consequential repercussions for regulatory legislation. It is therefore increasingly critical to bridge the "conservation genomics gap" for more effective exchange of knowledge among genomic researchers, conservation managers, and public stakeholders (Shafer et al., 2015; Taylor et al., 2017a; Kadykalo et al., 2020). This will necessitate an improvement in how scientists translate technical jargon for knowledge-users, and will ensure that the scientific interpretation of results accurately reflects the limitations of the genomic data or analyses. As an example of these persistent issues, we discuss the results, interpretation and conservation-related recommendations from a recently published phylogenomic study of chipmunks, which bear on a pending decision of U.S. federal protections under the Endangered Species Act (ESA; Puckett et al., 2021).

Least chipmunks (*Neotamias minimus*) are the most widespread species within a diverse mammalian genus collectively distributed throughout western North America (Piaggio and Spicer, 2001; Reid et al., 2012). There are currently 21 subspecies of least chipmunks that reflect a complex history of differentiation and diverse ecological and biogeographical associations (Verts and Carraway, 2001). The main focus of Puckett et al. (2021) was to evaluate the taxonomic validity of the Peñasco least chipmunk (*N. m. atristriatus*), a geographically isolated subspecies at the southernmost extent of the species' range, and which has been extirpated from most of its historical distribution (Hope and Frey, 2000; Frey and Boykin, 2007; McKibben and Frey, 2020). The taxon was described as a species by Vernon Bailey, a seminal mammalian taxonomist and naturalist, on basis of cranial and pelage characteristics (Bailey, 1913). Bailey stated of *Eutamias atristriatus* (= *N. m. atristriatus*), that "In cranial characters this chipmunk shows so little similarity to *E. operarius* (= *N. m. operarius*), its apparently nearest relative, that I have given it full specific rank. A thorough revision of the genus may show some other species to which it is more nearly related, but its range is widely separated from that of any other small species" (Bailey, 1913, p. 130). *Neotamias m. atristriatus* is not merely isolated from other populations, but is the most highly isolated of any least chipmunk population with a minimum distance of 200 km from the nearest population across unsuitable intervening habitat (see Puckett et al., 2021; Figure 1). This taxon is currently listed as Endangered within the State of New Mexico (NMDGF, 2016) and has been proposed to be listed as endangered under the ESA at the taxonomic level of subspecies (USFWS, 2021).

Puckett et al. (2021) performed a series of genomic data analyses of target-captured nuclear exons (DNA that codes for gene functions and which may or may not be subject to selection Luikart et al., 2018). They reported their data as 513 single nucleotide polymorphisms (SNPs) or 259 concatenated exon sequence loci, depending on the analysis. Their analyses also considered a mitochondrial genome dataset (maternally inherited haploid DNA). Taxonomic coverage included individuals representing 6 to 12 of the 21 recognized subspecies of least chipmunks (depending on analysis), as well as samples from several other species of chipmunk occurring in the southwestern U.S. Puckett et al. (2021, p. 9–10) stated that their "... genetic data do not support the current distinct subspecies designation for *N. m. atristriatus*." From this conclusion they made the taxonomic recommendation to synonymize *N. m. atristriatus* with two other subspecies (*N. m. caryi* and *N. m. operarius*), for which *N. m. operarius* has nomenclatural seniority. Their interpretations of results that led to this recommendation included: 1) "...the clustering analyses, nuclear phylogenomic tree, and mitogenome haplotype network *unequivocally* grouped *N. m. atristriatus* with *N. m operarius* and *N. m. caryi* in the southern clade"; and 2) "Neither mitochondrial nor nuclear datasets identified *reciprocally monophyletic* diversity between *N. m. atristriatus* and the geographically proximate *N. m. operarius* and *N. m. caryi*" (Puckett et al., 2021, p. 10; 13; emphases added by us). We refute the interpretation, principal conclusions, and taxonomic recommendations of Puckett et al. (2021; as outlined above). In this paper we identify four broad conceptual issues that led to errant recommendations: (1) interpretation of subspecies and diagnosability, (2) inappropriate use of reciprocal monophyly as a criterion for subspecies, (3) importance of geographic isolation, and (4) error in hypothesis testing and misinterpretation of results. We conclude that the data generated by Puckett et al. (2021) support recognition of *N. m. atristriatus* at the subspecies rank. In addition, we highlight that the conceptual oversights result in incorrect and misleading information for decision makers that can have profound impact on the conservation of taxa. Our primary intention here is to focus on furthering our collective understanding of how genomic analyses and evolutionary relationships should be interpreted, and their limitations for governing changes in infraspecific taxonomy.

INTERPRETATION AND DIAGNOSABILITY OF SUB-SPECIES

Subspecies are a rich concept. There is an extended literature on the definition of subspecies (for thorough reviews see Haig et al., 2006; Remsen, 2010; Patton and Conroy, 2017; and citations therein). Subspecies are characterized by heritable diagnostic traits including morphological or molecular differences that vary in frequency between geographically discrete but potentially interbreeding units of analysis (Hennig, 1966; Patten, 2010). An important criterion for assessing the validity of subspecies is an understanding of a taxon's biogeographic history. Given that subspecies are geographic variants, particularly among mammals, subspecies are described based on their geographic distribution coupled with diagnosable characters (Hall, 1981; Remsen, 2010). In contrast, Puckett et al. (2021) used a definition of subspecies devised specifically for cetaceans by Taylor et al. (2017b, p. 174): "...a population, or collection of populations, that appears to be a separately evolving lineage with discontinuities resulting from geography, ecological specialization, or other forces that restrict gene flow to the point that the population or collection of populations is diagnosably distinct." And with regards to diagnosability, Taylor et al. (2017a) invoked the definition of Archer et al. (2017, p. 104) as "...a measure of the ability to correctly determine the taxon of a specimen of unknown origin based on a set of distinguishing characteristics." Subsequently, Puckett et al. (2021, p. 11) stated (including emphasis) that "it is unclear what diagnosable, heritable character could be used to correctly determine that a least chipmunk specimen of *unknown origin* was *N. m. atristriatus*." Archer et al. (2017) did not provide insight to why diagnosis of cetaceans must be based on a specimen of unknown origin, but we presume that it reflects whale migration, and this is supported by their citation of Brambilla et al. (2010) with reference to diagnosability of migratory bird subspecies. This definition of subspecies used by Puckett et al. (2021) is not relevant for terrestrial non-migratory small mammals. The geographic origin of *N. m. atristriatus*, coupled with morphological or molecular characters, would allow for recognition of this subspecies. For instance, even photographs taken with remote cameras allow for accurate diagnosis of *N. m. atristriatus* when paired with information about location (McKibben and Frey, 2021).

Geographic origin aside, the statement of unclear diagnosability by Puckett et al. (2021) is misleading to decision-makers. This is coupled by a statement in the introduction that "…considerable scientific uncertainty surrounds the validity of *N. m. atristriatus* as a subspecies" (Puckett et al., 2021, p. 2), which is dismissive of the statistical support for morphological and ecological distinction of this taxon, based on previously published data. We contend that *N. m. atristriatus* is diagnosable via multiple characters investigated since its description: (1) significant genetic differentiation measured by the fixation index (F_{ST} values) between *N. m. atristriatus, N. m. operarius* and *N. m. caryi* (hereafter collectively the Southern group; i.e., Puckett et al., 2021 tested for significance of their F_{ST} values and provided these results in the Supporting Information Appendix S1); (2) unique mitochondrial diversity (Puckett et al., 2021, p. 11) based on unshared haplotypes within their haplotype network and deep estimated divergence times from their time-calibrated mitogenome tree; (3) unique pelage, unique cranial and bacular morphology, and unique allozymes (genetic variants; all assessed by Sullivan, 1985); and (4) unique ecological habitat associations (Sullivan, 1985). For context, we also point out that subspecies are based on population level diagnosability, not diagnosability of each individual in a population (Patten, 2015). This taxon *is* diagnosable.

USE OF RECIPROCAL MONOPHYLY

Reciprocal monophyly occurs when two or more clades are each monophyletic (genetically unique) with respect to the other, and given the genetic data being analyzed. This condition forms the basis of both the genealogical and phylogenetic species concepts (Wheeler and Meier, 2000; de Quieroz, 2007). At the genome scale, reciprocal monophyly would be indicative of a lack of gene flow between biological species. But, for subspecies, gene flow is expected, which would result in a lack of reciprocal monophyly (Patten, 2010; Patten and Remsen, 2017). By extension, reciprocal monophyly is explicitly not an acceptable criterion for defining subspecies (Braby et al., 2012; Patten, 2015). Puckett et al. (2021) viewed the unequivocal grouping of the three Southern group subspecies as support for their primary conclusion that lack of reciprocal monophyly between *N. m. atristriatus* and other subspecies justifies synonymy with *N. m. operarius*. This interpretation is both incorrect and oversimplified from an evolutionary perspective. The length of time that taxa have diverged from one another is ultimately reflected by how resolved genetic relationships are, but this also depends on both functional and stochastic processes that cause some parts of a genome to resolve as reciprocally monophyletic faster than others (Funk and Omland, 2012). The choice of data is therefore consequential for the power to resolve relationships. Phylogeny estimation might recover well-supported reciprocal monophyly between two recognized subspecies from a given genetic locus. Conversely, even fully reproductively isolated species may exhibit a lack of reciprocal monophyly at a given locus due to processes that include incomplete lineage sorting and ancient hybridization, both common phenomena among mammals, and in particular among western chipmunks (Sullivan et al., 2014). As an example, Puckett et al. (2021) did not recover well-supported reciprocal monophyly for *N. alpinus*, based on their exon data, although this is a recognized species based on more rapidly evolving loci coupled with other diagnostic characters (Rubidge et al., 2014). As such, although reciprocal monophyly is commonly used to indicate evolutionary independence of species under several species concepts, it is not a relevant criterion for assessing the validity of infraspecific taxonomy (Braby et al., 2012; Patten, 2015).

IMPORTANCE OF GEOGRAPHIC ISOLATION

Geography and geographic isolation are inextricably linked to the concept and delineation of subspecies (Vignieri et al., 2006; Patten, 2010). In addition to reproductive incompatibility, a lack of gene flow between populations can also be achieved simply through strict geographic isolation, and isolation is a key criterion for diagnosing independent evolutionary trajectories (Franklin, 1980; Sobel et al., 2010). From its most basic perspective, strict geographic isolation means that inheritance of genotypes from generation to generation, along with epigenetic factors (genotype-environment interactions) and any local adaptive pressures, is not influenced by any immigration and subsequent reproduction of related individuals from separate populations. Given isolation and local environmental conditions, the phenotype of a population will diverge through various evolutionary mechanisms including neutral genetic drift (particularly in small, declining, or demographically unstable populations) and the adaptive processes of natural selection in response to unique and particularly extreme environments. All of these dynamics are reflected by the ecology of *N. m. atristriatus* (Frey and Boykin, 2007). As such, geographic isolation of *N. m. atristriatus* for an extended timeframe, with evidence from both the divergence time estimates of Puckett et al. (2021) and by the relatively well-resolved biogeographic history of isolation and connectivity among the southwestern sky islands (e.g., Patterson, 1982; Frey et al., 2007; Hope et al., 2016; not discussed by Puckett et al., 2021) constitute primary lines of evidence for uniqueness of this subspecies. *Neotamias m. atristriatus* diverged from other subspecies of the Southern group between 190 thousand years ago (kya; Puckett et al., 2019) and 824 kya (Puckett et al., 2021), two mean divergence estimates based on nuclear species-tree analysis and mitogenome phylogeny reconstruction (under a Yule tree prior), respectively. The predicted distribution of *N. minimus* during the Last Glacial Maximum (∼18 kya) also demonstrates isolation of *N. m. atristriatus* from other Southern group subspecies (Puckett et al., 2021—Figure 8). All of the evidence presented supports prolonged isolation of *N. m. atristriatus* on an independent evolutionary trajectory. It then may be considered a matter of philosophical differences as to whether such a taxon represents a distinct subspecies (e.g., King et al., 2006; Ramey et al., 2007; Cronin et al., 2015; Weckworth et al., 2015), or indeed a distinct species (de Queiroz, 2020).

ERROR IN HYPOTHESIS TESTING AND OVERINTERPRETATION OF RESULTS

As expected by biogeographic history, there is a very close relationship between *N. m. atristriatus* and other populations in the Southern Rocky Mountains (Sullivan, 1985). However, this relationship has no bearing on the sub-specific status of *N. atristriatus*. It simply reflects that these individuals share a more recent common ancestor than they do with other populations of *Neotamias*. Puckett et al. (2021) accepted a lack of supported evidence for the genetic distinctness of *N. m. atristriatus* (e.g., a lack of strong nodal support of evolutionary relationships recovered from a phylogenetic tree) as conclusive evidence for synonymy of the three Southern group subspecies of least chipmunks. We agree with Puckett et al. (2021) that the evidence indicates that *N. m. atristriatus* is genetically aligned as a member of the Southern group. But, even if the recovered phylogenetic pattern was consistently well-supported as paraphyletic or polyphyletic among subspecies within the Southern group (i.e., evolutionary non-independence that suggests either that interbreeding is still occurring or that not enough time has passed for populations to exhibit fixed genetic differences), it would still not be appropriate to invalidate subspecies status. Subspecies are well-established as potentially interbreeding units of analysis and represent taxa on the continuum of the formation of species (Wilson and Brown, 1953; Padial et al., 2010; Patton and Conroy, 2017). Lack of strong support for a relationship does not signal strong support for the alternative (unless the alternative is strongly supported). The authors did not provide hypotheses or predictions to be tested, but the implicit null hypothesis they tested was that *N. m. atristriatus* is not a valid subspecies. Thus, their interpretation that *N. m. atristriatus* is not distinct from the other members of the Southern group opens them to a classic type II statistical error, wherein they accepted the null hypothesis as true based on the absence of information that the subspecies are different (Patten, 2010; Patten and Remsen, 2017).

None of the analyses used to assess distinction of *N. m. atristriatus*, including the mitogenome haplotype network, clustering analyses, and nuclear phylogenomic tree, provide any statistical support for independence or for non-independence. The Splitstree method for mitogenome haplotype network construction does not provide any statistical support for groups (Puckett et al., 2021—Figure 2), and is therefore only representative of the genetic distance between individuals (Huson et al., 2008); specimens of *N. m. atristriatus* appear to be grouped more closely to each other than to any other individuals of the Southern group, although distance values were not provided. The principal components clustering analyses do not provide K-values for number of clusters or 95% ellipses around discrete groups (Puckett et al., 2021—Figure 4). The first two components of this ordination within *N. minimus* only account for 9.3% of the observed genetic variation, indicating considerable variation among these taxa was not reported. The nuclear concatenated phylogenomic tree provides no bootstrap support for any relationship within the Southern group clade or even for monophyly of the Southern group (Puckett et al., 2021—Figure 6). Lack of support values means we can draw no conclusions about the strength of relationships among individuals within this clade. With these ambiguities, we cannot conclude that *N. m. atristriatus* is *not* distinct.

Given the lack of phylogenetic resolution recovered from exon capture data, Puckett et al. (2021) may have benefited by reporting additional analyses with their data, or minimally by discussing shortcomings, leaving the door open for further future analyses that might more accurately test hypotheses of uniqueness for *N. m. atristriatus* (Padial and De la Riva, 2021). For instance, exon data are known to evolve more slowly than intron data and other genomic elements including microsatellites, and may not be most suitable for resolving the tips of the tree of life (Bi et al., 2012). Exon data are most beneficial for quantifying adaptive processes (Luikart et al., 2018), including divergence among taxa, through analysis of non-neutral outlier loci, but assessments of this variation were not presented. Finally, from an explicit conservation standpoint, methods have recently been developed for hierarchical assessment and designation of conservation units including not just evolutionary significant units but also management units based on neutral loci and adaptive units based on loci under selection (Funk et al., 2012; Barbosa et al., 2018; Hohenlohe et al., 2021). Although none of these units are considered for mammalian listing under the ESA, they would surely bolster the importance of a recognized subspecies such as *N. m. atristriatus* in the context of the entire species.

GENOMICS AND CONSERVATION POLICY

We present this case study in response to a more general rapid expansion of genomic methods for assessing imperiled taxa associated with ESA listing. Such studies are inherently "applied research" and reach multiple stakeholders with variable levels of expertise for interpreting these complex datasets. Importantly, for those stakeholders not accustomed to translating genomic jargon, such data and analyses are not easily associable with their relevance to the ecology, biogeographic history, and contemporary demographic trends of the taxon of interest. Thus, some may rely on the conclusions presented without the knowledge of theory and molecular methods necessary to rigorously decipher data and results. Greater integration among disciplines is imperative (Godfray and Knapp, 2004; Padial et al., 2010). Molecular ecologists that have adopted genomic methods should invest in more comprehensive understanding of the biology of the study taxon and system. Studies focused on taxonomic assessments would benefit from collaboration with taxonomists (Pruett and Winker, 2010). And, extra care should be made to clearly explain what each analysis can or cannot confirm about the question of interest. Decisions by management agencies based on academic interpretations of complex datasets can be consequential for the maintenance of biodiversity. It is therefore equally important that decision makers have the information they need from both ecologists and evolutionary biologists to accurately assess the findings of genomic analyses. In addition, journal editors should assure that data and methods that relate to listing decisions be made available to ensure reproducibility, and should not accept for publication interpretations of reciprocal monophyly for qualifying subspecies status (e.g., Gilbert et al., 2012; Fanelli, 2018).

The proposal to list *N. m. atristriatus* as a subspecies under the ESA has recently undergone a 60-day public review period (USFWS, 2021), which makes the discussion about validity of its taxonomic status of critical importance. Since its inception, the ESA has always allowed listing of species and subspecies as these are formally recognized taxonomic entities (Haig et al., 2006). More recently, policy has also allowed the listing of Distinct Population Segments (DPSs) of vertebrates. DPSs are defined based on discreteness and importance relative to the remainder of the taxon, which means that interpretation of taxonomy can influence recognition of a DPS (Haig and D'Elia, 2010). Thus, although Puckett et al. (2021) promoted the Sacramento Mountains population as a unique DPS, their overarching conclusion that *N. m. atristriatus* taxonomy warrants revision casts doubt on the current evidence presented to the ESA as a basis for listing. Our account of the various misinterpretations of Puckett et al. (2021) reflects many of the same issues noted from other molecular genetic studies that have tested the validity of subspecies (e.g., Vignieri et al., 2006; Patten, 2015). *Neotamias m. atristriatus* is a Linnean trinomial taxon that was described by a professional taxonomist (Bailey, 1913) and has been validated by many subsequent analyses of its genetics, morphology, and ecology (Sullivan, 1985; Sullivan and Petersen, 1988). Protections for either DPSs or subspecies can potentially be legally rescinded. However, DPS is a rank that has arisen through legislative wildlife policy, and is prone to litigation and prolonged interpretation that can stall conservation efforts (Haig and D'Elia, 2010). Conversely, subspecies are a formal biological rank that describes nature, and as such may be contested based on appropriate biological evidence, but not through legal legislation (Haig et al., 2006).

CONCLUSION

We conclude that, rather than invalidating *N. m. atristriatus*, the results of Puckett et al. (2021) actually augment prior research demonstrating the validity of *N. m. atristriatus* as a subspecies. It has experienced long-term geographic isolation, and it is morphologically, genetically, and ecologically distinctive. We therefore recommend that *N. m. atristriatus* be considered for listing under the ESA at the subspecies level. The misinterpretation of genomic data as we have described can matter for endangered species listing. In some cases taxonomic disputes have ostensibly even been used in attempt to thwart or cast doubt on ESA listings (Vignieri et al., 2006). As such, it is imperative that studies centered on the principles of conservation genomics carefully consider the limitations of data, while also progressing to finer-scale diagnoses, for instance based on the genomics of local adaptation (e.g., Steiner et al., 2009). Although we vigorously disagree with their conclusions, Puckett et al. (2021) have provided the first focused genomic assessment of relationships among Southern subspecies of least chipmunks. Undoubtedly, future studies will benefit from their contributions for appropriate protections of declining wildlife.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AH led writing. JF substantially contributed to writing. Both authors contributed to the article and approved the submitted version.

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