SOFT TICKS AS PARASITES AND VECTORS

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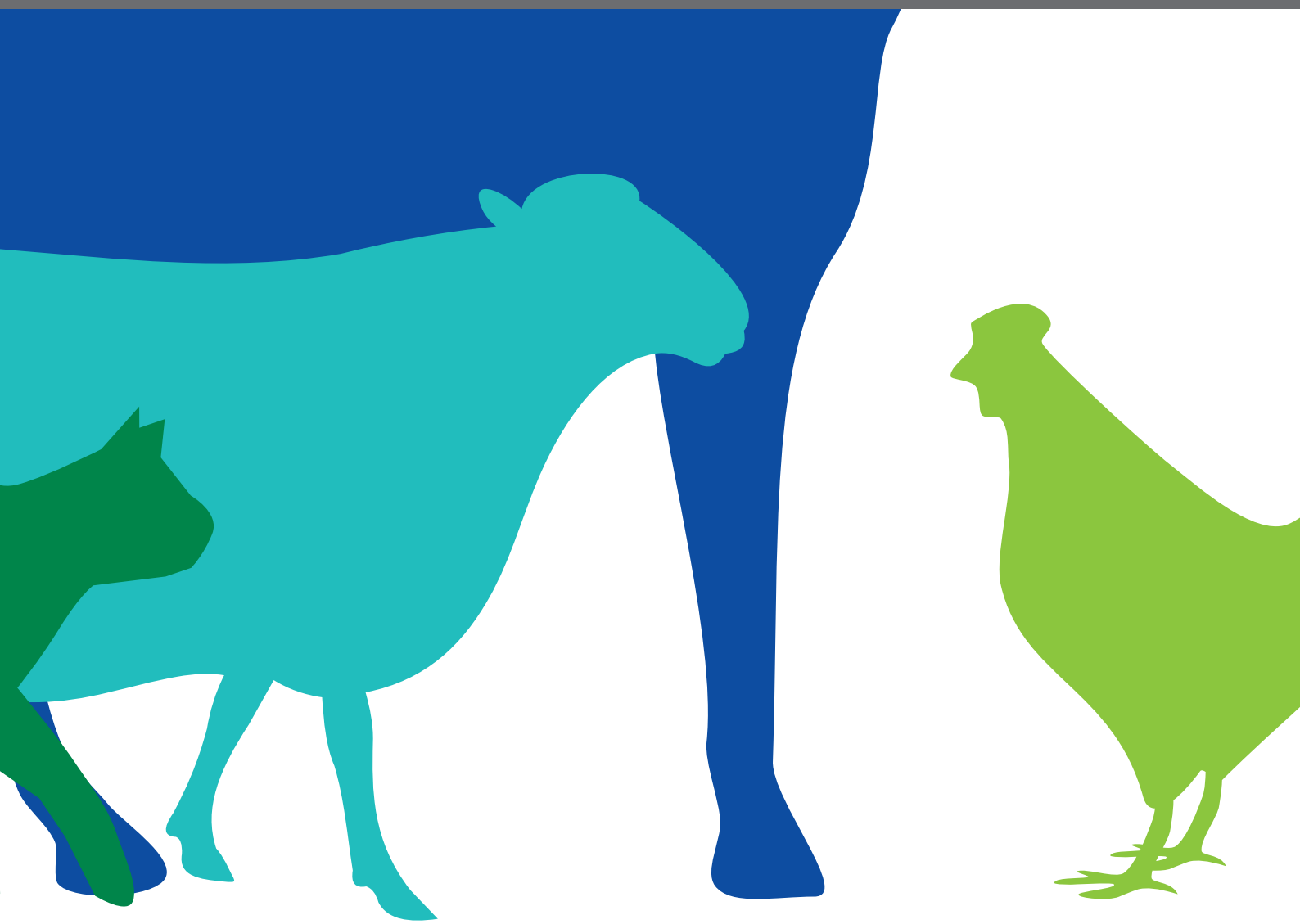
PUBLISHED IN: Frontiers in Veterinary Science and Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-83250-771-1
DOI 10.3389/978-2-83250-771-1

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SOFT TICKS AS PARASITES AND VECTORS

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Citation: Muñoz-Leal, S., Mans, B. J., Venzal, J. M., eds. (2022). Soft Ticks as Parasites and Vectors. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-771-1

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frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 31 October 2022
DOI 10.3389/fvets.2022.1037492

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

RECEIVED 05 September 2022
ACCEPTED 29 September 2022
PUBLISHED 31 October 2022

CITATION
Mans BJ, Venzal JM and Muñoz-Leal S (2022) Editorial: Soft ticks as parasites and vectors. *Front. Vet. Sci.* 9:1037492. doi: 10.3389/fvets.2022.1037492

Editorial: Soft ticks as parasites and vectors

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KEYWORDS

argasids, ecology, bloodmeal, salivary glands, distribution

Editorial on the Research Topic
Soft ticks as parasites and vectors

Soft ticks are interesting ectoparasites due to their ecological habits and unique blood-feeding biology. The wide-ranging nature of research into these parasites is reflected in the diverse set of papers captured in this special issue. Fundamental questions are addressed in the 9 articles included in this Research Topic that focuses on ecology, tick-host interaction, host associations, geographic distribution, and microbial endosymbionts.

Geographic dispersal of soft ticks

Adult and nymphal soft ticks feed quickly and are therefore not extensively associated with their hosts. In addition, they are generally nest- or burrow-dwelling parasites. Therefore, how they disperse to new hosts and burrows is an interesting question to tackle. The study by Rataud et al. investigated dispersal between yellow-legged gull (*Larus michahellis*) nests for the soft tick *Ornithodoros maritimus* (also known as *Alectorobius maritimus*) using a capture-mark-recapture strategy within a natural bird population during the breeding season. Overall dispersal rates were low, confirming the strong endophilic nature of this tick species. This contrasted with previous results on the random distribution of infectious organisms in this species that suggested extensive between-nest movement (1). The study considered the possibility that longer temporal scales of dispersal may be more relevant to argasid dispersal than short-term monitoring allows. The study certainly highlights great mysteries in soft tick biology that exist regarding geographic and temporal dispersal, and how life stage, seasonality, nest- or burrow-occupancy, and tick-feeding status may impact this.

Host identification through bloodmeal analysis

The ability of soft ticks to survive for extensive periods of time is linked with their slow metabolism and capability to store their bloodmeal for prolonged periods in an intact and undigested form. The fact that soft ticks can feed multiple times and that the previous bloodmeals survive feeding events in their guts offers the opportunity to utilize the bloodmeal to identify past hosts. This biological particularity would greatly expand the ability to associate soft ticks with their natural hosts, since the ticks are rarely collected on the host or dwell in burrows that harbor multiple vertebrate species, making host identification ambiguous. Two studies in this collection investigated various methods to detect hosts by bloodmeal analysis of the soft tick *Ornithodoros (Pavlovskyella) turicata*. On the one hand, the study by Busselman et al. validated the use of PCR and Sanger sequencing in ticks artificially fed on chicken and pig blood and could detect host DNA up to 1,105 days after feeding. Based on these parameters, they screened 19 field-collected ticks from a cave in Austin, Texas, and showed evidence of the presence of raccoon, black vulture, Texas black rattlesnake, and human blood in bloodmeals. On the other hand, the study by Kim et al. investigated species-specific qPCR analysis using ticks fed on various host species such as chicken, goat, and pig. According to their results, the specific origin of bloodmeals could be detected beyond 330 days post-feeding, through multiple molting events and multiple species in one bloodmeal. The authors also investigated the use of stable isotope analyses for the identification of bloodmeal origin in ticks, but their results suggest that this approach is less sensitive than qPCR. These studies highlighted the potential to use bloodmeal identification analyses as means to detect prior hosts and exploit the ability of soft ticks to retain intact host blood across molts and feeding events. While the strategies used, such as host-specific qPCR and direct Sanger sequencing may limit the ability to detect multiple hosts, future prospects such as cloning and sequencing of amplicons or implementing next-generation amplicon sequencing approaches will expand the opportunity to uncover the array of hosts parasitized by soft ticks in their natural habitats.

Domestic fowl as hosts of soft ticks

Most argasids parasitize wild animals and their impact on domestic animal or human health may be considered incidental, as found for the human that visited the cave frequented by *O. (P.) turicata* above (Busselman et al.). However, when infestations of argasids are experienced in human homes or domestic animal dwellings, the impact can be quite severe, especially when infected with pathogenic agents. For instance, *Argas persicus* parasitize poultry and has a worldwide distribution with a preference for tropical regions, and is generally the expected tick species when poultry dwellings are investigated. In the current collection, two studies geographically distant from each other investigated the infestation of poultry by argasids. Zahid et al. studied the infestation of domestic fowl in Khyber Pakhtunkhwa, Pakistan. From a large number of ticks collected from fowl dwellings ($n = 7{,}219$), the only tick found was *A. persicus*, as confirmed by morphological and molecular identification. The second study by López et al. from the Caribbean region of Colombia surprisingly found the exclusive infestation of fowl dwellings by *Ornithodoros puertoricensis* (also known as *Alectorobius puertoricensis*) as confirmed by morphological and molecular identification. This was the first association of this tick species with domestic fowl. Both studies highlight the importance of confirming which tick species parasitize domestic fowl in a given region since this will also impact on risk assessment of zoonosis and the possibility that high infestations can spill over to nearby human dwellings.

Birds as hosts of soft ticks

Palomar et al. collected soft ticks in wild birds from Spain. In this study, the authors were unable to identify the ticks to the species level using current available morphological keys. Species relationships were further investigated using mitochondrial 12S and 16S ribosomal RNA and cytochrome oxidase 1 genes. Phylogenetic analysis of the 16S rRNA indicated species identity with *A. persicus* and *Argas reflexus,* with at least two genotypes grouping with a clade formed by *Argas japonicus*, *Argas lagenoplastis*, *Argas polonicus,* and *Argas vulgaris* and a genotype that grouped with *Argas africolumbae*. The study pointed out the important need for the development of accurate keys for the genus *Argas*, given that this genus comprises 44 species with many potential new *Argas* species, as found in the Palearctic region. The study also detected *Rickettsia*, *Coxiella*, *Francisella,* and *Rickettsiella* species as well as a novel *Babesia* genotype, closely related to avian *Babesia* species. This highlights the potential role of *Argas* species in transmitting zoonotic bacterial and piroplasmid agents.

Bat-associated soft ticks

Sándor et al. investigated the associations between soft ticks and hosts, using mammals as study models. Five main tick species were identified, namely, *Carios vespertilionis*, *Chiropterargas boueti*, *Chiropterargas confusus*, *Reticulinasus salahi,* and *Secretargas transgariepinus*. Except for *C. vespertilionis* which showed the widest distribution, the distribution maps for these ticks are data sparse and indicate a need for more studies on bats and their associated argasid species. The maps presented will serve as important reference points in this regard. Moreover, it is highlighted that most

of these species would also parasitize humans and could be of zoonotic potential transmitting *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp., *Ehrlichia* spp., *Babesia* spp., and several nairo- and flaviviruses. Though this hypothesis still needs investigation. Integration of host association data shows little evidence of host specificity. This study will definitely serve as an important resource for argasids associated with Palearctic bats.

Soft ticks in China

Another meta-analysis of argasid ticks in China synthesized current knowledge on the species and associated pathogens for this important but neglected region (Chen et al.). Up to 15 argasid species were discussed including from the Argasinae: *Argas assimilis*, *Argas beijingensis*, *Argas japonicus*, *A. persicus*, *Argas pusillus*, *A. reflexus*, *Argas robertsi*, *Argas sinensis*, *A. vulgaris*, *Ornithodoros lahorensis* (also known as *Alveonasus lahorensis*); and from the Ornithodorinae: *Argas vespertilionis* (also known as *Carios vespertilionis*), *Ornithodoros capensis* (also known as *Alectorobius capensis*), *Ornithodoros* (*Ornithodoros*) *huajianensis*, *Ornithodoros* (*Pavlovskyella*) *papillipes,* and *Ornithodoros* (*Pavlovskyella*) *tartakovskyi*. The study brings to light old Chinese papers (1930s to 1960s) on soft ticks and highlights that the diversity of argasid species in this region could be by far underestimated.

Salivary gland transcriptomes of soft ticks

Lastly, the study by Reck et al. described the salivary gland transcriptome of *Ornithodoros* (*Pavlovskyella*) *brasiliensis* elucidated by next-generation sequencing. This study contributed a large number of genes from this species including secretory proteins and confirmed that these latter are highly abundant in argasid salivary glands. This gene catalog will be useful to identify toxins involved in toxicosis syndromes caused by this tick species in mammals.

Conclusions

This special issue highlighted the diversity of subject areas that can be illuminated by the study of argasid species and their biology. It also represents a summary of some areas of interest in contemporary argasid biology and promises that further study into these research lines will expand in the future.

Author contributions

BM: conceptualization and writing—original draft, and review and editing. JV and SM-L: writing—review and editing. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 October 2020
doi: 10.3389/fvets.2020.570157

Evaluating Functional Dispersal in a Nest Ectoparasite and Its Eco-Epidemiological Implications

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OPEN ACCESS

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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: 06 June 2020
Accepted: 27 August 2020
Published: 19 October 2020

Citation:
Rataud A, Dupraz M, Toty C, Blanchon T, Vittecoq M, Choquet R and McCoy KD (2020) Evaluating Functional Dispersal in a Nest Ectoparasite and Its Eco-Epidemiological Implications. Front. Vet. Sci. 7:570157. doi: 10.3389/fvets.2020.570157

Functional dispersal (between-site movement, with or without subsequent reproduction) is a key trait acting on the ecological and evolutionary trajectories of a species, with potential cascading effects on other members of the local community. It is often difficult to quantify, and particularly so for small organisms such as parasites. Understanding this life history trait can help us identify the drivers of population dynamics and, in the case of vectors, the circulation of associated infectious agents. In the present study, functional dispersal of the soft tick *Ornithodoros maritimus* was studied at a small scale, within a colony of yellow-legged gulls (*Larus michahellis*). Previous work showed a random distribution of infectious agents in this tick at the within-colony scale, suggesting frequent tick movement among nests. This observation contrasts with the presumed strong endophilic nature described for this tick group. By combining an experimental field study, where both nest success and tick origin were manipulated, with Capture-Mark-Recapture modeling, dispersal rates between nests were estimated taking into account tick capture probability and survival, and considering an effect of tick sex. As expected, tick survival probability was higher in successful nests, where hosts were readily available for the blood meal, than in unsuccessful nests, but capture probability was lower. Dispersal was low overall, regardless of nest state or tick sex, and there was no evidence for tick homing behavior; ticks from foreign nests did not disperse more than ticks in their nest of origin. These results confirm the strong endophilic nature of this tick species, highlighting the importance of life cycle plasticity for adjusting to changes in host availability. However, results also raise questions with respect to the previously described within-colony distribution of infectious agents in ticks, suggesting that tick dispersal either occurs over longer temporal scales and/or that transient host movements outside the breeding period result in vector exposure to a diverse range of infectious agents.

Keywords: capture-mark-recapture (CMR), multi-state model, vector, tick, Argasidae, colonial seabirds, *Larus michahellis*, *Ornithodoros maritimus*

INTRODUCTION

Dispersal is a fundamental process influencing the ecology and evolutionary trajectory of species. It is a major determinant of a species' population dynamics and genetic structure, and as such, conditions the ability of organisms to adapt to new environments (1). True dispersal requires the physical movement of an individual from one patch to another (i.e., functional dispersal), followed by successful reproduction (i.e., effective dispersal). Genetic studies have been extremely useful for measuring effective dispersal, particularly in organisms that are hard to follow directly [e.g., (2)], but these studies can only provide estimates of dispersal rates when genetic structure occurs and cannot inform us about physical movement when post-movement reproduction is not successful. Functional dispersal is nevertheless essential to understand when one wants to predict expansion/invasion dynamics and associated colonization success (1), or when examining disease circulation in cases when the transient presence of an individual is enough for pathogen transmission to occur. However, measuring functional dispersal can be difficult because the ability to follow individual animals depends on their biology and ecology.

Capture-mark-recapture (CMR) studies have contributed much to our understanding of movement and are frequently used to study population dynamics and dispersal of vertebrates (3). These methods are only rarely applied to invertebrates (4–8). Although many studies have successfully marked and released arthropods to determine dispersal distances [e.g., (9, 10)], obtaining sufficient data for subsequent statistical analyses is difficult, limiting our ability to make robust inferences on movement in many groups. The present study focuses on the functional dispersal of the seabird tick, *Ornithodoros maritimus*, a member of the Argasidae or soft tick family, using CMR methodology.

Ticks are among the most important disease vectors worldwide, transmitting a wide variety of infectious agents including bacteria, viruses, and eukaryotic parasites (11) to a multitude of vertebrate hosts including birds, reptiles, and mammals (12). There is a general lack of knowledge on tick biology and population dynamics under natural conditions, and this is particularly true for soft ticks which, because of their more endophilic lifestyle and feeding habits, frequently go undetected in host populations (13). Here, we use *O. maritimus* as a model soft tick species to examine functional dispersal at a small spatial scale, among nests within a breeding colony of its host, in order to better understand its role in local population expansion, genetic structure and the transmission of infectious agents among host individuals.

Ornithodoros maritimus is commonly found in seabird breeding colonies in the Mediterranean Sea and eastern North Atlantic Ocean (14–17) and may act as vector to numerous infectious agents including diverse bacteria, protozoans and viruses (18–20). Like most argasid ticks, *O. maritimus* has a nidicolous lifestyle and feeds on the host rapidly (several minutes) in nymphal and adult life stages, usually at night when the host is largely immobile (13). This limited contact with the host should result in low among-colony dispersal, and may have a cascading effect on pathogen spread (21). At the within-colony scale, among nest dispersal should mainly depend on the intrinsic movements of the tick itself, as the seabird hosts are generally territorial during the breeding season. However, active dispersal in endophilous ticks like *O. maritimus* is thought to be limited (13). A need for specific environmental conditions could further induce strong site fidelity and homing behavior to specific microhabitats in these ticks. However, a recent study on the among-nest distribution of infectious agents carried by *O. maritimus* found no spatial structure in their presence in ticks (19). As gulls are territorial during the breeding season and tend to show high nest site fidelity between years (22), all ticks in a nest should be exposed to the same infectious agents. If ticks move independently of their host, but only short distances, neighboring nests should have a higher probability of sharing infectious agents than more distant nests. As these patterns were not found, it was suggested that ticks move among host nests frequently enough to disseminate infectious agents across the colony (19).

Here, we test this hypothesis by characterizing functional dispersal of *O. maritimus* within a colony of yellow-legged-gulls during the breeding period. By integrating an experimental field study with detailed CMR data and a multi-state statistical framework (23, 24), we also test if functional dispersal differs according to host nest success, i.e., whether a lack of chicks in the nest may motivate ticks to move more readily, and tick life stage. We only consider nymphal and adult ticks in our study for two reasons. First, applying CMR methods to larvae in the field could not be done for methodological reasons because larvae are too small to repeatedly mark. Second, larval ticks are more susceptible to environmental conditions (25) and are thus less likely to successfully move independently of the host. Based on our current knowledge, we expected higher among nest tick dispersal in failed nests, higher dispersal of male ticks because of lower blood meal requirements and their quest for sexual partners and, higher dispersal in adults than in nymphal ticks because adults are more resistant to environmental conditions (26). By translocating ticks from nearby nests to focal nests, we also tested for homing behavior, which could illustrate site fidelity in *O. maritimus*.

MATERIALS AND METHODS

Biological System

Ornithodoros maritimus is a member of the soft tick (Argasidae) complex *Ornithodoros capensis* sensu lato which is currently composed of eight described species that exploit colonial seabirds in the tropical and sub-tropical areas of the world (15). Like other soft tick species, *O. maritimus* has a polyphasic life cycle composed of three active stages: a single larval stage, several nymphal instars and a sexual adult stage (27). Unlike hard ticks (Ixodidae), these ticks feed rapidly on the host (from several minutes in the nymphal and adult stages to several hours in the larval stage) when the host is resting, usually at night (28). Total time on the host is therefore much shorter in soft ticks compared to hard ticks. Dispersal in these ticks can occur by active movement of the ticks themselves, and/or via their hosts.

FIGURE 1 | Map showing the position of the 40 tracked nests on Carteau (43°22′39″N 4°51′28″E). Nests are identified according to their status: focal nests (successful = yellow circles, failed = yellow circles with a black star) are labeled with a capital letter followed by a number; peripheral nests (orange circles) are labeled with the name of the focal nest of the same nest group followed by a lower-case letter.

The latter is the only mechanism for inter-colony dispersal for *O. capensis* s.l. ticks. Within colonies, both passive and active dispersal could occur. As mentioned in the introduction, both are expected to be low because of the nidicolous nature of these ticks (13), the territoriality of the gulls, and the fact that ticks do not exploit hosts during the active periods of the day (21). No quantification of dispersal at either spatial scale currently exists for ticks of this group.

Ornithodoros maritimus is known to exploit a wide range of colonial seabird host species including cormorants, terns, and gulls from southern Great Britain to the Mediterranean Sea (16). In the Mediterranean region, this tick often exploits breeding yellow-legged gulls (19). Yellow-legged gulls are the most common and widespread seabird of the western Mediterranean (29) and tend to show high ecological adaptability (30). At adulthood, these birds typically breed in dense colonies, laying 2–3 eggs per year in nests built on the ground or on cliff ledges. During the breeding season, they have limited movements, going from feeding areas to the nest territory (31). Outside breeding, *L. michahellis* remains gregarious, concentrating around ports, harbors, and dumps (31). Because of its longevity, nest site fidelity, and seasonal breeding (22), the presence of this bird in the colony area is highly predictable for nest parasites like *O. maritimus* (32).

Despite the limited time that this tick is in contact with the host during the bloodmeal, the repeated nature of these meals may increase the transmission probability of infectious agents carried by the birds and, as a consequence, their prevalence within local populations (21). Indeed, although few investigations exist to date, ticks of the *O. capensis* complex are known vectors of several infectious agents, such as *Borrelia* spp bacteria responsible for relapsing fever in humans (33) and the Soldado virus which can induce high mortality rates in bird populations and pruritus in humans (18, 20, 34). Numerous infectious agents have also been identified in *O. maritimus* in the focal colony of the present study: bacteria including *Anaplasma* spp, *Bartonella henselae*, *Borrelia* sp., *Coxiella* sp., *Francisella* sp., and *Rickettsia* spp.; protozoan *Babesia* sp., and a virus closely related to the West Nile virus (19). The pathogenic effect of these infectious agents for birds and humans are largely unknown as of yet [e.g., (35)].

Study Location

Field work was conducted in the yellow-legged gull colony of Carteau (43°22′39″N 4°51′28″E), a small islet in the Gulf of Fos in the Camargue area of southern France (**Figure 1**). This flat islet of 1.36 km^2 (210 m long by 65 m wide) is entirely occupied by breeding yellow-legged-gulls. During the 2018 regional population survey, 275 breeding pairs were counted on Carteau (Tour du Valat, Association des Marais du Vigueirat). *Ornithodoros maritimus* was identified morphologically and genetically from gull nests in past studies and was the only tick species found on Carteau (15, 19).

Experimental Procedures

Field sampling took place once per week over 5 weeks from April to May 2017. To estimate inter-nest dispersal and the factors that affect it, we selected, marked and recorded the GPS coordinates of 10 nest groups across the islet (**Figure 1**). Each nest group included four nests: a focal nest and the three closest nests (peripheral nests). The average distance between nests of a group was 6.29 (±3.21) m, whereas the average distance between nest groups was 25.22 (±13.13) m. One half of the focal nests were manipulated for breeding success during egg incubation: five were left in success and five were put in failure (eggs removed). At the time of manipulation, the average clutch size of the studied nests was 2.6. One successful nest failed at the egg stage and one failed nest relaid; the category of these nests was reversed for the analyses. Otherwise all successful nests produced chicks.

At each field visit, each nest was searched for 3 min by two people (6 min total search time per nest); one person examined the upper nest materials in a white tray while the other searched directly inside the nest. Thirty adult and nymphal ticks from the

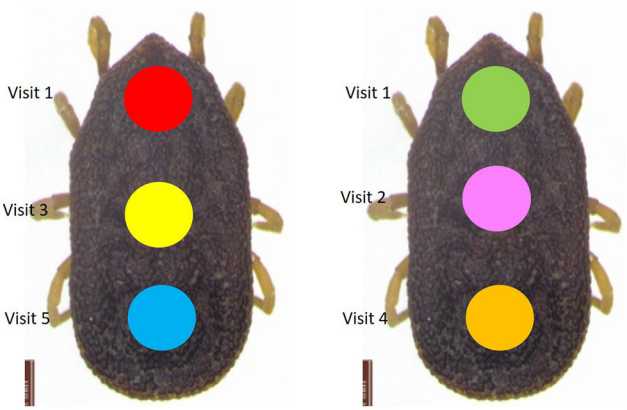

FIGURE 2 | Marking protocol for *Ornithodoros maritimus*. Ticks were initially marked with the color of their nest of origin (visit 1). At each consecutive visit, a date-specific color was used to track capture histories. For example, the individual on the left originated in the peripheral nest B4a and was moved to focal nest B4 during visit 1 (red mark corresponding to the nest of origin B4a). It was then found in focal nest B4 in visits 3 (date-specific yellow mark) and 5 (date-specific blue mark). It was not seen during visits 2 and 4. Its recapture history was thus 10101 (see **Supplementary Material**). The individual on the right was found in focal nest A1 during visit 1 (green mark corresponding to the nest of origin A1), then again in the same nest in visits 2 (date-specific pink mark) and 4 (date-specific orange mark). In visit 5, it was found in peripheral nest A1a and was collected. It was not found in visit 3. Its recapture history was thus 11012 (see **Supplementary Material**).

focal nests and 30 adult and nymphal ticks from the peripheral nests were marked with a spot of acrylic paint (**Figure 2**) at the first sampling occasion. To test for homing behavior, the 30 ticks from the peripheral nests were placed in the focal nest, such that a minimum of 60 ticks were present in each focal nest. Based on previous studies, this number corresponds to natural infestation levels in moderate to highly infested nests (17, 19). An individual color was attributed to each focal nest and a different color to the three peripheral nests of the same group (20 colors overall). During subsequent sampling occasions, all ticks found during the timed searches were counted, but only the initially marked cohort was followed in detail. At each visit, these marked ticks received a date-specific color to indicate their recapture history (**Figure 2**). The life stage and sex of the ticks were recorded at each visit. Any ticks that dispersed to the peripheral nests were collected.

CMR Modeling

To estimate dispersal rates of *O. maritimus* within the colony, we applied a multistate CMR model to the dataset (36). CMR modeling is based on individually marking part of a population. Marked individuals are followed over time during several recapture occasions. The recapture history of an individual is composed of a succession of detection and non-detection events, respectively, noted 1 and 0. For example, 10100 indicates that the individual was detected on the first and third occasions, but not on the second, fourth, and fifth. CMR modeling has the particularity of taking the probability of detection into account in order to obtain unbiased demographic estimates (survival, dispersal). "Events" code the observations made at time t (i.e., detection or not during the sampling occasion), whereas "states" define physiological or geographical states (i.e., individual alive or dead) between time t and $t+1$. In this study, encounter histories were coded with 4 events. For each recapture occasion, ticks were either not observed (coded 0), observed in a focal nest (coded 1), observed in a peripheral nest (coded 2), or found dead (coded 3). Thus, events were: {not observed (0), observed in site 1 (1), observed in site 2 (2), found dead (3)}. Moreover, four states were defined to describe the data. Indeed, ticks could be present in the focal nests (noted site 1), present in the peripheral nests (noted site 2), just dead (since the last weekly visit, noted $\dagger$) or dead (over a week, noted†). We assumed "just dead" individuals were ticks found dead in the nest, whereas "dead" individuals were ticks that were no longer capturable (because they died some time ago). As no tick was found dead in site 2, we did not need to specify the site for the state "dead." States were thus: {site 1, site 2, $\dagger$, †}. The multistate model is described in more detail in the **Supplementary Materials**.

As individuals could differ according to characteristics like life stage or sex, the effect of these covariates on demographic parameters were directly included in the model sets (see below). Model selection was performed using AIC values corrected for sample size (QAICc), with the best fit model providing information on the relative influence of different included factors.

Model Set

Model 1: Tick Life Stage

First, we tested whether survival (S), detection probability (P) and inter-nest dispersal (Ψ) varied in relation to tick life stage. In the null model, survival and detection were coded as being constant across tick stages and nest success; these variables were then added in alternative models. No effect of tick origin (tick from focal or peripheral nest) was expected on these two parameters and this factor was therefore not included in the model set. We modeled dispersal in relation to tick stage, origin and nest success.

Model 2: Tick Sex

We then tested if survival (S), detection probability (P), and inter-nest dispersal (Ψ) varied in relation to adult tick sex. In the null model, survival and detection were again coded as constant across sexes and nest success, and then added to alternative models. Likewise, no effect of tick origin (tick from focal or peripheral nest) was expected on these two parameters and this factor was therefore not included in the model set. We modeled dispersal in relation to adult tick sex, origin, and nest success.

Model selection and parameter estimation were performed using Program E-SURGE 1.8 (37, 38). The selected model in each model set had the smallest QAICc and two models were deemed to be equivalent when they differed by <2 (39).

RESULTS

Tick Sampling

At the first field visit, 578 ticks (189 adult males, 249 adult females, and 140 nymphs) were marked. In total, 138 ticks (30

TABLE 1 | Model selection results for model 1, taking into account different life stages of *Ornithodoros maritimus*.

Model	Number of parameters	Deviance	QAIC	QAICc
S_{cst} P_{cst} $\Psi_{nestsuccess.tickorigin}$	7	1,109.5988	1,123.5988	1,123.7529
$S_{nestsuccess}$ $P_{nestsuccess}$ $\Psi_{nestsuccess.tickorigin}$	9	1,105.8826	1,123.8826	1,124.1309
$S_{nestsuccess}$ P_{cst} $\Psi_{nestsuccess.tickorigin}$	8	1,108.0607	1,124.0607	1,124.2591
S_{cst} $P_{tickstage}$ $\Psi_{nestsuccess.tickorigin}$	8	1,109.0804	1,125.0804	1,125.2787
$S_{tickstage}$ P_{cst} $\Psi_{nestsuccess.tickorigin}$	8	1,109.5364	1,125.5364	1,125.7347

Survival (S) and detection probability (P) were modeled as constant (cst) and depending on tick stage and nest success. Dispersal was modeled as a constant (cst), and depending on tick stage, nest success and tick origin. Only the top five of 128 models are presented. The number of parameters and the deviance were used to calculate QAICc (Akaike Criterion corrected for sample size) of each model. The selected model has the smallest QAICc. The complete model set is available at https://doi.org/10.5281/zenodo.2591254.

TABLE 2 | Model selection results for model 2 that considers only adult *Ornithodoros maritimus*.

Model	Number of parameters	Deviance	QAIC	QAICc
$S_{nestsuccess}$ $P_{ticksex.nestsuccess}$ $\Psi_{nestsuccess.tickorigin}$	11	844.0884	866.0884	866.5692
$S_{nestsuccess}$ $P_{ticksex.nestsuccess}$ $\Psi_{nestsuccess}$	9	850.2589	868.2589	868.5856
$S_{ticksex.nestsuccess}$ $P_{nestsuccess}$ $\Psi_{nestsuccess.tickorigin}$	11	847.1897	869.1897	869.6706
$S_{nestsuccess}$ $P_{ticksex}$ $\Psi_{nestsuccess.tickorigin}$	9	852.1825	870.1825	870.5092
$S_{tickstage.nestsuccess}$ $P_{nestsuccess}$ $\Psi_{nestsuccess}$	9	853.3609	871.3609	871.6876

*Survival (S) and detection probability (P) were modeled as constant (cst) and depending on tick sex and nest success. Dispersal was modeled as a constant (cst), and depending on tick sex, nest success and tick origin. Only the top five of 128 models are presented. Model selection was performed ass outlined in **Table 1**. The complete model set is available at https://doi.org/10.5281/zenodo.2591254.*

adult males, 77 adult females, and 31 nymphs) were recaptured at least once, representing 23.9% of the initial number. Three ticks were found dead in focal nests and nine were recaptured in peripheral nests and collected.

Model Selection

Model 1: Tick Life Stage

The QAICc values of the different models were all very close for model set 1, and no one model was selected. However, the models with smallest QAICc suggested an effect of tick origin and nest success on dispersal (**Table 1**); no effect of tick life stage was evident. As no one model best described the data, we did not attempt to estimate demographic parameters for this analysis.

Model 2: Tick Sex

The selected model from the model 2 set revealed a difference in tick survival according to nest success and an effect of sex and nest success on the detection probability. There was also a difference in tick dispersal according to origin and nest success, but not according to tick sex (**Table 2**).

Estimated Parameters From Model 2

The survival probability of *O. maritimus* differed according to nest success. As expected, the one week survival probability of ticks in successful nests [0.609, $IC_{95\%}$ = (0.495; 0.712)] was higher than that in failed nests [0.381, $IC_{95\%}$ = (0.295; 0.475)].

The detection probability varied with tick sex and nest success. Detection of females in failed nests was higher than that of females in successful nests [females, failed = 0.459 [$IC_{95\%}$ = (0.286; 0.642)]; females, successful = 0.289 ($IC_{95\%}$ = [0.189; 0.414])]. Detection of males was lower in general, but followed the same trend in relation to nest success (males, failed = 0.37 ($IC_{95\%}$ = [0.207; 0.575]) and males, successful = 0.119 ($IC_{95\%}$ = [0.063; 0.214]); **Figure 3**).

Overall, inter-nest dispersal rates of ticks were very low, but some dispersal did occur. Surprisingly, ticks in successful nests tended to disperse more than ticks in failed nests. Moreover, in successful nests, ticks from focal nests tended to disperse more than ticks from peripheral nests. The probability that ticks were present and alive on a site at time *t* and present and alive on the same site at time *t+1* was 1,00 ($IC_{95\%}$ = not available) in focal and peripheral nests in failure, 0.846 ($IC_{95\%}$ = [0.709; 0.925]) in focal nests in success and 0.980 ($IC_{95\%}$ = [0.873; 0.998]) in peripheral nests in success (**Figure 4**).

DISCUSSION

In this study, we characterize functional dispersal in the soft tick *O. maritimus* at a small spatial scale, among nests within a colony of yellow-legged gulls, using capture-mark-recapture data. Estimated inter-nest dispersal rates of ticks were very low overall, indicating a low tendency for *O. maritimus* to move among nests and confirming a strongly endophilous lifestyle in this tick species.

Few studies to date have attempted to measure arthropod movements using CMR modeling, largely due to the difficulty in marking and recapturing individuals. Here, we focused on a short time period when ticks could be followed with a low probability of losing their marks (over a few weeks during the host breeding season). Given the relatively large size of the ticks (about 4 mm; 11), we were also able to place multiple marks

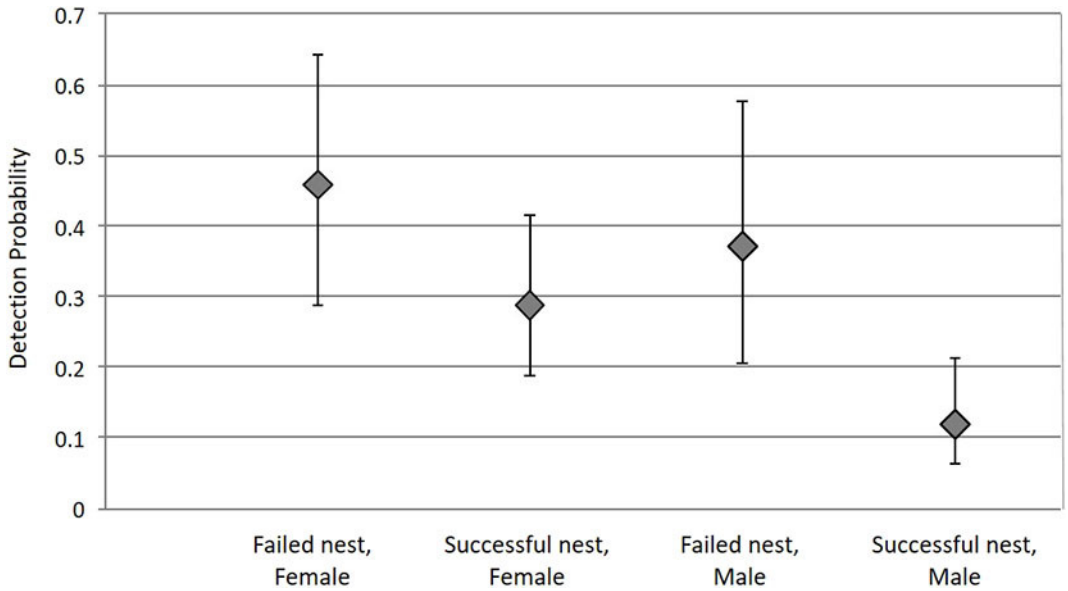

FIGURE 3 | Tick detection probability depends on the interaction between nest success (failed or successful) and tick sex (female or male). Bars represent 95% confidence intervals.

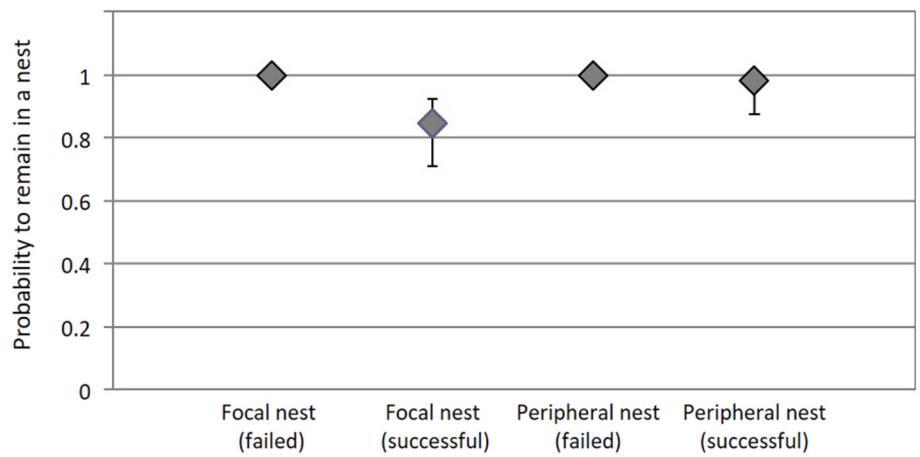

FIGURE 4 | The probability that a tick remains at a nest site (Ψ_{11}) depends on the interaction between nest success (failed or successful) and tick origin (focal or peripheral nest). Bars represent 95% confidence intervals.

that enabled us to directly follow the capture history of each individual. Using this data, we observed a recapture rate of 24%. This rate is relatively high for an arthropod model (5), providing us with enough data to estimate demographic parameters. Other studies that marked ticks found similar recapture rates in adult female ticks, including in one soft tick, *Ornithodoros moubata* (25%) (4, 7, 8, 10)], suggesting that this approach may work well for these arthropods. According to the selected model from the model 2 analysis, which considered only adult ticks, the detection probability of *O. maritimus* seems to depend on tick sex; there was a significantly lower detection probability for male ticks (0.245 for males compared to 0.374 for females). This difference could either be due to the smaller body size of male ticks, or to sex-specific behavioral differences. In the latter case, making predictions about behavior and detection are not obvious because there are several reasons that time spent in the nest may differ between the sexes, depending, for example, on where females prefer to lay eggs and where copulation takes place; these elements are unknown for *O. maritimus*. Surprisingly, the detection probability of soft ticks also seemed to depend on nest success, with higher detection in failed nests. However, again this may be due to behavior, where engorged ticks leave the nest area to molt or lay eggs. Detailed behavioral studies are now required to test these hypotheses.

As the top ranking models did not include an effect of tick life stage (see model 1 results), we did not estimate detection

probabilities for nymphal ticks. Although one of the five top models suggested a potential effect of life stage on detection, the observed proportion of global recaptures for nymphs (22.1%) and adults (24.4%) was similar. This suggests that detection may not differ strongly between two life stages and mark loss due to nymphal molts may not occur within the studied period. However, the overall lower proportion of followed nymphs (∼24%) compared to adults (∼76%) could have lowered our ability to detect an effect.

Neither tick life stage nor tick sex was found to impact survival probability. However, survival probability of *O. maritimus* did differ according to nest success and was higher in nests when chicks were present. This was expected as the ability to have ready access to a host for the bloodmeal should improve tick fitness. Indeed, the quality of the bloodmeal is known to influence the success and duration of the life cycle in argasid ticks (13). However, the survival probability of *O. maritimus* does not seem to depend only on feeding ability, as it was still estimated at 38% in failed nests. Nest-associated parasites often have to survive long periods without hosts, and those parasites associated with pelagic seabirds may represent an extreme [e.g., (40)]. Indeed, colonial seabirds are frequently only present for a few months per year at the nest site, during the breeding season. The rest of the time, they can wander over vast zones and remain largely (or completely) at sea and are therefore unavailable for exploitation (32). In such cases, dormancy behavior becomes essential for parasite survival, allowing them to wait, sometimes under extreme environmental conditions, until the host is available again. *Ornithodoros* ticks are known to survive long periods (years) without a host if microclimatic conditions are appropriate (13). We therefore feel that our survival estimates are robust. However, one could also postulate that these estimates are distorted by the presence of transient ticks (41), individuals that are considered dead, but which were simply unrecapturable because they permanently emigrated outside the studied area. Analyses realized on data collected in 2018 have shown that this hypothesis does not have high support (42).

In contrast to predictions based on the distribution of infectious agents in ticks within the colony (19), overall inter-nest dispersal rates of *O. maritimus* were very low. However, tick dispersal depended on their origin (focal or peripheral nest), with ticks from focal nests tending to disperse more than ticks from peripheral nests. This was unexpected, and particularly so if ticks have a homing response, i.e., a preference to return to a specific, known microhabitat. Ticks displaced from peripheral to focal nests could have had less energy to allocate to dispersal than local ticks because of the energetic costs of acclimating to another nest environment or having access to fewer bloodmeals post-dispersal. From our results, there is absolutely no indication of homing behavior in displaced ticks.

We expected the dispersal of *O. maritimus* to depend on nest success, with ticks in failed nests dispersing more than ticks in successful nests. Contrary to this prediction, dispersal of soft ticks does not seem to be induced by the quest for a bloodmeal. This may reflect the ability of these ticks to survive long periods of time without a host (13) and highlights the importance of a flexible dormancy strategy where quiescence can offset the costs of limited dispersal in endophilous species. We also found that ticks in successful nests dispersed more than ticks in failed nests. This could again be because ticks in failed nests, unable to feed, may lack enough energy to move.

Here, we examine active tick movement, but dispersal of *O. maritimus* via host movement is of course possible. Given the short duration and timing of the tick bloodmeal and the limited movements of yellow-legged gulls within the colony during the breeding period (43), we considered this unlikely. Indeed, no effect of chick presence on the dispersal of *O. maritimus* was indicated in analyses from 2018, tick movement did not increase at the time that chicks started to move around the colony (42). However, the role of host movements in tick dispersal later in the year and at different spatial scales remains unknown. A population genetic study of ticks at the among-nest scale could shed light on the role of the host in local dispersal events.

We also expected dispersal in *O. maritimus* to depend on tick sex, with higher dispersal in male ticks, due to their reduced need for bloodmeals and their quest for sexual partners. Although past studies have documented male-biased dispersal in ticks (44), we found no support for this. Future population genetic analyses would also enable us to test the hypothesis of sex-biased dispersal in *O. maritimus*.

We found that *O. maritimus* has low functional dispersal rates among nests within the host breeding colony. This result is consistent with the general idea that soft ticks tend to be strongly endophilous (13). The sedentary lifestyle of *O. maritimus* should restrict gene flow among natural populations resulting in high phenotypic variability and genetic structure among populations (13, 21). A lack of gene flow could mean a limited role of this soft tick in the circulation of associated infectious agents. Although transmission may occur more readily in soft tick systems compared to hard tick systems, because soft ticks repeatedly feed in nymphal and adult life stages (21), without dispersal an infected tick can only transmit its infectious agents to hosts breeding in the same nest site (i.e., family members). Again, a genetic approach examining dispersal could help us determine the role of tick dispersal in the transmission of associated infectious agents, particularly for larval ticks which feed for longer periods of time on the host compared to nymphal and adult stages (several hours compared to several minutes). It would also allow us to infer whether seabird presence in the colony outside the breeding season could result in exposure of active ticks to novel host individuals, potentially explaining observed patterns in pathogen prevalence in ticks (19).

CONCLUSIONS

Knowledge on functional dispersal, describing physical movements of individuals from one patch to another, is essential to understand population dynamics and to predict ecological and evolutionary changes in a species. Functional dispersal can be particularly important to take into account in the case of vectors like ticks, because these ectoparasites affect host reproduction and can transmit infectious agents. Our capture-mark-recapture (CMR) study has allowed us to identify some of the factors influencing inter-nest dispersal probability of the soft tick *O. maritimus* at a small spatial scale, within a colony of yellow-legged gulls, taking into account both

tick survival and detection probability. These first results have highlighted a weak dispersal propensity in this tick and suggest a limited role of active tick movement in the circulation of associated infectious agents at the within-colony level. Although survival and inter-nest dispersal of *O. maritimus* seem to depend on nest success (host availability), analyses did not indicate homing behavior. The detection probability of *O. maritimus* also depended on nest success and tick sex, but not in the predicted directions. More in-depth knowledge on the biology of this tick is now required to fully interpret these results and should prove useful for future work on this biological system. Although the present study represents one of the first applications of CMR modeling to an arthropod system using multiple recapture events, more information on tick dispersal at larger spatial and temporal scales is now necessary to better understand its population dynamics, the potential impact of these dynamics for the seabird host, and the circulation of infectious agents within the Mediterranean Basin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories: https://doi.org/10.5281/zenodo.2591254.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the only manipulation presented in the study involved the modification of gull reproductive success-i.e., egg removal. This species is under control programs and approval for this manipulation of reproductive success was given by the local autorities (Grand Port Maritime de Marseille and DDTM 13/Service Mer Eau Environnement/Pôle Nature et Territoires n°13-2018-02-2-003).

AUTHOR CONTRIBUTIONS

KM, RC, and MV designed the initial study. KM, MV, TB, CT, and MD contributed to the practical implementation of the design and carried out the field work. AR and RC organized the data and performed the CMR analyses. All authors contributed to data interpretation. AR, RC and KM wrote the first draft of the manuscript and all authors contributed to revisions.

FUNDING

Funding for this study was provided by the ANR grant ESPEVEC (ANR-13-BSV7-0018) and by an exploratory research grant DISTIC from the Labex CeMEB (Center Méditerranéen de l'Environnement et de la Biodiversité).

ACKNOWLEDGMENTS

We thank Olympe Tritto, Maxime Duhayon, Denis Bourguet, Justine Rouchon, Hugo Ferreira, Irene Badone, Aurore Fayard, Cloé Joly, Carole Leray, and Marie Buysse for their help in the field. We also thank Adele Mennerat, Shelly Lachish and three reviewers for comments that helped improve this work. Experiments in the colony were performed under the approval of Grand Port Maritime de Marseille and DDTM 13/Service Mer Eau Environnement/Pôle Nature et Territoires n°13-2018-02-22-003. A preprint of this work was reviewed and recommended by *Peer Community In Ecology* (45).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.570157/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with the authors KM and CT.

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BRIEF RESEARCH REPORT
published: 15 February 2021
doi: 10.3389/fvets.2021.639400

Host Bloodmeal Identification in Cave-Dwelling *Ornithodoros turicata* Dugès (Ixodida: Argasidae), Texas, USA

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OPEN ACCESS

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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: *08 December 2020*
Accepted: *20 January 2021*
Published: *15 February 2021*

Citation:
Busselman RE, Olson MF, Martinez V, Davila E, Briggs C, Eldridge DS, Higgins B, Bass B, Cropper TL, Casey TM, Edwards T, Teel PD, Hamer SA and Hamer GL (2021) Host Bloodmeal Identification in Cave-Dwelling Ornithodoros turicata Dugès (Ixodida: Argasidae), Texas, USA. Front. Vet. Sci. 8:639400. doi: 10.3389/fvets.2021.639400

Tick-host bloodmeal associations are important factors when characterizing risks of associated pathogen transmission and applying appropriate management strategies. Despite their biological importance, comparatively little is known about soft tick (Argasidae) host associations in the United States compared to hard ticks (Ixodidae). In this study, we evaluated a PCR and direct Sanger sequencing method for identifying the bloodmeal hosts of soft ticks. We collected 381 cave-associated *Ornithodoros turicata* near San Antonio, Texas, USA, and also utilized eight colony-reared specimens fed artificially on known host blood sources over 1.5 years ago. We correctly identified the vertebrate host bloodmeals of two colony-reared ticks (chicken and pig) up to 1,105 days post-feeding, and identified bloodmeal hosts from 19 out of 168 field-collected soft ticks, including raccoon (78.9%), black vulture (10.5%), Texas black rattlesnake (5.3%), and human (5.3%). Our results confirm the retention of vertebrate blood DNA in soft ticks and advance the knowledge of argasid host associations in cave-dwelling *O. turicata*.

Keywords: soft ticks, Argasidae, *Ornithodoros turicata*, blood meal, host identification

INTRODUCTION

The identification of arthropod host-feeding patterns through bloodmeal analysis can provide key information for vertebrate host contact and pathogen transmission networks (1–4). Bloodmeal analysis methods based on the detection of vertebrate DNA left in the residual bloodmeal are widely used across diverse arthropod taxa. For example, reservoir hosts of *Leishmania* were identified by studying previous bloodmeals of sand flies (5), and host-feeding patterns in mosquitoes allowed for an enhanced understanding of the reservoirs of West Nile virus (6, 7). Molecular analysis of bloodmeals has also been used to identify a broad host community for *Culicoides*, vectors of avian Haemosporida infections (8), and of triatomines, vectors of *Trypanosoma cruzi*, agent of Chagas disease (9).

Bloodmeal analysis, applied to ticks, has repeatedly been associated with limited success (10, 11), likely owing to DNA degradation during the molt and many months since prior bloodmeal acquisition. Given their importance as vectors of human pathogens, several studies have conducted bloodmeal analysis of hard ticks (Ixodidae), identifying vertebrate hosts in 20–93% of analyzed ticks (12, 13). Given the challenges of PCR-Sanger sequencing-based bloodmeal analysis of hard ticks, alternative strategies have been evaluated to identify bloodmeal hosts, including analysis of the variation in stable isotopes in fed ticks (10, 14, 15), reverse line blot (16, 17), and proteomics (18). In comparison, relatively few studies have attempted to identify the bloodmeal hosts of argasid ticks (soft ticks).

Ornithodoros turicata, found in the southwestern United States and Florida (19), is a vector of human and animal pathogens. *O. turicata* is a known vector of tick-borne relapsing fever caused by *Borrelia turicatae* (20), and is also a putative vector for transmission of African swine fever virus, an emerging disease in Africa, Europe, and most recently Asia (21, 22). This DNA virus is transmitted by soft ticks of the *Ornithodoros* genus and is highly pathogenic to domestic swine (23). While African swine fever has yet to be detected in the U.S., recent studies have identified *O. turicata* as a most likely vector should the virus reach the US (24, 25).

Ornithodoros turicata is found in caves or burrows occupied or visited by diverse vertebrate hosts (19). Larvae, nymphs and adults attach, blood-feed, and drop from a host quickly (typically 15–20 min); thus, they are seldom collected from hosts during blood feeding events, complicating knowledge of tick-host associations (26). Further, they can survive for years between bloodmeals as nymphs and adults (27).

Identification of the host community that supports *O. turicata* populations could be useful in providing an ecological basis for vector control and disease management. We previously conducted a bloodmeal analysis study using quantitative PCR for the identification of vertebrates in experimentally fed *O. turicata* (28). The results demonstrated vertebrate DNA could be detected 330 days post-feeding and through multiple molts, suggesting longer retention of bloodmeal DNA in soft ticks compared to hard ticks. The current study builds on these prior results by conducting a PCR-Sanger sequencing bloodmeal analysis protocol on *O. turicata* fed experimentally on known hosts as well as field-collected specimens from cave environments in Texas near the location of recent outbreaks of TBRF in humans (29, 30).

MATERIALS AND METHODS

Tick Collections

We obtained eight adult *O. turicata* specimens (five male, three female) from an established colony at Texas A&M University, previously described (31). The feeding history of colony *O. turicata* was known only to one author (PDT) while the rest of the authors remained blinded to the bloodmeal species identification. We collected soft ticks from three caves in Government Canyon State Natural Area, San Antonio, TX, USA, (Lat: 29.549316, Lon:−98.764715) in March 2019 (**Figure 1**). Seven dry-ice baited sticky traps were set out inside or near the openings of three caves over 2 days for 18 and 21.5 h, respectively (**Figure 2**). These caves are closed to park visitors and were selected based on a previous study which demonstrated robust *O. turicata* populations in these caves (31). Traps consisted of 1.9L coolers filled with dry ice (cooler spout open) bolted through the bottom to a 0.41 m^2 untreated 3-ply pine plywood with edges cut at 45° angles to improve tick access to the surface of the board. Double sided carpet tape (Roberts, Boca Raton, FL) was applied to the surface of the board, and insect glue boards (Bell Laboratories, Madison, WI) were cut into strips and applied to the carpet tape and under the corners of the plywood board. The ticks were removed from the sticky tape on-site and placed into ethanol-filled 1.5 ml tubes; each vial contained 3–10 ticks depending on the number of ticks caught at each trap each day. Samples were transported to the laboratory and stored at 4°C until DNA extraction.

Tick Processing and DNA Extraction

O. turicata specimens were measured, identified to species and life stage by morphological features, and sexed if adults (32). A subset of 124 ticks were photographed to serve as a reference for confirming life stage and sex (**Supplemental Data Sheet 1**). Photos were taken early in the identification process and throughout tick cataloging as needed when life stage or sex was difficult to determine. We attempted to record the size of the bloodmeal based on the shape of the abdomen and presence of blood; however, the storage in ethanol produced a dark red color in all specimens and visual bloodmeal scoring was not reliable.

The first 20 ticks collected at each site, followed by equal numbers of the largest ticks from each cave (based on length), were processed further using the following methods until a threshold of 40% of the total number of collected ticks had been processed. To minimize exogenous DNA on the exterior surface of ticks, ticks were washed in ethanol for 5 s, then a 10% bleach solution for 15 s, and finally two consecutive 15 s DNA-free water rinses immediately after (10, 33, 34). On a sterile microscope slide over ice, the legs were then removed, placed in ethanol, and stored at −40°C to decrease the amount of tick DNA processed and to preserve samples of each tick for future use. The tick bodies were placed in clean tubes, flash-frozen in liquid nitrogen, and crushed with a sterile pestle (Wards Sciences, Rochester, NY), which was discarded after each use. The crushed tissue was then lysed, and DNA was extracted using the MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems ThermoFisher Scientific, Waltham, Massachusetts) according to the manufacturer's protocol. Negative and positive controls, including blood from sheep, tiger, and crane (vertebrate species not expected to occur around the cave environment), were included during DNA extraction procedures. The eluted tick DNA from each tick was stored in two tubes at −40°C until PCR amplification. For a small subset of samples from two early extractions ($n = 6$), we quantified DNA using a spectrophotometer (Epoch, BioTek Instruments, Inc.) to confirm the presence of DNA in the extracted samples (average = 32.39 ng/μL, range = 9.07–68.07 ng/μL).

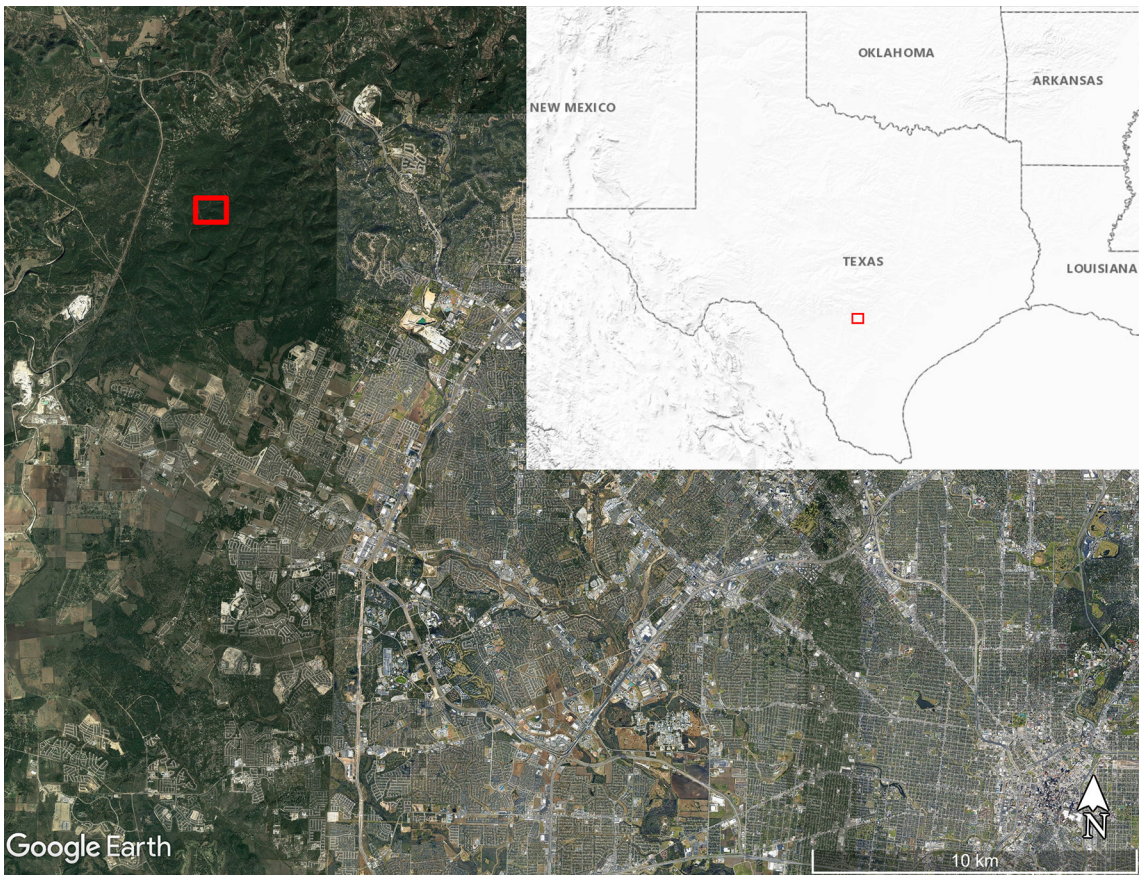

FIGURE 1 | Map of soft tick collection location along Government Canyon Creek (red box) inside Government Canyon State Natural Area on the northwest side of San Antonio, Texas, USA. Map made using Google Earth Pro version 7.3.3.7786. Inset map shows the United States Geological Survey Shaded Relief map of the region around Texas with the map boundary near San Antonio outlined with the red box.

FIGURE 2 | Pictures of dry ice-baited soft tick traps deployed inside and outside of caves in Government Canyon State Natural Area, Texas, USA. (**A**, left) Soft tick traps were placed with a cooler full of dry ice in the mouths of caves, and the cooler spout was left open for dry ice sublimation. (**B**, bottom middle) *O. turicata* soft ticks can be seen attached to the perimeter of the sticky tape. (**C**, right) Additional portions of sticky tape were added to parts of the perimeter of the trap to enhance collections.

PCR Amplification and Sanger Sequencing

DNA extracts from ticks were subjected to multiple vertebrate barcoding primers targeting different genes in an iterative process to identify the most successful primers (**Supplementary Table 1**). The Failsafe PCR Enzyme Mix with PreMix E (Epicenter Biotechnologies, Madison, WI) and primer pairs from Integrated DNA Technologies (IDT) were used for PCR amplification. Primers were used at varying concentrations of 0.33 or 0.4 μM (see **Supplementary Table 1**) in 25 μL reactions, including 2 μL of tick DNA. PCR products were visualized on e-gels (ThermoFisher Scientific, Waltham, Massachusetts) and all products were purified by Exo-SAP-IT (Applied Biosystems, Foster City, CA) and sequenced in forward directions by Eton Biosciences (San Diego, California). Sequences were trimmed to at least 215 base pairs long, and chromatographs were manually scrutinized for quality. Sequences were blasted to the NCBI database using Geneious software (Newark, New Jersey) to identify the closest match of the unknown sequence to a known organism. Sequences with >90% similarity were interpreted as a match, in which case the bloodmeal host was identified. All samples that produced sequences with double peaks were re-run with the "mammal c" primer pair. Because of the chance for contamination of samples with human DNA, any sample that produced a sequence that matched to human was tested a second time with an independent PCR, and in these cases, two matches to the same species were needed to confirm a result.

RESULTS

Colony Ticks

Bloodmeal analysis was conducted on eight *O. turicata* from a colonized population with known prior bloodmeals, with personnel conducting the molecular work blinded to the vertebrate species. We successfully detected chicken (*Gallus gallus*) in an adult tick 1,105 days (last fed 27 January 2016) post bloodmeal and pig (*Sus scrofa*) in an adult tick 622 days (last fed 24 May 2017) days post bloodmeal. We were unable to obtain a PCR amplicon or sequence from six ticks that had fed 622–1,109 days post-bloodmeal (last fed on dates through January 23, 2016 to May 24, 2017).

Field-Collected Ticks

A total of 381 soft ticks were collected in March 2019, and all were identified by morphology as *O. turicata*. Mad Crow Cave yielded the highest number of soft ticks trapped ($n = 184$), followed by Bone Pile Cave ($n = 109$) and Little Crevice Cave ($n = 88$). We identified 32 females, 55 males, 285 nymphs, four adults that were damaged and unable to be sexed, and five other specimens that were damaged and we were unable to determine either sex or life stage (**Table 1**). The average length of males was 3.53 mm ($n = 54$, *SD*: 0.98), 5.28 mm for females ($n = 32$, *SD*: 1.70), and 2.23 mm ($n = 284$; *SD*: 0.82) for nymphs. One male and one nymph were damaged and unable to be measured.

A subset of 168 field-collected ticks were processed for bloodmeal analysis. Most PCR primers amplified exclusively *Ornithodoros* sp. DNA, including the primer pairs mammal c, 0066/0067, 0035/0049, 0033/0049, and Herp/BM1. The primer pair which had the best success at minimizing tick DNA amplification and maximizing vertebrate DNA amplification was "mammal c" targeting a 395 base pair region of cytochrome b (4, 35). The bloodmeals from 19 ticks (11.3%) were identified to species using the "mammal c" primer pair (**Table 2**). Of this subset, 15 ticks (78.9%) contained raccoon (*Procyon lotor*) DNA, two ticks (10.5%) contained black vulture (*Coragyps atratus*) DNA, one tick (5.3%) contained black-tailed rattlesnake (*Crotalus molossus*) DNA, and one tick (5.3%) contained human (*Homo sapiens*) DNA. Of the 168 field-collected samples subjected to bloodmeal analysis, 69 were adults (39 male, mean size = 3.62 mm; 26 female, mean size = 5.67 mm), 97 were nymphs (mean size = 2.64 mm, $n = 96$ as one tick length was unreliable), and two were unable to be determined. The 19 ticks with identifiable bloodmeals included four males (mean size = 3.48 mm), one female (5.6 mm), and 14 nymphs (mean size = 2.96 mm).

DISCUSSION

This study builds on prior results, which demonstrated that vertebrate DNA detected by quantitative PCR in prior bloodmeals of *O. turicata* persists for long periods post-feeding and through molts (27, 31). In the current study, we adopted PCR and direct Sanger sequencing and confirmed that, for experimentally fed ticks in the laboratory, we were able to detect bloodmeals that were up to 1,105 days old. However, a challenge encountered by the molecular approach used in this study was that *Ornithodoros* sp. DNA was amplified consistently using five different primer pairs (mammal c, 0066/0067, 0035/0049, 0033/0049, and Herp/BM1). Amplification of vector DNA has not been an issue during mosquito bloodmeal analysis studies, which served as a main source of bloodmeal primers used in this study (3, 34, 36). Many of the chromatographs from the sequences suggested double-nucleotide peaks in the amplicons that matched to *Ornithodoros* sp., and repeated PCRs with the same or different primers were unable to resolve the amplified sequences. We suspect this non-target amplification of tick DNA is attributed to the barcoding primer design that minimizes non-target amplification of Insecta but perhaps not Ixodida. The size of the argasid tick genome (1.2 Gbp) is also 2.2x larger than the genome of *Culex pipiens* (0.54 Gbp), a common mosquito in which bloodmeal primers are developed, which further increases the opportunity for non-target amplification (35, 37, 38).

Despite the challenges posed by the non-target amplification of soft tick DNA, we were still able to produce repeatable bloodmeal host identification results in 19 samples. We suspect the success in the vertebrate ID in these samples was possible when sufficient blood was present. The most common vertebrate ID for these cave-dwelling soft ticks was raccoon, followed by black vulture, black-tailed rattlesnake, and human. The presence of these species in these caves was confirmed during a prior camera trapping study (31). Although these caves are off-limits to the public, camera traps documented unauthorized human access to these caves, supporting the potential for a human bloodmeal in this study and also identifying a risk associated

TABLE 1 | Demographic data of field-collected *Ornithodoros turicata* collected from Government Canyon State Natural Area, San Antonio, TX, 2019.

Cave name	Adult male	Adult female	Adult unknown	Life stage unknown	Nymphs	Total
Mad Crow Cave	20 (10.9%)	13 (7.1%)	1 (0.5%)	5 (2.7%)	145 (78.8%)	184
Bone Pile Cave	10 (9.2%)	14 (12.8%)	1 (0.9%)	0	84 (77.1%)	109
Little Crevice Cave	25 (28.4%)	5 (5.7%)	2 (2.3%)	0	56 (63.6%)	88

TABLE 2 | Demographic data of ticks and their identified bloodmeal sources.

Cave/location name	Tick ID number	Life stage	Sex	Length (mm)	Vertebrate bloodmeal result (% match)
Colony	A2	A	M	3.7	*Gallus gallus* (99.7%)
Colony	B2	A	F	7	*Sus scrofa* (95.4%)
Mad Crow Cave	ST-02A	N	U	2	*Procyon lotor* (92%)
Mad Crow Cave	ST-02B	N	U	3.5	*Procyon lotor* (100%)
Mad Crow Cave	ST-05B	N	U	4.3	*Coragyps atratus* (99.3%)
Bone Pile Cave	ST-18E	N	U	2.5	*Procyon lotor* (90%)
Bone Pile Cave	ST-19B	N	U	3	*Procyon lotor* (96%)
Bone Pile Cave	ST-19C	N	U	2	*Procyon lotor* (99.7%)
Bone Pile Cave	ST-20B	N	U	2.5	*Procyon lotor* (100%)
Bone Pile Cave	ST-21E	A	M	3	*Procyon lotor* (100%)
Bone Pile Cave	ST-22B	A	M	4.5	*Crotalus molossus* (98.9%)
Bone Pile Cave	ST-40B	N	U	2.5	*Procyon lotor* (96.2%)
Bone Pile Cave	ST-46B	N	U	2.1	*Procyon lotor* (90.3%)
Little Crevice Cave	ST-65E	N	U	3	*Homo sapien* (99.7%)
Little Crevice Cave	ST-68A	A	M	2	*Procyon lotor* (100%)
Mad Crow Cave	ST-72H	N	U	3.25	*Coragyps atratus* (100%)
Mad Crow Cave	ST-74E	A	F	5.6	*Procyon lotor* (99.7%)
Mad Crow Cave	ST-78C	N	U	4.8	*Procyon lotor* (99.7%)
Mad Crow Cave	ST-78I	A	M	4.4	*Procyon lotor* (99.7%)
Mad Crow Cave	ST-80B	N	U	3	*Procyon lotor* (99.7%)
Mad Crow Cave	ST-80H	N	U	3	*Procyon lotor* (95.1%)

All bloodmeals were identified using the mammal c primer pair. A, Adult; N, Nymph; M, Male; F, Female; U, Unknown.

with exposure to *B. turicatae,* which has been documented for central Texas (29, 39, 40). A study in Iowa documented large infestations of the soft tick, *Carios kelleyi,* in human dwellings using capillary electrophoresis to conduct bloodmeal analysis and identified one nymphal soft tick that had fed on a female human (1). Additionally, *Argas cooleyi* invaded a hospital in Arizona from their resident bird nests outside and fed on humans, with 17% of the analyzed bloodmeals belonging to humans (41). These studies, along with our study of a wild *O. turicata* population, indicate the importance of studying soft ticks and their potential associations with humans as hosts.

This cross-sectional study also informs the population structure of *O. turicata* in the caves of the region. Of the 381 collected soft ticks, 74.8% were found to be in the nymphal stage and no larvae were collected. These skewed demographics of the soft tick community composition illustrate a large proportion of immature ticks in early March compared to adults. Some soft tick species molt to future nymphal instars based on ambient temperature, and in some species, larvae do not need to feed on a bloodmeal, and receive all the nutrients they need to molt into first instar nymphs from the egg (26). However, in colony, *O. turicata* have been recorded to feed between larval and nymphal stages, and nymphs reared in similar environmental conditions can molt to adults as either 4, 5, or 6th nymphal instars (42). Due to the limited literature available on soft tick ecology, this study may indicate that in spring months, nymphs are more abundant than adults. Alternately, larvae may have been present in the caves yet less likely to be trapped using the dry ice/sticky trap stations that we deployed. Population demographic data is key in understanding the natural cycles of soft tick populations.

Limited prior studies have documented vertebrate host feeding patterns of soft ticks collected in the field. A study in Portugal performed bloodmeal analysis on *Ornithodoros erraticus* collected in two pig pens and were able to identify vertebrate hosts including pigs, humans, bovines, sheep, rodents, and birds through bloodmeal analysis in 23% of the analyzed ticks (4). We used many of the same primer pairs from this previous study, and

the lower success of the bloodmeal identification was likely also due to non-target amplification of tick DNA.

The challenges of non-target amplification of tick DNA could be resolved by multiple modifications in the future. All specimens collected in this study were host-seeking as they approached the CO_2-baited traps. Accordingly, very few likely had fresh bloodmeals, which made it difficult to distinguish bloodmeal contents in the abdomen, especially when ethanol added a red coloration to all specimens. Future studies processing field-collected soft ticks should consider the use of morphological features such as the size and depth of inter-mammillary grooves to judge the state of repletion. A future sampling approach could use an aspirator (43) which would increase the chances of obtaining specimens with fresh bloodmeals. One modification would be to specifically design primers to avoid tick genomes and amplify exclusively vertebrate DNA (35, 44). Another modification could be to insert a clone of the amplicon into a bacterial vector, and then select several colonies per sample to sequence, in hopes of detecting the vertebrate host sequence even if tick DNA was preferentially amplified. This technique is used routinely in bloodmeal analysis studies (45), although this method is labor intensive and limited in resolution. A third option would be to perform amplicon deep sequencing, which would provide thousands of sequences of each amplicon and is an approach recently adopted for arthropod bloodmeal analysis studies (46–48). This method of metabarcoding and deep sequencing would be advantageous given soft tick biology, including multiple bloodmeals obtained during immature development and multiple gonotropic cycles of adults. The amplicon deep sequencing approach would allow the ID of not just the most recent bloodmeal but also the potential to detect prior vertebrate bloodmeals (49, 50).

Given the continued emergence of human and animal diseases vectored by soft ticks, further studies of the ecology of argasid ticks- including their vertebrate host associations- are critical for informing tick-host-pathogen transmission networks, vector management efforts, and disease risk assessment.

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 at: https://hdl.handle.net/1969.1/191880, OAKTrust, Texas A&M University Libraries.

AUTHOR CONTRIBUTIONS

PT, SH, and GH conceived study, supervised field, and lab work. RB, VM, ED, TE, TLC, TMC, and GH conducted field collections. RB, MO, VM, ED, CB, DE, BH, and BB conducted lab work. PT provided colony ticks. RB, VM, and ED wrote initial manuscript. All authors contributed to revisions.

FUNDING

Funding for this study was provided in part by the Montague Center for Teaching Excellence Scholar Program, Texas A&M University Department of Veterinary Integrative Biosciences Teaching Enhancement Funds, Texas A&M University Department of Entomology, and a Seed Fund from the Coalition for Epi Response, Engagement, and Science (CERES).

ACKNOWLEDGMENTS

We appreciate permission from Texas Parks and Wildlife Division and Government Canyon State Natural Area for providing access to the campgrounds and caves to facilitate this study. We would also like to acknowledge the students of VIBS/ENTO 426/626 that participated in field work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.639400/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fvets.2021.620441

Identification of Host Bloodmeal Source in *Ornithodoros turicata* Dugès (Ixodida: Argasidae) Using DNA-Based and Stable Isotope-Based Techniques

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Edited by:
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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: 22 October 2020
Accepted: 11 January 2021
Published: 17 February 2021

Citation:
Kim HJ, Hamer GL, Hamer SA, Lopez JE and Teel PD (2021) Identification of Host Bloodmeal Source in Ornithodoros turicata Dugès (Ixodida: Argasidae) Using DNA-Based and Stable Isotope-Based Techniques. Front. Vet. Sci. 8:620441. doi: 10.3389/fvets.2021.620441

The ecology and host feeding patterns of many soft ticks (Ixodida: Argasidae) remain poorly understood. To address soft tick–host feeding associations, we fed *Ornithodoros turicata* Dugès on multiple host species and evaluated quantitative PCR (qPCR) and stable isotope analyses to identify the vertebrate species used for the bloodmeal. The results showed that a qPCR with host-specific probes for the *cytochrome b* gene successfully identified bloodmeals from chicken (*Gallus gallus* L.), goat (*Capra aegagrus hircus* L), and swine (*Sus scrofa domesticus*) beyond 330 days post-feeding and through multiple molting. Also, qPCR-based bloodmeal analyses could detect multiple host species within individual ticks that fed upon more than one species. The stable isotope bloodmeal analyses were based on variation in the natural abundance of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes in ticks fed on different hosts. When compared to reference isotope signatures, this method discerned unique $\delta^{13}C$ and $\delta^{15}N$ signatures in the ticks fed on each host taxa yet could not discern multiple host species from *O. turicata* that fed on more than one host species. Given the significance of soft tick-borne zoonoses and animal diseases, elucidating host feeding patterns from field-collected ticks using these methods may provide insight for an ecological basis to disease management.

Keywords: *Ornithodoros turicata*, bloodmeal analysis, stable isotope, soft tick, DNA-based technique

INTRODUCTION

The identification of host bloodmeal sources in arthropod vectors provides vital information in vector-borne disease ecology, which enables vector-specific control and a proactive vector–host–pathogen risk assessment (1–7). Numerous bloodmeal analyses have evolved over time. Serological methods, such as the precipitin test, which rely on antibody reaction to host blood date back to the 1940s (8, 9), and the advent of polymerase chain reactions (PCRs) and quantitative PCR (qPCR) have enabled a suite of DNA-based arthropod bloodmeal analysis techniques to be developed (10). DNA-based techniques for the bloodmeal analysis have been used successfully for a wide variety

of arthropod vectors, including mosquitoes, kissing bugs, and black flies (10). However, some arthropod vectors with a prolonged generation time, starvation periods, and molting between life stages pose challenges to the DNA-based bloodmeal analyses.

The primary challenge of conducting bloodmeal analyses in arthropod vectors enduring starvation periods lasting months to years, such as in ticks, is that the DNA obtained from previous life stage bloodmeals is degraded during molting (11, 12). To overcome this challenge, techniques such as reverse line-blot hybridization have shown potential due to its improved sensitivity to low quantities of DNA and the ability to use a large panel of host-specific probes (13, 14). However, this approach to bloodmeal analysis may be limited by availability of existing host blood probe data and optimization procedures (10, 13, 14).

The application of stable isotopes (SIs) to tick bloodmeal analyses offers the advantage of not needing to rely on host blood DNA detection. SI analyses determine the relative ratios of heavy to lighter elements within the organism that are influenced by its diet (3, 15–18). Proof-of-concept studies indicate that SI ratios of carbon and nitrogen from host bloodmeals are detectable in hard ticks after feeding on known hosts and molting (19), with the ability to discern among host taxa for up to 34 weeks post-molt in *Amblyomma americanum* (L.) (7). However, differentiation between ticks fed on ecologically similar hosts (*Peromyscus leucopus* mice vs. *Tamias striatus* chipmunks) was not possible, suggesting that SI analyses for bloodmeal host detection may be useful in sorting ticks to the level of the feeding guild, but not to the host species (20). Further, SI analyses require different vertebrate hosts' unique SI profiles to interpret the results from arthropods (15, 18, 21).

Bloodmeal analysis studies for argasid ticks are relatively rare (22–27) compared to those conducted for ixodid species. Some argasid ticks, such as *Ornithodoros turicata* Dugès (Ixodida: Argasidae), are known to survive for years without a bloodmeal (28, 29). The medical–veterinary importance of *O. turicata* and the ease with which it can be reared and maintained in the laboratory make it a good model for evaluating DNA- and SI-based tools for host bloodmeal identification.

O. turicata is a well-established vector and reservoir of *Borrelia turicatae*, one of the spirochetes that can cause tick-borne relapsing fever (TBRF) (30–36) and a potential vector of the African swine fever virus (37, 38). Collection records indicate that the distribution of *O. turicata* ranges from southern Mexico to the southwestern United States, as well as the state of Florida (39–41). Despite its early description in 1876 and subsequent studies linking its vector potential to TBRF as early as the 1930s, the vector ecology and host preferences of *O. turicata* remain relatively unstudied. This knowledge gap may be a result of biological and behavioral attributes of *O. turicata* that pose challenges to conducting surveillance in its native environment. For example, *O. turicata* is generally considered a nocturnal organism with an affinity toward microhabitats found in caves, burrows, nests, and cavities with host activity and is seldom found in relatively accessible open environments (39, 42–44). It is a generalist with a broad host range including taxa of mammals, birds, and reptiles; has up to seven nymphal instars; and may require one or more bloodmeals at each life stage before molting to the next (40, 43). With each host feeding event lasting just a few minutes (45), *O. turicata* is rarely found attached to its host. Therefore, current survey methods for *O. turicata* and other argasid ticks are limited to labor-intensive and time-consuming techniques such as the CO_2 baiting, debris-filtering methods (40, 46), and animal burrow vacuuming techniques (47).

The objective of this study is to compare DNA- and SI-based bloodmeal analyses on colonized *O. turicata* with known bloodmeals on vertebrates at different days post-feeding. The development of reliable techniques to identify host bloodmeals would provide valuable methods and applications to ecological studies and surveillance programs.

MATERIALS AND METHODS

Tick (*O. turicata*) Colony

Adults and late-instar nymphs of *O. turicata* used in this study were obtained from a colony maintained at the Tick Research Laboratory, Texas A&M AgriLife Research, College Station, TX, United States. The colony originated from specimens collected in a natural cavern in Travis County, TX, United States in 1992. The *O. turicata* colony has been maintained under a 14:10 (light: dark) photoperiod, 25.0 ± 3.0°C, and 80–85% relative humidity and fed approximately once a year using young cockerels (*Gallus gallus* L.) as bloodmeal hosts according to procedures approved by the Institutional Animal Care and Use Committee of Texas A&M University (AUP no. 2014-255).

Cohort Preparation

Timelines and protocol overview are outlined in **Table 1**. Four *O. turicata* cohorts, each consisting of approximately 600 larvae, were prepared by transferring 30 larvae from the progeny of each of 20 female ticks using a camel-hair brush. The cohorts were reared to the fourth-instar nymph stage using different combinations of chicken (*G. gallus* L.), goat (*Capra aegagrus hircus* L.), and swine (*Sus scrofa domesticus*) blood. The first group was labeled "EC," short for "Exclusively fed on Chicken," and was reared exclusively on live chickens for four bloodmeals. The second group was labeled "CG," short for fed on "Chicken and Goat," and was reared on live chickens for three bloodmeals and a final bloodmeal on commercially acquired, mechanically defibrinated goat blood (Rockland Immunochemicals Inc., Limerick, PA, United States) via an artificial membrane protocol developed by Kim et al. (48). The third group was labeled "CS," short for fed on "Chicken and Swine," and was reared on live chickens for three bloodmeals and final bloodmeal on commercially acquired, mechanically defibrinated swine blood (Rockland Immunochemicals Inc., Limerick, PA, United States) via an artificial membrane. The fourth group was labeled "ES," short for "Exclusively fed on Swine," and was reared solely on commercially acquired, mechanically defibrinated swine blood via an artificial membrane. At 30-day intervals after their final bloodmeal (up to 9 months), a group of 10 ticks was harvested from each cohort, with five ticks serving as replicates for DNA-based analysis and five ticks serving as replicates for SI-based bloodmeal analysis shown in **Table 1**.

TABLE 1 | Timeline for the development and sampling scheme of four experimental *Ornithodoros turicata* cohorts fed on different kinds of host blood.

Experiment (days)	*O. turicata* (state)	Note
-	Larvae to 2N	EC, CG, and CS cohorts reared to 2N using chicken blood. ES cohort reared to 2N using swine blood
0	2N (engorged)	EC, CG, and CS cohorts fed on chicken blood. ES cohort fed on swine blood
30	3N (unfed)	Samples collected for qPCR and SI analyses from each cohort
60	3N (engorged)	Final bloodmeal for all cohort EC cohort fed on chicken blood CG fed on goat blood CS fed on swine blood SS fed on swine blood Samples collected for qPCR and SI analyses from each cohort
90	4N 0M (freshly molted)	Samples collected for qPCR and SI analyses from each cohort
120	4N 1M (1 month post molt)	Samples collected for qPCR and SI analyses from each cohort
150	4N 2M (2 months post molt)	Samples collected for qPCR and SI analyses from each cohort
180	4N 3M (3 months post molt)	Samples collected for qPCR and SI analyses from each cohort
210	4N 4M (4 months post molt)	Samples collected for qPCR and SI analyses from each cohort
240	4N 5M (5 months post molt)	Samples collected for qPCR and SI analyses from each cohort
270	4N 6M (6 months post molt)	Samples collected for qPCR and SI analyses from each cohort
330	4N 7M (9 months post molt)	Samples collected for qPCR and SI analyses from each cohort

All O. turicata cohorts were maintained under a 14:10 (light: dark) photoperiod, 25.0 ± 3.0°C, and 80–85% relative humidity.
N, instar nymph; M, month.

DNA Extraction and qPCR Analysis

Ticks from each sample group ($n = 5$) outlined in **Table 1** were used for DNA extraction and qPCR analysis. In summary, five ticks from each cohort (EC, CG, CS, and ES) were collected immediately after their last bloodmeal, after a molt (∼30 days post last bloodmeal), for six consecutive months (∼4-week interval), and at 9 months post-molt. Surface contaminants on each tick were removed by briefly placing the tick in a 50% bleach solution for 30 s and then washing with water as outlined by Graham et al. (49). The whole-body DNA extraction was performed per the manufacturer's instructions using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, Unite States) with overnight lysis. DNA was extracted from each *O. turicata* by cutting it into two equal segments in a sterile centrifuge tube, exposing its midgut content to a lysis buffer solution with a final elution volume of 50 µl. DNA from aliquots of each host blood (15 µl per each host blood) used to feed *O. turicata* treatment groups for this study was also extracted to serve as positive controls for their respective treatment groups. Both water-template and no-template wells served as negative controls.

The qPCR analysis was performed to detect the *cytochrome b* (*cytb*) gene in the extracted DNAs using host blood-specific primers and probes, as previously described by Cupp et al. (50). The *cytb* gene was selected as the appropriate molecular marker for the bloodmeal analyses because the primers are vertebrate-specific and would not amplify *O. turicata* DNA (50). Primer and probe sequences used for qPCR are listed in **Table 2**. Moreover, each probe was tagged with a unique reporter dye to aid in differentiating between vertebrate hosts. The unmodified *cytb* primers and probes for chicken and goat were used as described by Woods et al. (51). The primer and probe for the swine blood designed in this study used the Beacon Designer 8.0 software (Premier Biosoft, Palo Alto, CA, United States) based on *Sus scrofa* mitochondrion genome (GenBank accession #AF034253.1).

The LightCycler® 96 system (Roche Diagnostics Corporation., Indianapolis, IN, United States) was used for all qPCR analyses with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. Subsequently, nuclease-free water and whole-blood DNA extracts were used as the qPCR negative and positive controls, respectively. Each qPCR assay used a 25-µl total reaction volume that had 400 nM of forward and reverse primers each, 200 nM of probe, and 4.5 µl of DNA template (concentration unknown) using the Bio-Rad iTaq Universal Master Mix (Bio-Rad, Hercules, CA, United States). A total of 750 qPCR assays were conducted on DNA extracts. DNA extracted from host blood (chicken: 211.7 ng/µl, swine: 9.26 ng/µl, or goat: 17.4 ng/µl) was used as positive controls in the assays. Among the 750 assays, 365 qPCR assays were labeled as "unmatched samples," which denoted DNA extracts from *O. turicata* sample groups tested using host-specific primer and probes of blood that was not used to rear them. This was done to further assess any cross-reactivity across host blood. The remaining 385 qPCR assays were labeled as "matched samples," denoting DNA extracts from *O. turicata* sample groups tested using host-specific primers and probes of blood used by the corresponding cohort. The matched samples included the ticks reared on two host species when the primer–probe set was specific to one of the hosts. The qPCR results were interpreted as positive when a DNA sample cycle threshold (Ct) value was <35. This threshold value was determined conservatively based

TABLE 2 | Primer and probe sequences for host-specific *cytb* gene used as a molecular marker in qPCR assays to identify host blood source in colony-reared *Ornithodoros turicata*.

Host blood	Gene	Amplicon size	Primer and probe sequences	References/GenBank accession number
Chicken	*cytb*	162	Forward primer 5′-CCTCTACAAGGAAACCTCAAACAC-3′ Reverse primer 5′-GACTAGGGTGTGTCCAATGTAGG-3′ Probe 5′-ROX-CGCCATAGTCCACCTGCTCTTCCTCCA-BHQ-3′	(51)
Goat	*cytb*	125	Forward primer 5′-TCCTCCCATTCATCATCACAGC-3′ Reverse primer 5′-TGGTGTAGTAAGGGTGAAATGGG-3′ Probe 5′-ROX-CGCCATAGTCCACCTGCTCTTCCTCCA-BHQ-3′	(51)
Swine	*cytb*	176	Forward primer 5′-CTACGGTCATCACAAATCTACTATCAG-3′ Reverse primer 5′-GTGCAGGAATAGGAGATGTACG′ Probe 5′-Cy5-ATCGGAACAGACCTCGTAGAATGAATC-BHQ-3′	This study/AF034253.1

on preliminary trials that showed that late and non-specific amplification of negative controls occurred at or above 36 cycles.

SI Analysis

SI analysis was conducted using *O. turicata* from each tick sample group ($n = 5$) collected concurrently as those gathered for qPCR. Elemental analysis isotope ratio mass spectrometry (EA-IRMS) at the Stable Isotope Geosciences Facility at Texas A&M University, College Station, TX, United States, was used to analyze individual ticks for carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopic values as described by Hamer et al. (7). The EA combusted the tick and blood samples at 1,200°C, separating CO_2 and N_2 gases, and analyzed on the EA-IRMS. The standard delta (δ) notation $\delta X = [(Rsample/Rstandard) - 1] \times 1{,}000$, where R was the ratio of the heavy to light SI in the sample and standard, was used to represent the results. Next, results were referenced according to the Vienna Pee Dee Belemnite (VPDB) carbonate standard for $\delta^{13}C$ and relative to air for $\delta^{15}N$. Finally, the range of $\delta^{13}C$ and $\delta^{15}N$ values of samples for a 2-point calibration and internal laboratory standards every ∼12 unknowns was used to measure analytical precision as described by Hamer et al. (7). Samples from each host blood type used to feed corresponding cohorts were used to generate reference isotope signatures in the SI analysis.

Statistical Data Analysis

The statistical program JMP® Pro 12 (SAS Co., Cary, NC, United States) was used for all statistical analyses. The exact Cochran–Armitage trend test was conducted to assess any difference in qPCR assay results of each *O. turicata* sample group ($n = 5$) based on the experiment days (length of starvation). A chi-square test was conducted to determine the associations between each tick's host feeding history (EC, CG, CS, and ES) and the qPCR results (positive or negative for each assay). Pillai's trace multivariate analysis of variance (MANOVA) was used to compare $\delta^{13}C$ and $\delta^{15}N$ values of host blood, all unfed third-instar nymphs, all engorged third-instar nymphs, sample groups from each cohort, and combined sample group values for all cohorts. When MANOVA indicated a significant difference, a *post-hoc* test using Tukey's honestly significant differences was conducted to assess pairwise differences in both $\delta^{13}C$ and $\delta^{15}N$ based on an alpha level of 0.05.

RESULTS

qPCR Analysis

All 365 "unmatched samples" tested negative in the qPCR, which denotes no significant cross-reactions ($P < 0.0001$, CI = 0, 0.01) among host *cytb* genes and non-corresponding host primers and probes. The chicken *cytb* gene was detected in at least one replicate within each five-tick sample group fed on chicken blood in all EC, CG, and CS cohorts across the entire experiment period (**Table 3**). The *O. turicata* samples from the EC cohort had the highest overall qPCR-positive prevalence of 98%, followed by the samples from the CG cohort with 76% and the samples from the CS group with 60%. There were no significant differences in qPCR results of *O. turicata* samples based on the experiment day (length of starvation) in the EC cohort ($P = 0.10$, Cochran–Armitage trend test) and CG cohort ($P = 0.35$, Cochran–Armitage trend test). However, a significant difference was observed in the qPCR array results in tick samples from the CS cohort based on experiment days ($P < 0.01$, Cochran–Armitage trend test) (**Table 3**), indicating that the starvation period had affected the qPCR outcome.

The goat *cytb* gene was detected in at least one replicate within each five-tick sample group fed on goat blood in the CG cohort during the entire experiment period (**Table 3**). The overall average qPCR-positive prevalence for goat DNA in *O. turicata* samples from the CG cohort was 64.4%. There were no significant differences in the qPCR assay results of *O. turicata* samples based on the length of starvation in the CG cohort ($P = 0.38$, Cochran–Armitage trend test) (**Table 3**). The swine *cytb* gene was detected in at least one replicate within each five-tick sample group fed on swine blood in both CS and ES cohorts during the entire experiment period (**Table 3**). The *O. turicata* samples from the ES cohort had a higher overall average qPCR-positive prevalence of 82.0%, followed by the samples from the CS cohort with 75.6%. There were no significant differences in the qPCR assay results of *O. turicata* samples based on the length of starvation in the ES cohort ($P = 0.27$, Cochran–Armitage trend test). However, a

TABLE 3 | Summary of qPCR assays (Ct value < 35) based on experiment days per *Ornithodoros turicata* tick sample group ($n = 5$) fed on chicken (*Gallus gallus*), goat (*Capra aegagrus hircus*), and swine (*Sus scrofa domesticus*) blood.

***O. turicata* state**	**Experiment days**	**EC**	**CG**	**CS**	**CG**	**CS**	**ES**
		qPCR positives out of 5 samples (%) using chicken *cytb* primer and probe			**qPCR positives out of 5 samples (%) using goat *cytb* primer and probe**	**qPCR positives out of 5 samples (%) using swine *cytb* primer and probe**	
2N (engorged)	0	5 (100)	4 (80)	5 (100)	0 (0)*	0 (0)**	5 (100)
F 3N (engorged)	60	5 (100)	2 (40)	5 (100)	5 (100)	5 (100)	5 (100)
4N 0M (freshly molted)	90	5 (100)	4 (80)	4 (80)	4 (80)	5 (100)	3 (60)
4N 1M (1 month post molt)	120	5 (100)	5 (100)	2 (40)	2 (40)	4 (80)	5 (100)
4N 2M (2 months post molt)	150	5 (100)	4 (80)	3 (60)	2 (40)	4 (80)	3 (60)
4N 3M (3 months post molt)	180	5 (100)	5 (100)	3 (60)	2 (40)	5 (100)	3 (60)
4N 4M (4 months post molt)	210	5 (100)	2 (40)	1 (20)	2 (40)	1 (20)	5 (100)
4N 5M (5 months post molt)	240	5 (100)	4 (80)	1 (20)	5 (100)	3 (60)	5 (100)
4N 6M (6 months post molt)	270	5 (100)	3 (60)	4 (80)	1 (20)	5 (100)	2 (40)
4N 9M (9 months post molt)	330	4 (80)	5 (100)	2 (40)	5 (100)	2 (40)	5 (100)
Range of % positive per cohort		80–100	40–100	20–100	20–100	20–100	40–100
Mean % positive per cohort		98	76	60	64.44	75.56	82.00
SD per cohort		6.32	22.71	29.81	31.27	29.63	23.94
Mean % positive across cohorts			71		-		75.71
SD per all cohort			29.68		-		28.99
Exact Cochran–Armitage trend test		$P = 0.1$	$P = 0.35$	$P < 0.01$	$P = 0.38$	$P < 0.01$	$P = 0.27$

*N, instar nymph; EC, exclusively fed on chicken blood; CG, fed on chicken and goat blood; CS, fed on chicken and swine blood; ES, exclusively fed on swine blood. *2N CG cohort not fed on goat blood and excluded from statics analysis. **2N CS cohort not fed on swine blood and excluded from statistical analysis.*

TABLE 4 | Summary of qPCR assay results based on host blood-specific primers and probes.

***Ornithodoros turicata* cohort**	**Primer–probe**	**Positive (%)**	**Total samples**
Fed on chicken blood	Chicken	142 (71.0)	200
Fed on goat blood	Goat	29 (64.4)	45
Fed on swine blood	Swine	106 (75.7)	140
		277 (71.9)	385
	n		385
	Chi-square df		2
	Chi-square value		2.33
	Chi-square *P*		$P = 0.31$

significant difference was observed in the tick samples from the CS cohort ($P < 0.01$, Cochran–Armitage trend test) (**Table 3**). Finally, there were no differences between the qPCR results of the tick samples based on the host blood ($X^2 = 2.33$, df = 2, $P = 0.31$) (**Table 4**), indicating that the host blood did not affect the qPCR outcome.

SI Analysis

SI analysis results of $\delta^{13}C$ and $\delta^{15}N$ for each host blood were significantly different ($F = 57.20$; df = 4, 24; $P < 0.01$) (**Figure 1**). The *post-hoc* tests showed significant differences in $\delta^{13}C$ for all pairwise combinations ($P < 0.01$ each), in which swine had the highest $\delta^{13}C$ and goat had the lowest $\delta^{13}C$. The *post-hoc* test showed $\delta^{15}N$ in swine blood was significantly higher than that in goat and chicken ($P < 0.01$ each).

SI analysis results of $\delta^{13}C$ and $\delta^{15}N$ for unfed third-instar *O. turicata* nymph samples from each cohort showed significant differences ($F = 4.65$; df = 6, 32; $P < 0.01$) (**Figure 1**). The *post-hoc* tests showed no significant differences in either $\delta^{13}C$ or $\delta^{15}N$ between EC, CG, and CS cohorts. On the other hand, the *post-hoc* tests for $\delta^{13}C$ and $\delta^{15}N$ showed that the ES cohort was significantly different from the EC, CG, and CS cohorts ($P < 0.01$ each). Similarly, in engorged third-instar *O. turicata* nymph samples, there were significant differences among the cohorts in $\delta^{13}C$ and $\delta^{15}N$ ($F = 29.46$; df = 6, 32; $P < 0.01$) (**Figure 1**). The *post-hoc* tests for $\delta^{13}C$ and $\delta^{15}N$ showed significant differences ($P < 0.01$ each), except between ES and CS cohorts ($P = 0.55$).

SI analysis results of $\delta^{13}C$ and $\delta^{15}N$ for EC, CG, CS, and ES fourth-instar *O. turicata* nymph samples based on the time since starvation showed significant differences among sample groups [($F = 3.04$; df = 16, 72; $P < 0.01$) for EC, ($F = 3.10$; df = 16, 72; $P < 0.01$) for CG, ($F = 3.91$; df = 16, 72; $P < 0.01$) for CS, and ($F = 3.22$; df = 16, 72; $P < 0.01$) for ES] (**Figure 1**). The *post-hoc* test for $\delta^{13}C$ showed no significant differences between all cohort samples. However, the *post-hoc* test for $\delta^{15}N$ generally indicated significant differences between engorged third-instar nymphs and all post-molt fourth-instar nymphs. There were significant differences between engorged third-instar nymphs and all post-molt fourth-instar nymphs ($P < 0.01$ each) in EC and CS, between engorged third-instar nymphs and 3, 4, and 6 months post-molt fourth-instar nymphs ($P < 0.01$ each) in CG, and between engorged third-instar nymphs and 1–6

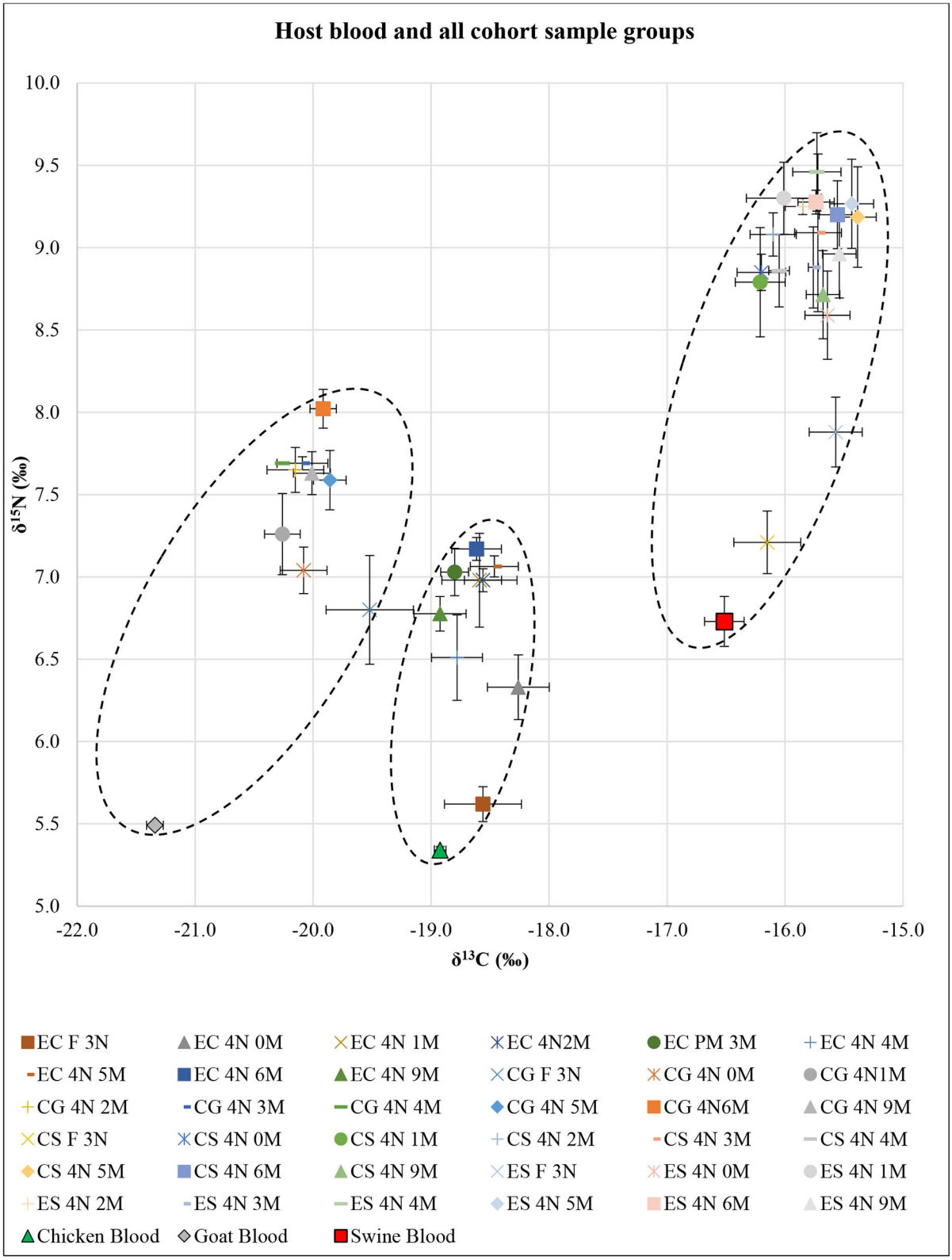

FIGURE 1 | Isotopic results of all *Ornithodoros turicata* cohort sample groups based on post-molt time ($n = 5$ each) represented as $\delta^{13}C$ and $\delta^{15}N$ superimposed over the host blood results. X- and Y-axis error bars represent SEs around means. Dotted oval shapes encircle $\delta^{13}C$ and $\delta^{15}N$ values for each of the EC, CG, and CS+ES cohorts. EC, exclusively fed on chicken blood; CG, fed on chicken and goat blood; CS, fed on chicken and swine blood; ES, exclusively fed on swine blood; F 3N, third-instar nymph immediately after feeding; 4N 0M, fourth-instar nymph immediately after a molt; 4N 1M–4N 9M, fourth-instar nymph 1 month post-molt to fourth-instar nymph 9 months post-molt.

and 9 months post-molt fourth-instar nymphs ($P < 0.01$ each) in ES.

The combined $\delta^{13}C$ and $\delta^{15}N$ SI values comparing all fourth-instar *O. turicata* nymph cohorts regardless of their post-feeding time (average SI value, $n = 45$ per cohort) showed significant differences among the cohorts ($F = 117.46$; df = 6, 352; $P < 0.01$) (**Figure 1**). The *post-hoc* tests for both $\delta^{13}C$ and $\delta^{15}N$ showed significant differences between cohorts ($P < 0.01$ each) except between CS and ES cohorts. There were three clusters of $\delta^{13}C$ and $\delta^{15}N$ values observed (shown by dotted oval shapes) that represented EC, CG, and CS+ES cohorts (**Figure 1**). An isotopic shift of increasing $\delta^{13}C$ and $\delta^{15}N$ with tick age was observed for all treatment groups.

DISCUSSION

This study reports the first in-depth bloodmeal analysis of an experimentally fed argasid tick, *O. turicata*, using DNA- and SI-based techniques. The DNA-based bloodmeal analysis in this study accurately differentiated host blood in *O. turicata* cohorts including those receiving bloodmeals from different vertebrate species. Unexpectedly, the host-specific *cytb* gene was detectable in at least some replicates of each study group across the entire experiment period of 330 days using the DNA-based technique. Similarly, the SI-based bloodmeal analysis technique was proven capable of discerning the difference among *O. turicata* cohorts which fed on different single vertebrate host blood taxa (chicken, goat, or swine). However, the SI-based bloodmeal analysis failed to discern the difference between cohorts that fed only on blood from a single host taxon (e.g., swine only) from cohorts that fed on blood from two host taxa when the latter included the same vertebrate host blood as the single-host-blood cohorts (e.g., chicken then swine vs. swine only).

The SI analysis generated distinctive $\delta^{13}C$ and $\delta^{15}N$ values for each host blood as well as for the *O. turicata* cohorts that fed on different kinds of host blood. While the SI values of the host blood did not overlap directly with *O. turicata* that fed on the same host blood, a previous study has shown that the isotopic discrimination (i.e., the tick-blood spacing) was invariable, and an increase in nitrogen composition in the tick is expected relative to the composition of the blood on which the tick fed, given the increase in the level of the food chain (19). Furthermore, we observed an isotopic shift (increasing $\delta^{13}C$ and $\delta^{15}N$) with tick age, which has also been observed in prior studies (7, 19) and which complicates the utility of this approach for tick bloodmeal analysis. SI analyses may be limiting when a large number of vertebrate species are expected as bloodmeal hosts. However, SI bloodmeal analysis could be viable if, during field or laboratory experiments, the vertebrate host community has restricted species richness and if the time post-feeding (e.g., >1 year) exceeds the ability of DNA-based approaches. However, sample cost is a consideration. The inclusion of an additional SI, sulfur (^{34}S), is now available given that many laboratories have the ^{13}C, ^{15}N, and ^{34}S combined analysis that can be used on the sample. However, the cost for the dual ^{13}C-and-^{15}N analysis is $8.50 per sample at the UC-Davis Stable Isotope Facility, while the cost of the ^{13}C, ^{15}N, and ^{34}S triplex is $73.00 (https://stableisotopefacility.ucdavis.edu/index.html).

Prolonged detectability of vertebrate host DNA within *O. turicata* suggests that the processing and storing of host blood in *O. turicata* may be considerably different between argasid and ixodid species. Hamer et al. (7) conducted host-specific qPCR-based bloodmeal analysis on *A. americanum* and reported that the assay began to fail to detect host-specific *cytb* as early as 42 days post-feeding in adult sample groups and rarely detected the host beyond 40 weeks post-feeding. There are no clear explanations for differences in *cytb* integrity observed between these studies. Nonetheless, exploring the bloodmeal digestion process between *A. americanum* and *O. turicata* may elucidate a plausible explanation.

Bloodmeal processing in both ixodid and argasid species is composed of three phases. Hemolysis takes place during the first phase, which occurs immediately upon feeding and lasts 2 to 15 days. The second phase, also called the "rapid" digestion, takes place in the midgut of ticks and can last from several weeks to 3 months. Finally, the third phase, also called the "slow" digestion, occurs mainly in the apical branches of the diverticula and can last for years (52). The difference between ixodid and argasid tick digestion is the third digestion phase. Bloodmeal digestion in ixodid ticks occurs uniformly, and the ingested bloodmeal is evenly stored and consumed at a steady rate in the midgut as well as in the diverticula (52). On the other hand, bloodmeal digestion in the third phase of argasid ticks occurs at a variable rate because a substantial amount of bloodmeal is stored in the peripheral regions of the midgut diverticula with no digestive activity (52). This slow and uneven digestion of the bloodmeal allows argasid ticks to endure starvation that could last for years, as observed in *O. turicata* (45). In this study, *O. turicata* samples from CG and CS cohorts were able to maintain a detectable level of chicken *cytb* gene throughout two molts and starvation periods exceeding 9 months (**Table 3**). Indeed, the rate of biochemical processes (i.e., no digestive activity) in the peripheral regions of the midgut diverticula can slow down the digestion of the bloodmeal, thus prolonging the overall bloodmeal consumption (52). Nevertheless, the physical capacity of the peripheral regions of the midgut diverticula that store a previous bloodmeal may also force subsequent and newly acquired bloodmeal to be kept in the medial regions of the midgut where active digestion occurs (52). This "blocking" of storage space by the previous bloodmeal may allow residual bloodmeal from earlier feedings to remain for the entire tick life span of *O. turicata*, enabling qPCR analysis to detect multiple host *cytb* genes. In contrast, the qPCR analysis was useful in simultaneously detecting two host-specific *cytb* genes correctly across all *O. turicata* fed on multiple kinds of host blood. However, this documentation was based on the detection of a chicken bloodmeal which was taken prior to the goat or swine bloodmeal in the mixed-species sample groups. We did not attempt the reverse order by feeding the ticks on goat or swine and then chicken bloodmeals, which is important to note given that the bird blood likely had more DNA than the mammal blood.

The detectability of multiple host bloodmeals within *O. turicata* that experimentally fed on two species varied depending on the bloodmeal analysis techniques employed. The SI

technique could not be used to discern the difference between single-host and multihost blood-fed *O. turicata* cohorts. For example, there were no differences between the overall $\delta^{13}C$ and $\delta^{15}N$ values of the CS and ES cohorts (**Figure 1**). Moreover, engorged third-instar nymphs from CS and ES cohorts showed no significant difference in their $\delta^{13}C$ and $\delta^{15}N$ values (**Figure 1**), despite each cohort being fed different kinds of host blood previously. This further strengthens the argument that the last bloodmeal *O. turicata* acquired determines the outcome of the SI analysis. In contrast, the qPCR analysis was useful in simultaneously detecting two host-specific *cytb* genes correctly across all *O. turicata* cohorts fed on multiple host blood. However, the detection of DNA in the different *O. turicata* cohorts ranged between one of five ticks to five of five ticks, indicating variation in either the retention of the DNA in the ticks or variation in the methodology (DNA extraction or qPCR). For example, we used 4.5 µl of template DNA from all samples and did not adjust based on the quantity of DNA, which was likely variable. Additionally, quantifying sample DNA and running these qPCR reactions in replicates would likely have strengthened the results and should be considered in future studies.

The duration of starvation could influence the outcomes of each type of bloodmeal analysis technique. For example, qPCR results for the CS cohort using swine-specific primers and probes seem to be influenced by the duration of starvation (**Table 3**). There was no logical explanation for this since the qPCR results of other groups, such as the ES cohort, which were also reared using swine blood were not affected by the duration of starvation. Since there were only five tick replicates per group, increasing the number of replicates per sample group may reduce the inconsistency observed in qPCR results for future studies. However, no sample tick group failed to retain detectable DNA at any time during the entire experiment period. Thus, it is uncertain how much improvement in the reproducibility of qPCR results can be made by increasing the number of ticks per sample group.

Different amounts of DNA that exist in different kinds of host blood provide another plausible explanation for the apparent influence of the starvation period on the outcomes of each type of bloodmeal analysis technique. Chicken blood, which consists of both immature and mature nucleated erythrocytes (53), presumably had higher overall DNA concentration in ticks. In contrast, goat and swine blood (and other mammals) are known to have immature nucleated erythrocytes that become anucleated once matured, attributing to relatively low DNA extract yield (54). While this study did not examine the proportion of *cytb* gene within the total DNA extract of host blood, an inference can be made based on the ubiquitous presence of the *cytb* genes in vertebrate hosts as part of their mitochondria, in that the relative proportions of *cytb* gene in the chicken, goat, and swine blood would be similar to that of total DNA extract (50, 55–57). Therefore, the *O. turicata* CS cohort, which fed on swine blood once, may not have had the chance to acquire and maintain the adequate amount of swine *cytb* gene throughout the entire experiment period compared to the *O. turicata* ES cohort, which had four opportunities to feed on swine blood. Moreover, the fact that the host blood type had no effect on the overall qPCR results of any tick sample groups (**Table 4**) further denigrates the significance of different qPCR results seen in the CS cohort. Therefore, an argument can be made that the inconsistency observed in qPCR results based on the length of starvation seen in CS cohort may not be attributed to a single reason but due to combinations of low sample number, lower DNA extract yield in swine blood, and variable rate of bloodmeal digestion of *O. turicata*.

Starvation duration influenced the outcomes of SI analysis differently than that of qPCR analysis. First, patterns of increased $\delta^{13}C$ and $\delta^{15}N$ values in engorged third-instar nymphs in each cohort compared to their corresponding host blood were observed. This observation could be due to SI (e.g., nitrogen) being enriched (15, 17, 58). The increase of $\delta^{13}C$ and $\delta^{15}N$ values in engorged *A. americanum* was also previously observed (7). However, the SI analysis failed to provide conclusive evidence for the SI fractionation, which occurs due to nutrient stress such as starvation. Such physiological stresses cause nitrogen fractionation via changes in the rate of amino acid consumptions, uric acid formations, and secretions (17, 21). Indeed, Hamer et al. (7) also reported changes in $\delta^{13}C$ over time in *A. americanum* fed on chicken; however, data from this study were inconclusive to make such inference. This may be due to the inconsistent digestion rate in *O. turicata* mentioned above.

The applicability and limitation of the bloodmeal analysis techniques used in this study must be carefully considered in the contexts of *O. turicata* biology and ecology. For example, the qPCR analysis will require a catalog of specific host genes, primers, and probes. In contrast, SI analysis will require blood SI signatures of the animals circulating in the *O. turicata* habitat in order to conduct bloodmeal analyses accurately. Furthermore, the longevity of *O. turicata* ticks may pose unique considerations for understanding the vertebrate hosts that are important for feeding ticks. The longevity of *A. americanum* is typically <3 years, and overlapping generations found in their population structure may rarely consist of more than two generations (59). In this case, tick host feeding patterns observed in the population may closely resemble the actual host utilization as the host population dynamic may not change drastically within the typical generation time. On the other hand, the longevity of *O. turicata* may be measured in decades (28, 45). Thus, ticks may outlive their hosts or live through the drastic changes in host population composition. For example, progressive feral swine invasion (60) has increased tick host diversity and abundance (61), impacting exposure to and interactions with *O. turicata* (62, 63). Consequently, overlapping *O. turicata* generations may occur. In this case, bloodmeal analysis may not accurately depict host utilization of older generation ticks that may have had exclusive access to hosts that are no longer available or diminished to younger-generation ticks.

In summary, the bloodmeal analysis techniques evaluated in this study demonstrated promising tools for determining host utilization of *O. turicata*. The DNA-based bloodmeal analysis underscored the feasibility to discern multiple-host

utilization by *O. turicata* and confirmed the applicability of the *cytb* gene as a host-specific molecular marker. The SI-based bloodmeal analysis was able to distinguish host blood, *O. turicata* cohorts fed on different kinds of host blood, and nitrogen enrichment in *O. turicata* post-bloodmeal consumption, although the utility of this approach in the future may be limited to unique circumstances. Our future work is applying the DNA-based bloodmeal analysis to *O. turicata* collected in the field [(64) in review]. A comprehensive understanding of vector ecology, including host utilization, is important for studying the natural history of soft ticks, their associated tick-borne pathogens, and applications of techniques for surveillance and intervention strategies.

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 Institutional IACUC committee reviewed and approved the protocol.

AUTHOR'S NOTE

The content of this manuscript has been published in part, as a chapter in the 2017 dissertation of Hee J. Kim (65).

AUTHOR CONTRIBUTIONS

All authors made substantial intellectual contributions to the project including experimental design, protocol execution, assessment of results, and preparation of the manuscript.

FUNDING

This study was supported in part by the USDA Formula Animal Health program project no. TEX09534 and by Texas A&M AgriLife Research Project TEX08911.

ACKNOWLEDGMENTS

We thank Lisa Auckland for laboratory assistance, the Stable Isotope Geosciences Facility at Texas A&M University for conducting the stable isotope analysis, and Dr. Mark Olson for critically reviewing the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 March 2021
doi: 10.3389/fvets.2021.637837

Novel Genotypes of Nidicolous *Argas* Ticks and Their Associated Microorganisms From Spain

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The knowledge of the distribution, richness and epidemiological importance of soft ticks of the genus *Argas* is incomplete. In Spain, five *Argas* species have been recorded, including three ornitophilic nidicolous ticks, but their associated microorganisms remain unknown. This study aimed to investigate ticks from bird nests and their microorganisms. Ticks were collected extensively from natural cavities and nest-boxes used by European rollers (*Coracias garrulus*) and little owls (*Athene noctua*) in Southeastern and Central Spain. Ticks were morphologically and genetically identified and corresponding DNA/RNA tick extracts were analyzed [individually ($n = 150$) or pooled ($n = 43$)] using specific PCR assays for bacteria (Anaplasmataceae, *Bartonella*, *Borrelia*, *Coxiella*/*Rickettsiella,* and *Rickettsia* spp.), viruses (Flaviviruses, Orthonairoviruses, and Phenuiviruses), and protozoa (*Babesia*/*Theileria* spp.). Six *Argas* genotypes were identified, of which only those of *Argas reflexus* ($n = 8$) were identified to the species level. *Two* other genotypes were closely related to each other and to *Argas vulgaris* ($n = 83$) and *Argas polonicus* ($n = 33$), respectively. These two species have not been previously reported from Western Europe. Two additional genotypes ($n = 4$) clustered with *Argas persicus*, previously reported in Spain. The remaining genotype ($n = 22$) showed low sequence identity with any *Argas* species, being most similar to the African *Argas africolumbae*. The microbiological screening revealed infection with a rickettsial strain belonging to *Rickettsia fournieri* and *Candidatus* Rickettsia vini group in 74.7% of ticks, mainly comprising ticks genetically related to *A. vulgaris* and *A. polonicus*. Other tick endosymbionts belonging to *Coxiella*, *Francisella* and *Rickettsiella* species were detected in ten, one and one tick pools, respectively. In addition, one *Babesia* genotype, closely related to avian *Babesia* species, was found in one tick pool. Lastly, Anaplasmataceae, *Bartonella, Borrelia*, and viruses were not detected. In conclusion, five novel *Argas* genotypes and their associated microorganisms with unproven pathogenicity are reported for Spain. The re-use of nests between and within years by different bird species appears to be ideal for the transmission of tick-borne microorganisms in cavity-nesting birds of semiarid areas. Further work should be performed to clarify the taxonomy and the potential role of soft *Argas* ticks and their microorganisms in the epidemiology of zoonoses.

Keywords: soft ticks, *Argas* spp., nidicolous, cavity-nesting birds, tick-borne bacteria, tick-borne viruses, tick-borne protozoa, Spain

OPEN ACCESS

Edited by:
Sebastián Muñoz-Leal, University of Concepcion, Chile

Reviewed by:
José M. Venzal, Universidad de la Republica, Uruguay
Quan Liu, Foshan University, China

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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: *04 December 2020*
Accepted: *24 February 2021*
Published: *29 March 2021*

Citation:
Palomar AM, Veiga J, Portillo A, Santibáñez S, Václav R, Santibáñez P, Oteo JA and Valera F (2021) Novel Genotypes of Nidicolous Argas Ticks and Their Associated Microorganisms From Spain. Front. Vet. Sci. 8:637837. doi: 10.3389/fvets.2021.637837

INTRODUCTION

Soft ticks of the genus *Argas* Latreille, 1795 (Ixodida; Argasidae) are distributed worldwide and include around 60 species (1). Of them, only eight species have been described in the Western Palearctic region, specifically, *Argas gilcolladoi*, *Argas persicus*, *Argas reflexus*, *Argas transgariepinus*, *Argas vespertilionis*, *Argas macrostigmatus*, *Argas vulgaris,* and *Argas polonicus* (2–5). All but the latter three species have been reported in Spain (Southwestern Europe) as parasites of birds or bats (6). The majority of *Argas* spp. are nidicolous and birds are exclusive vertebrate hosts for several species, mainly those of *Persicargas* subgenera, while humans are accidental hosts (7, 8). The genus *Argas* includes species responsible for the transmission of pathogens of medical and veterinary interest. Apart from conditions caused directly by soft ticks, such as toxicosis and anaphylaxis (9, 10), these ticks carry microorganisms that could be agents of infectious diseases. Specifically, *Argas* species can vector bacterial pathogens such as *Borrelia anserina* and *Aegyptianella* spp. and viruses such as Issyk-kul virus (11, 12). Other microorganisms with unproved pathogenicity have been detected in *Argas* ticks: bacteria from genera *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, *Rickettsia,* and *Rickettsiella*, viruses belonging to Flaviviridae, Orthomyxoviridae, Orthonairoviridae, Phenuiviridae, and Reoviridae families, and protozoans such as *Babesia* and *Hemolivia* spp. (7, 13–17).

The lack of information on the natural history and distribution of various *Argas* species, their incorrect or incomplete taxonomic description, and the fact that some species share morphological features but have not been molecularly examined, are responsible for the poor knowledge of *Argas* ticks in Spain. Moreover, their role in the epidemiology of tick-borne microorganisms has not been studied in this country. Here, we aim at describing soft ticks from natural and artificial nests occupied by different cavity-nesting birds in Spain and the prevalence of selected tick-borne microorganisms.

MATERIALS AND METHODS

Study Area and Study System

The main study area (∼50 km^2) lies in the Desert of Tabernas (Almería province, SE Spain, 37.08°N, 2.35°W). The landscape mostly consists of open shrubland with olive and almond groves interspersed among numerous dry riverbeds—ramblas. The climate in this area is semiarid Mediterranean with a marked water deficit during long, hot summer months. The mean annual rainfall is ∼ 230 mm, with high inter- and intra-annual variability (18). Tick samples also were collected in Segovia and Guadalajara provinces (both in the interior of the Iberian Peninsula), whose climate is Mediterranean with some continental characteristics.

In the main study area in Almería, natural cavities in sandstone cliffs, seminatural cavities in stone bridges and abandoned farmhouses and nest boxes provide nest sites for cavity-nesting birds, namely the European roller (*Coracias garrulus*, hereafter roller), the little owl (*Athene noctua*) and the rock/feral pigeon (*Columba livia*, hereafter pigeon). In this study, we sampled ticks in cavities occupied by rollers and little owls. The roller is a migratory bird that arrives at its breeding grounds in the study area during the second fortnight of April whereas the little owl is a resident bird. Both species rear a single brood per year (19). In contrast to these species, the pigeon is a resident bird that breeds at any time of the year in our study area and does not use nest boxes. Other species breeding in natural and seminatural cavities mainly include jackdaws (*Corvus monedula*), and common kestrels (*Falco tinnunculus*), whereas Scops owls (*Otus scops*), spotless starlings (*Sturnus unicolor*), and house sparrows (*Passer domesticus*) can breed in nest boxes.

Given nest-site limitation in the study area, both intra- and interspecific competition for suitable nesting holes occur and individual cavities can be re-used by different species both within and between years. This is frequently the case in Almería, so that many samples were collected from natural and seminatural cavities of rollers and little owls previously used by pigeons. The samples from Segovia were collected from a natural tree hole occupied by rollers but excavated by the Iberian green woodpecker (*Picus sharpei*), whereas the samples from Guadalajara were taken from rollers breeding in nest boxes.

Tick Collection and Preservation

In the framework of a long-term project of cavity-nesting birds in the Desert of Tabernas, cavities and nest boxes have been routinely inspected during each breeding season since 2005 and both nestlings and nest material periodically examined for ectoparasites. Ticks were collected from cavities occupied by breeding rollers and little owls during 2009, 2012, 2015, and 2018–2020. Additionally, four tick individuals were obtained from roller nests in Central Spain (Guadalajara and Segovia) in 2004 (**Table 1**). Ticks collected until 2018 were preserved in ethanol, while ticks obtained in 2019 and 2020 were kept fresh upon delivery to the Centre of Rickettsiosis and Arthropod-borne Diseases (CRETAV). Before frozen at −80°C until later analysis, fresh ticks were identified and a single leg of each specimen was dissected.

Tick Identification

The taxonomic identification of the ticks was carried out using morphological keys (20–23). Tick individuals were surface-sterilized and DNA was individually extracted from a single leg of each tick specimen using incubations with ammonium hydroxide (24). The obtained DNA templates were used for genetic characterization by the amplification and sequencing of the 16S rRNA fragment gene (25). Two other mitochondrial genes, 12S rRNA, and cytochrome oxidase subunit I (COI), were also used in analyses (**Supplementary Table 1**).

Microbial Screening

Ticks were pooled (from 1 to 7 specimens; whole larvae and body halves for the other life stages) according to tick species or genotype, origin and date of collection and, when possible, tick developmental stage. DNA extracts from pools of ticks preserved in ethanol were obtained using the Qiagen DNA DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. Ticks of each

TABLE 1 | Tick samples used in this study.

Province	Municipal boundary	Nest Coordinates	Date of collection	Host	Preservation method	Developmental stage/Gender	No. of specimens (No. of pools)	Tick species
Almería	Tabernas	Diego tronco 37°3'58.43"N;2°21'19.38"W	17/06/2018	*Coracias garrulus*	Ethanol	Larvae	2 (1)	*Argas* sp. EEZA-CRETAV3
		Redondo Paloma 37° 3'58.39"N;2°21'19.48"W	27/06/2020	*C. garrulus*	Fresh-Frozen	Larvae	7 (1)	*Argas* sp. EEZA-CRETAV3
			28/06/2020	*C. garrulus*	Fresh-Frozen	Nymph	1 (1)	*Argas reflexus*
					Fresh-Frozen	Larvae	10 (2)	*Argas* sp. EEZA-CRETAV3
		RG 2M 37°4'14.86"N;2°20'27.65"W	17/06/2009	*C. garrulus*	Ethanol	Nymphs	2 (1)	*Argas* sp. EEZA-CRETAV3
		RH Grieta 37°3'53.50"N,2°20'48.89"W	2012	*C. garrulus*	Ethanol	Male	1 (1)	*Argas* sp. EEZA-CRETAV2
		RH SV 37°3'55.46"N;2°20'34.25"W	31/05/2012	*C. garrulus*	Ethanol	Nymph	1 (1)	*Argas* sp. EEZA-CRETAV1
		Tapadera alberca 37°3'54.53"N;2°21'30.43"W	01/06/2018	*Athene noctua*	Ethanol	Nymphs	7 (2)	*A. reflexus*
		Tapadera cueva 37° 3'56.71"N;2°21'24.29"W	08/06/2015	*C. garrulus*	Ethanol	Nymph	1 (1)	*Argas* sp. EEZA-CRETAV3
			09/05/2018	*A. noctua*	Ethanol	Adults or nymphs (last stage)	3 (1)	*Argas* sp. EEZA-CRETAV1
						Nymphs	6 (2)	*Argas* sp. EEZA-CRETAV1
						Nymphs	3 (1)	*Argas* sp. EEZA-CRETAV2
			08/06/2018	*A. noctua*	Ethanol	Adult	1 (1)	*Argas* sp. EEZA-CRETAV1
						Nymphs	3 (1)	*Argas* sp. EEZA-CRETAV1
			23/05/2019	*A. noctua*	Fresh-Frozen	Nymphs	12 (2)	*Argas* sp. EEZA-CRETAV1
						Nymphs	4 (1)	*Argas* sp. EEZA-CRETAV2
			30/05/2019	*A. noctua*	Fresh-Frozen	Nymph	1 (1)[a]	Argas sp. EEZA-CRETAV1
						Nymphs	2 (1)[b]	*Argas* sp. EEZA-CRETAV2
			10/06/2019	*A. noctua*	Fresh-Frozen	Male	1 (1)[a]	*Argas* sp. EEZA-CRETAV1
						Nymph	1 (1)[b]	*Argas* sp. EEZA-CRETAV2
			17/06/2019	*A. noctua*	Fresh-Frozen	Larva	1 (1)[a]	*Argas* sp. EEZA-CRETAV1
						Nymphs	2 (1)[a]	*Argas* sp. EEZA-CRETAV1
			13/05/2020	*A. noctua*	Fresh-Frozen	Nymphs	5 (1)	*Argas* sp. EEZA-CRETAV1
						Male	1	*Argas* sp. *
						Females	2	*Argas* sp. *
			27/05/2020	*A. noctua*	Fresh-Frozen	Nymphs	7 (1)	*Argas* sp. EEZA-CRETAV1
						Nymph	1 (1)[c]	*Argas* sp. EEZA-CRETAV2
			11/06/2020	*A. noctua*	Fresh-Frozen	Adult	1 (1)[c]	*Argas* sp. EEZA-CRETAV2
						Nymphs	5 (1)[c]	*Argas* sp. EEZA-CRETAV2
						Adult	1 (1)[d]	*Argas* sp. EEZA-CRETAV1
						Nymphs	12 (2)[d]	*Argas* sp. EEZA-CRETAV1

(Continued)

TABLE 1 | Continued

Province	Municipal boundary	Nest Coordinates	Date of collection	Host	Preservation method	Developmental stage/Gender	No. of specimens (No. of pools)	Tick species
			15/06/2020	*A. noctua*	Fresh-Frozen	Larva	1	*Argas* sp. *
						Female	1	*Argas* sp. *
						Nymphs	12 (2)	*Argas* sp. EEZA-CRETAV2
						Nymphs	25 (5)	*Argas* sp. EEZA-CRETAV1
						Nymphs	8	*Argas* sp.*
	Tahal	Tahal cantera 37°8'7.13"N;2°14'22.39"W	18/06/2018	*C. garrulus*	Ethanol	Nymphs	1 (1)	*Argas* sp. EEZA-CRETAV2
	Ulelia del Campo	Aguador NB cjo 37° 9'52.69"N;2°12'13.49"W	14/06/2015	*C. garrulus*	Ethanol	Males	2 (2)	*Argas* sp. EEZA-CRETAV1
						Nymph	1 (1)	*Argas* sp. EEZA-CRETAV2
						Female	1 (1)	*Argas* sp. EEZA-CRETAV2
Guadalajara	Illana	Chopera Illana 40°12'20.37"N;2°58'39.92"W	05/07/2004	*C. garrulus*	Ethanol	Males	2 (2)	*Argas* sp. EEZA-CRETAV5
Segovia	Pinarejos	Pinarejos 41°14'53.78"N;4°18'31.67"W	15/07/2004	*C. garrulus*	Ethanol	larvae	2 (2)	*Argas* sp. EEZA-CRETAV4

*not processed; [a] [b] [c] [d]; same pool.

Data about sample origin (province, municipality, coordinates of the nest where individuals were captured), collection date, avian host, method of tick preservation, developmental stage, and gender (when possible), No. of ticks and No. of pools formed, and tick taxon name are given.

pool that were preserved frozen were homogenized in 600 μL of culture medium with antibiotics [Dulbecco's Modified Eagle Medium (DMEM) with 100 units/mL penicillin and 100 μg/mL streptomycin, Sigma]. Four hundred μL of the homogenate were used for nucleic acid extraction (DNA and RNA) and the remaining 200 μL were preserved at −80°C for future analysis. The DNA and RNA were extracted using the DNeasy blood and tissue kit and RNeasy Mini kit (Qiagen, Hilden, Germany), respectively, following the manufacturer's recommendations.

The quality of nucleic acid extraction was checked using the 16S rRNA PCR assay (25). Positive samples were subjected to microbial screening using specific PCR assays for the analysis of (i) bacteria: Anaplasmataceae family, *Bartonella* spp., *Borrelia* spp., *Coxiella* spp., *Rickettsiella* spp., and spotted fever group (SFG) *Rickettsia* spp., (ii) viruses: Flaviviridae, Orthonairoviridae and Phenuiviridae families, and (iii) protozoans: *Babesia* and *Theileria* spp. Negative and positive controls (DNA or cDNA extracts of *Anaplasma phagocytophilum*, *Bartonella henselae*, *Borrelia spielmanii, Borrelia miyamotoi*, *Coxiella*-like of *Rhipicephalus bursa*, *Rickettsia amblyommatis*, Crimean-Congo haemorrhagic fever virus, Japanese encephalitis virus, Uukuniemi uukuvirus virus, and *Babesia* sp. from *Rhipicephalus microplus*) were included in all the PCR assays performed. Primers and PCR conditions are described in the **Supplementary Table 1**. The SFG *Rickettsia* was screened in tick legs for all ticks. In addition, pools formed by specimens that gave negative results were also tested for *Rickettsia* spp. Moreover, all the pools were screened for the presence of the remaining bacteria and protozoans. Lastly, the viral screening was performed on tick pools comprising specimens of fresh/frozen ticks (**Table 1**).

Prevalence of Infection

The prevalence of infection (PI) was estimated by:

PI = (No. of positive ticks/total No. of ticks analyzed) × 100%. When microorganisms were amplified from pools of more than one tick, prevalence was calculated assuming that each positive pool contained only one positive tick. This estimate, known as minimum infectious rate (MIR), is calculated as follow:

MIR = (No. of positive pools/total No. of individual ticks analyzed) × 100%.

Analysis of Nucleotide Sequences

Nucleotide sequences were analyzed using the BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the resulting sequences were submitted to GeneBank. The Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for multiple sequence alignment. Phylogenetic analyses were conducted with MEGA X (http://www.megasoftware.net) using the maximum likelihood method including all sites. The nucleotide substitution model was selected according to the Akaike information criterion implemented in MEGAX. Confidence values for individual branches of the resulting trees were determined by bootstrap analysis with 500 replicates.

TABLE 2 | Highest similarities of the *Argas* genotypes detected in this study reached with public sequences from GenBank.

	Fragment gene; GenBank accession No.	Identity (%)	Tick species (GenBank accession No.)
Argas reflexus	16S rRNA; MW289075[a]	100	*A. reflexus* (L34322)
	12S rRNA; MW289084	96.6	*A. reflexus* (U95865)
	COI; MW288388[b]	81.5	*A. walkerae* (KJ133584)[h]
Argas sp. EEZA-CRETAV1	16S rRNA; MW289069	90.0	*A. vulgaris* (AF001404)
	12S rRNA; MW289077	94.1	*A. lagenoplastis* (KC769587)[i]
	COI; MW288380[c]	88.0	*A. lagenoplastis* (KC769587)[i]
Argas sp. EEZA-CRETAV2	16S rRNA; MW289070[d]	98.8	*A. polonicus* (AF001403)
	12S rRNA; MW289078[e]	93.8	*A. lagenoplastis* (KC769587)[i]
	COI; MW288382[f]	88.3	*A. lagenoplastis* (KC769587)[i]
Argas sp. EEZA-CRETAV3	16S rRNA; MW289072	92.1	*A. africolumbae* (JQ665720)
	12S rRNA; MW289081[g]	92.1	*A. africolumbae* (KJ133580)
	COI; MW288385	85.3	*A. africolumbae* (KJ133580)
Argas sp. EEZA-CRETAV4	16S rRNA; MW289073	96.5	*A. persicus* (MT012684)
	12S rRNA; MW289083	95.3	*A. persicus* (MT012684)
	COI; MW288386	90.1	*A. persicus* (KJ133581)
Argas sp. EEZA-CRETAV5	16S rRNA; MW289074	97.3	*A. persicus* (MT012684)
	12S rRNA; Not obtained		
	COI; MW288387	90.7	*A. persicus* (MT012684)

[a] *One more sequence with a single nucleotide substitution was obtained (MW289076).*
[b] *One more sequence with two nucleotide substitutions was obtained (MW288389).*
[c] *One more sequence with four nucleotide substitutions was obtained (MW288381).*
[d] *One more sequence with a single nucleotide substitution was obtained (MW289071).*
[e] *Two more sequences with one and three nucleotide substitutions were obtained (MW289079; MW289080).*
[f] *Two more sequences with 20 and 16 nucleotide substitutions were obtained (MW288383; MW288384).*
[g] *One more sequence with two nucleotide substitutions was obtained (MW289082).*
[h] *There are not public sequences for A. reflexus.*
[i] *There are not public sequences for A. vulgaris and A. polonicus.*

RESULTS

Tick Identification

One hundred and sixty-three ticks, mainly nymphs, were collected from bird nests in Almería ($n = 159$), Guadalajara ($n = 2$), and Segovia ($n = 2$). Arthropods were obtained from nest material in cavities occupied by little owl ($n = 129$) and roller ($n = 34$) or, in few cases, from nestlings of these species (**Table 1**). All the specimens were morphologically identified as *Argas* spp. and 150 specimens were further studied molecularly. Examination of morphological characters enabled the identification of eight nymphs as *A. reflexus*, but the morphological identification of the remaining ticks could not be accurately performed with available keys. The *A. reflexus* identification was corroborated molecularly based on 16S rRNA fragment gene (**Table 2**). The molecular identification was not conclusive for the remaining 142 tick samples, which were grouped based on the 16S rRNA results into five different genotypes, designated as *Argas* spp. EEZA-CRETAV1–5 (**Tables 1**, **2**). Based on 16S rRNA gene analyses, *Argas* sp. EEZA-CRETAV1 ($n = 83$) and *Argas* sp. EEZA-CRETAV2 ($n = 33$) were closest to *A. vulgaris* and *A. polonicus*, respectively (**Table 2**). In turn, the *Argas* sp. EEZA-CRETAV3 genotype ($n = 22$) shared the highest identity (<92.2%) with *Argas africolumbae*. Lastly, *Argas* sp. EEZA-CRETAV4 ($n = 2$) and *Argas* sp. EEZA-CRETAV5 genotypes ($n = 2$) shared highest identities with *A. persicus* (**Table 2**). The phylogeny inferred from 16S rRNA analysis corroborates the BLAST results (**Figure 1**). Phylogenetic analyses based on 12S rRNA and COI fragment genes could not be performed because of the lack of homologue sequences for the majority of *Argas* spp.

Argas spp. EEZA-CRETAV1–3 specimens were collected in Almería, whereas those of *Argas* spp. EEZA-CRETAV4–5 were obtained in Segovia and Guadalajara. Also, some of the ticks of *Argas* spp. EEZA-CRETAV1–2 genotypes were collected from the same nests (**Table 1**).

Bacterial Screening

All the DNA extracts (individual samples and pools) gave positive results for the tick-16S rRNA PCR assay and, consequently, were screened for bacteria (**Table 3**). Amplicons for *ompA* gene were obtained from 112 DNA extracts from tick legs (PI = 74.7%). Specifically, *Rickettsia* was amplified from 81 and 31 samples belonging to *Argas* sp. EEZA-CRETAV1 (PI = 97.6%) and *Argas* sp. EEZA-CRETAV2 (PI = 93.9%) samples, respectively. DNA extracts corresponding to individual tick-leg samples from *A. reflexus* and *Argas* sp. EEZA-CRETAV3–5 specimens were negative, but body-halve extracts of these specimens were further analyzed using pooled samples. The nucleotide extracts from these pools were negative for all *A.*

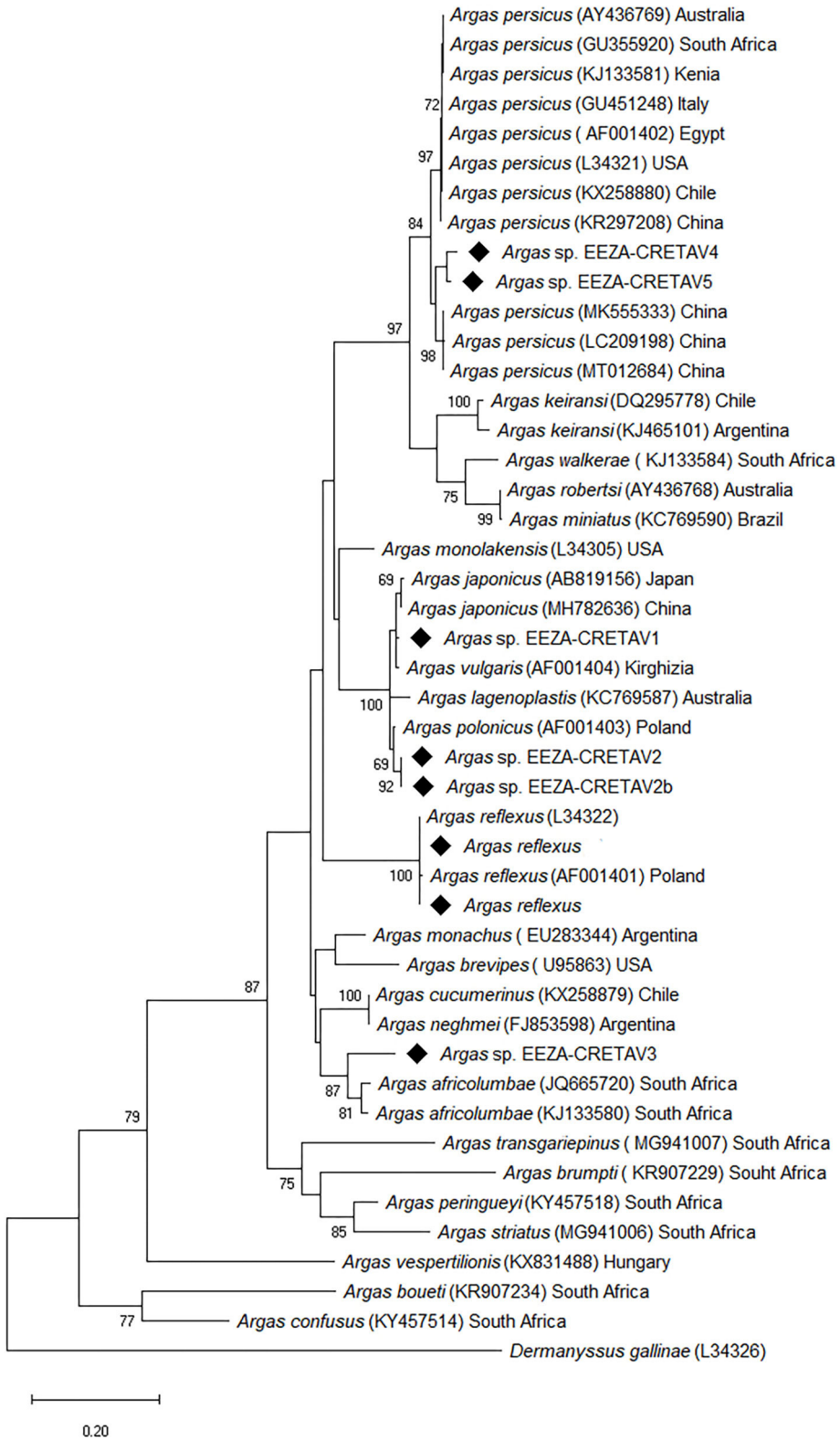

FIGURE 1 | Phylogenetic tree based on 16S rRNA analysis showing the relationships between tick species and genotypes identified in this study and published validated *Argas* species. The evolutionary analysis was inferred using the maximum likelihood method and general time reversible + G model with Mega X. The

(Continued)

FIGURE 1 | analysis involved 46 nucleotide sequences and a total of 441 positions in the final dataset. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers (>65%) shown at the nodes correspond to bootstrapped percentages (for 500 repetitions). The GenBank accession number of sequences used in the analysis is shown in brackets after *Argas* taxon name and before sample origin. Sequences obtained in this study are marked with diamond. *Dermanysus gallinae* is used as outgroup.

reflexus specimens, but yielded positive results for two out of six pools of *Argas* sp. EEZA-CRETAV3 specimens (MIR = 9.1%) (comprising a nymph collected from a roller nest in Almería in 2015 and larvae that were attached to a roller nestling in Almería in 2020), and all the pools of *Argas* sp. EEZA-CRETAV4–5 specimens (MIR = 100% for the two pools) (**Table 3**).

All the *ompA* gene sequences obtained were identical and showed the highest identity with *Rickettsia fournieri* (**Table 4**). Selected *Rickettsia*-positive samples were further genetically characterized by the amplification of five more rickettsial fragment genes (26). Nucleotide sequences for the respective genes were identical and showed highest identities with *R. fournieri* and *Candidatus* Rickettsia vini (**Table 4**). The phylogenetic tree based on the concatenated fragment genes of the *Rickettsia* strain detected, designated as *Rickettsia* sp. EEZA-CRETAV, corroborated the close relation with both *R. fournieri* and *Ca*. R. vini (**Figure 2**).

A total of 27 nucleotide sequences were obtained using the *rpoB* PCR assay selected for the *Coxiella*/*Rickettsiella* detection but highest identities with validated bacterial species reached < 85% for 14 samples. The genetic analysis of the amplicon obtained from an *Argas* sp. EEZA-CRETAV1 nymph, collected in Almería in 2012, showed the highest identity with *Francisella persicus* (PI = 0.7% and PI = 1.2% for *Argas* sp. EEZA-CRETAV1) (**Tables 3**, **5**). The novel *Francisella* strain molecularly described in this study was designated as *Francisella* sp. EEZA-CRETAV. *Coxiella*-like strains were successfully amplified from 10 pools (MIR = 6.7%). Two out of three *A. reflexus* pools (with ticks collected in a little owl nest in Almería in 2018) showed infection with a *Coxiella* strain previously detected in this tick species (MIR = 25%) (**Table 5**). A new strain of *Coxiella* spp., designated as *Coxiella* sp. EEZA-CRETAV1, was amplified in all the pools of *Argas* sp. EEZA-CRETAV3 specimens ($n = 6$, MIR = 27.3%). One more *Coxiella* strain, *Coxiella* sp. EEZA-CRETAV2, was detected in two pools of different tick genotypes, an *Argas* sp. EEZA-CRETAV2 (MIR = 3%) female collected in Almería in 2015 and an *Argas* sp. EEZA-CRETAV4 (MIR = 50%) larva collected in Segovia in 2004 (**Table 3**). Nucleotide sequences corresponding to *rpoB* an *groEL* genes of these two novel strains shared 95.3 and 97% identity, respectively, and reached highest identities with *Coxiella* strains detected in *Ornithodoros* ticks (**Table 5**). Moreover, pools integrated over a single specimen were also submitted for *groEL* analysis and a nymph belonging to *Argas* sp. EEZA-CRETAV2, collected in Almería in 2018, showed infection with *Rickettsiella* sp. (PI = 0.7% and PI for *Argas* sp. EEZA-CRETAV2 = 3%; **Table 3**). The corresponding amplicon, designated as *Rickettsiella* EEZA-CRETAV, showed highest identities with *Rickettsiella* species amplified from *Ornithodoros normandi* (**Table 5**).

All the tick pools were examined by PCR assays for the presence of Anaplasmataceae, *Bartonella,* and *Borrelia* species and all gave negative results.

Viral Screening

Twenty-one pools originated from fresh/frozen ticks [$n = 111$ ticks: 1 tick (1 pool) of *A. reflexus*, 67 (12) of *Argas* sp. EEZA-CRETAV1, 26 (5) of *Argas* sp. EEZA-CRETAV2 and 17 (3) of *Argas* sp. EEZA-CRETAV3 specimens; **Table 1**] were analyzed for the presence of viruses belonging to families Flaviviridae, Orthonairoviridae, and Phenuiviridae. No sequences were amplified using the selected PCR assays.

Protozoan Screening

Tick DNA from 43 pools was analyzed using a PCR assay that amplifies 18S rRNA gene of *Babesia* and *Theileria* spp. (**Table 3**, **Supplementary Table 1**). *Babesia* sp. was detected from a male *Argas* sp. EEZA-CRETAV1 collected in Almería in 2015 (MIR = 0.7%, MIR = 1.2% for *Argas* sp. EEZA-CRETAV1) (**Table 3**). Three more ticks (one *Argas* sp. EEZA-CRETAV1 and two *Argas* sp. EEZA-CRETAV2 ticks) collected simultaneously from the same nest gave negative results. Based on the analysis of 18S rRNA gene, the closets *Babesia* sp. from the detected strain, designated as *Babesia* sp. EEZA-CRETAV, was *Babesia ardeae* (KY436057; 95.8% identity) (**Figure 3**). Two more genes, ITS1 and ITS2, were also examined, but currently there are no *B. ardeae* sequences available for these markers. The analysis of these genes showed highest (<82%) identities with *Babesia poelea* (accession no. DQ200887). A pool formed by 4 *Argas* sp. EEZA-CRETAV3 larvae, collected in Almería in 2020, showed presence of a coccidian parasite *Adelina bambarooniae* (AF494059) (MIR = 0.7%) (**Table 3**).

Sequences Submission to a Public Database

The nucleotide sequences of ticks and microorganisms detected in this study ($n = 42$) were deposited in the GenBank database under accession numbers showed in **Table 6**.

DISCUSSION

Soft ticks are important vectors of microbial agents of animal and human diseases. Despite this, the knowledge of argasid tick species and their associated microorganisms is generally scarce. Herein, the occurrence of five novel *Argas* spp. genotypes, in addition to *A. reflexus*, collected in the Iberian Peninsula (Spain) in nests occupied by little owls and European rollers is reported. Moreover, we detected the presence of tick-borne microorganisms belonging to genera *Rickettsia*, *Coxiella*, *Francisella*, *Rickettsiella,* and *Babesia* in *Argas* ticks. In contrast,

TABLE 3 | Microorganisms detected in this study.

Tick species	Developmental stage/Gender	No. of specimens (No. of pools)	Host	Origin	Date of collection	*Rickettsia*		*Coxiella*	*Rickettsiella*	*Francisella*	*Babesia*	*Adelina*
						No. specimens (PI%)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)
Argas reflexus	Nymphs	7 (2)	*Athene noctua*	Tabernas (Almería)	01/06/2018	0 (0)	0 (0)	**2 (28.6)**	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)	*Coracias garrulus*	Tabernas (Almería)	28/06/2020	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV1	Nymph	1 (1)	*C. garrulus*	Tabernas (Almería)	31/05/2012	**1 (100)**	NP	0 (0)	0 (0)	**1 (100)**	0 (0)	0 (0)
	Males	2 (2)	*C. garrulus*	Uleila del Campo (Almería)	14/06/2015	**2 (100)**	NP	0 (0)	0 (0)	0 (0)	**1 (50)**	0 (0)
	Adults or nymphs (last stage)	3 (1)	*A. noctua*	Tabernas (Almería)	09/05/2018	**3 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	6 (2)	*A. noctua*	Tabernas (Almería)	09/05/2018	**6 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Adult	1 (1)	*A. noctua*	Tabernas (Almería)	08/06/2018	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	3 (1)	*A. noctua*	Tabernas (Almería)	08/06/2018	**3 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	12 (2)	*A. noctua*	Tabernas (Almería)	23/05/2019	**12 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)[a]	*A. noctua*	Tabernas (Almería)	30/05/2019	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Male	1 (1)[a]	*A. noctua*	Tabernas (Almería)	10/06/2019	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Larva	1 (1)[a]	*A. noctua*	Tabernas (Almería)	17/06/2019	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	2 (1)[a]	*A. noctua*	Tabernas (Almería)	17/06/2019	**1 (50)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	5 (1)	*A. noctua*	Tabernas (Almería)	13/05/2020	**5 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	7 (1)	*A. noctua*	Tabernas (Almería)	27/05/2020	**7 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Adult	1 (1)[d]	*A. noctua*	Tabernas (Almería)	11/06/2020	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	12 (2)[d]	*A. noctua*	Tabernas (Almería)	11/06/2020	**11 (91.6)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	25 (5)	*A. noctua*	Tabernas (Almería)	15/06/2020	**25 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

(Continued)

TABLE 3 | Continued

Tick species	Developmental stage/Gender	No. of specimens (No. of pools)	Host	Origin	Date of collection	*Rickettsia*		*Coxiella*	*Rickettsiella*	*Francisella*	*Babesia*	*Adelina*
						No. specimens (PI%)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)
Argas sp. EEZA-CRETAV2	Male	1 (1)	*C. garrulus*	Tabernas (Almería)	2012	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)	*C. garrulus*	Uleila del Campo (Almería)	14/06/2015	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Female	1 (1)	*C. garrulus*	Uleila del Campo (Almería)	14/06/2015	**1 (100)**	NP	**1 (100)**	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	3 (1)	*A. noctua*	Tabernas (Almería)	09/05/2018	**3 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	1 (1)	*C. garrulus*	Tahal (Almería)	18/06/2018	**1 (100)**	NP	0 (0)	**1 (100)**	0 (0)	0 (0)	0 (0)
	Nymphs	4 (1)	*A. noctua*	Tabernas (Almería)	23/05/2019	**4 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	2 (1)[b]	*A. noctua*	Tabernas (Almería)	30/05/2019	**2 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)[b]	*A. noctua*	Tabernas (Almería)	10/06/2019	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)[c]	*A. noctua*	Tabernas (Almería)	27/05/2020	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Adult	1 (1)[c]	*A. noctua*	Tabernas (Almería)	11/06/2020	**1 (100)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	5 (1)[c]	*A. noctua*	Tabernas (Almería)	11/06/2020	**4 (80)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Nymphs	12 (2)	*A. noctua*	Tabernas (Almería)	15/06/2020	**11 (91.6)**	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV3	Nymphs	2 (1)	*C. garrulus*	Tabernas (Almería)	17/06/2009	0 (0)	0 (0)	**1 (50)**	0 (0)	0 (0)	0 (0)	0 (0)
	Nymph	1 (1)	*C. garrulus*	Tabernas (Almería)	08/06/2015	0 (0)	**1 (100)**	**1 (100)**	0 (0)	0 (0)	0 (0)	0 (0)
	Larvae	2 (1)	*C. garrulus*	Tabernas (Almería)	17/06/2018	0 (0)	0 (0)	**1 (50)**	0 (0)	0 (0)	0 (0)	0 (0)
	Larvae	7 (1)	*C. garrulus*	Tabernas (Almería)	27/06/2020	0 (0)	0 (0)	**1 (14.3)**	0 (0)	0 (0)	0 (0)	0 (0)
	Larvae	10 (2)	*C. garrulus*	Tabernas (Almería)	28/06/2020	0 (0)	**1 (10)**	**2 (20)**	0 (0)	0 (0)	0 (0)	**1 (10)**

(Continued)

TABLE 3 | Continued

Tick species	Developmental stage/Gender	No. of specimens (No. of pools)	Host	Origin	Date of collection	*Rickettsia*		*Coxiella*	*Rickettsiella*	*Francisella*	*Babesia*	*Adelina*
						No. specimens (PI%)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)	No. of pools (MIR %)
Argas sp. EEZA-CRETAV4	larvae	2 (2)	*C. garrulus*	Pinarejos (Segovia)	15/07/2004	0 (0)	**2 (100)**	**1 (50)**	0 (0)	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV5	Males	2 (2)	*C. garrulus*	Illana (Guadalajara)	05/07/2004	0 (0)	**2 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total						**112 (74.7)**	–	**10 (6.7)**	**1 (0.7)**	**1 (0.7)**	**1 (0.7)**	**1 (0.7)**
A. reflexus		8 (3)				0 (0)	0 (0)	**2 (25)**	0 (0)	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV1		83 (20)				**81 (97.6)**	NP	0 (0)	0 (0)	**1 (1.2)**	**1 (1.2)**	0 (0)
Argas sp. EEZA-CRETAV2		33 (10)				**31 (93.9)**	NP	1 (3)	**1 (3)**	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV3		22 (6)				0 (0)	**2 (9.1)**	**6 (27.3)**	0 (0)	0 (0)	0 (0)	**1 (4.5)**
Argas sp. EEZA-CRETAV4		2 (2)				0 (0)	**2 (100)**	**1 (50)**	0 (0)	0 (0)	0 (0)	0 (0)
Argas sp. EEZA-CRETAV5		2 (2)				0 (0)	**2 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

PI, Prevalence of infection = (No. of positive ticks/total No. of individual ticks); MIR, Minimun infectious rate = (No. of positive pools/total No. of individual ticks) × 100; NP, not processed; [a,b,c,d]*: same pool. Positive results are shown in bold.*

TABLE 4 | Identities between fragment genes of *Rickettsia* sp. EEZA-CRETAV detected in the present study and published sequences from *Rickettsia fournieri* and *Candidatus* Rickettsia vini.

	Identity (%) GenBank accession No.				
	ompA	***ompB***	***gltA***	**16S rRNA**	**17-KDa**
Rickettisia fournieri	99.8 KF666477	99.7 KF666469	99.8 KF666473	99.7 KF666471	99.8 OFAL01000006
Candidatus Rickettsia vini	99.5 JF758828	99.6 MT062906	99.1 JF758829	99.8 JF803266	99.8 JF758827

Anaplasmataceae, *Bartonella,* and *Borrelia* bacterium species and viruses belonging to the families Flaviviridae, Orthonairoviridae, and Phenuiviridae have not been detected.

Tick Identification

Of the six *Argas* genotypes detected in this study, only one could be identified to the species level, namely, *A. reflexus*. The morphological identification of ticks is challenging, even for experts (27), while the molecular approach appears to be an accurate tool for tick identification (25, 27). Nevertheless, increased effort for molecular characterization of more *Argas* species is needed for a reliable taxonomic inference based on molecular tools. In order to confirm if the genotypes identified in this study represent validated or potentially novel tick species, a further morphological analysis including unfed larva specimens, should be performed along with rigorous molecular analyses of *Argas* ticks from diverse geographical locations.

Argas reflexus specimens, known as the pigeon tick, have been collected in two bird nests in Almería (Southern Spain), one occupied by little owl ($n = 7$) and one by roller ($n = 1$). This species occurs in Spain, and it is a well-known ectoparasite of little owls, whereas information regarding roller infestations is scarce (6, 20, 28). *Argas* sp. EEZA-CRETAV4 and *Argas* sp. EEZA-CRETAV5, genotypes amplified in Central Spain, clustered molecularly with *A. persicus* from China (29), but in a different branch than other *A. persicus* specimens (**Figure 1**). Nevertheless, the broad genetic divergence of this group (29, 30), also revealed in the phylogeny inferred herein (**Figure 1**), suggests that cryptic species could occur in this taxon known as fowl tick. This worldwide distributed tick has been previously reported in Spain and is known to infest wild birds also in other countries (6, 28). In addition, ticks of three more *Argas* genotypes have been identified, all of them in Almería. The *Argas* sp. EEZA-CRETAV1–2 genotypes clustered together and appear to be closely related with *A. vulgaris* and *A. polonicus*, respectively (**Figure 1**). Neither of these two tick species have been previously reported from western Europe and their occurrence is only documented in a few eastern European countries (20, 21, 31). These genotypes have been found in the nest material of roller and little owls, occurring in the same nests in several cases (**Table 1**). In the study area, there is a high competition among cavity-nesting birds for suitable cavities and the same cavity can be successively used by little owls, pigeons and rollers (32). This fact could explain the infestation of both bird species by the same nidicolous tick taxa, i.e., *A. reflexus* and *Argas* sp. EEZA-CRETAV1–2. In contrast, *Argas* sp. EEZA-CRETAV3, closely related to *A. africolumbae*, has been found only in nests occupied by rollers. The roller is a long-distant migrant species (trans-Saharan migrant) and the Spanish populations overwinter in different African regions (33). It is worth mentioning that *A. africolumbae* is an ornithophilic tick that occurs in Africa: South Africa, Kenya, Tanzania and Burkina Faso (34, 35). It is well-known that birds can serve as dispersers of ticks and tick-borne microorganisms, even though this information pertains mainly to hard ticks (36, 37). Some studies suggest that the role of birds as dispersers of soft ticks is less important, due to the biology of these ticks (nidicolous behavior and shorter blood-feeding time), but the role of migratory birds as reservoirs or amplifiers of tick-borne microorganisms associated with soft ticks remains to be better investigated (38, 39).

Tick-Borne Microorganisms

The microbiological screening of ticks is important to identify the local risks of emergence of tick-borne diseases. *Argas* species, including *A. reflexus* and *A. persicus*, have been described as important pests and vectors of diseases in poultry and wild birds, being responsible for high economic losses (7, 11, 40). These tick species have also been recorded biting humans and causing anaphylaxis episodes (8, 10). Although humans are accidental hosts of *Argas* ticks and the ticks carry numerous microorganisms, human pathogens among them, the role of these ticks as vectors of human infectious agents has not been proven.

The most prevalent microorganisms amplified in this study belong to the *Rickettsia* genus (α-Proteobacteria; Rickettsiaceae). The high prevalence of *Rickettsia* spp. found in ticks of *Argas* sp. EEZA-CRETAV1–2 specimens suggests that the bacterium is a common endosymbiont of ticks of the two genotypes. The detection of the newly described *Rickettsia* strain in tick-body samples, but not in tick-leg samples of ticks of other genotypes (*Argas* sp. EEZA-CRETAV3–5), suggests that this *Rickettsia* species may not be a true intracellular endosymbiont and its presence in the former genotypes could be acquired through feeding on infected hosts or by cofeeding. The phylogenetic analysis of *Rickettsia* sp. EEZA-CRETAV reveals its close relation with *R. fournieri* and *Ca.* R. vini, both *Rickettsia* species associated with ornithophilic nidicolous ticks (41, 42). While *R. fournieri*

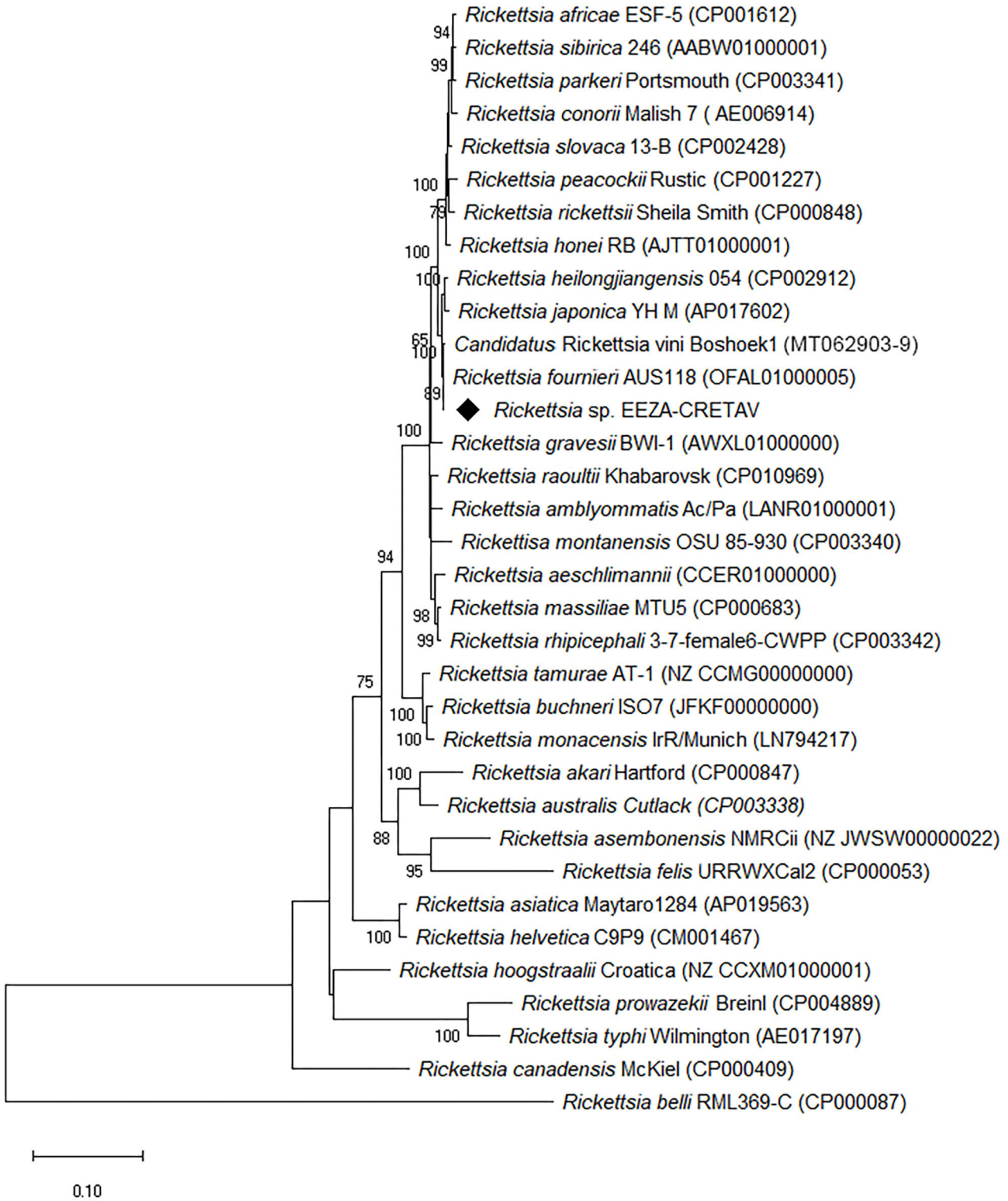

FIGURE 2 | Phylogenetic tree showing the relationships between *Rickettsia* sp. EEZA-CRETAV and published *Rickettsia* spp. taxa. The evolutionary analysis was inferred using the maximum likelihood method and general time reversible + G model with Mega X, by concatenating fragments of six genes (*sca4*, 16s rRNA, *ompB*, *ompA*, 17-kDa, and *gltA*). The analysis involved 34 nucleotide sequences and a total of 4,120 positions in the final dataset. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers (>65%) shown at the nodes correspond to bootstrapped percentages (for 500 repetitions). The GenBank accession number of the sequences used in the analysis is shown in brackets after *Rickettsia* taxon name and the corresponding strain. Sequences obtained in this study are marked with diamond.

TABLE 5 | Highest identities reached between fragment genes of *Francisella*, *Coxiella,* and *Rickettsiella* spp. detected in the present study and published sequences.

Bacteria	Gene (GenBank accession No.)	Identity (%) Species (GenBank accession No.)
Francisella sp. EEZA-CRETAV	*rpoB* MW287617	97.4 *Francisella persica* ATCC (CP013022, CP012505)
Coxiella sp. of *Argas reflexus*	*rpoB* MW287616	100 *Coxiella*-like endosymbiont of *Argas reflexus* (KY677983, KY677982)
Coxiella sp. EEZA-CRETAV1	*rpoB* MW287614	96.7 *Coxiella*-like endosymbiont of *Ornithodoros rostratus* (KP985288-91)
	groEL MW287611	97.0 Uncultured *Coxiella* sp. (KJ459055-6; detected in *Ornithodoros capensis*) *Coxiella*-like endosymbiont of *Ornithodoros peruvianus* (KP985466-7; KP985476-7)
Coxiella sp. EEZA-CRETAV2	*rpoB* MW287615	96.5 *Coxiella*-like endosymbiont of *Ornithodoros rostratus* (KP985288-91)
	groEL MW287612	97.4 *Coxiella*-like endosymbiont of *Ornithodoros amblus* (KP985447-8)
Rickettsiella sp. EEZA-CRETAV	*groEL* MW287613	99.7 *Rickettsiella* endosymbiont of *Ornithodoros normandi* (*KP985530*, KP985531)

has been described only once from *A. lagenoplastis* in Australia (41), *Ca.* R. vini has been detected in several European countries in *Ixodes* spp. (36, 42–45). The single study performed suggests that *Ca.* R. vini is not pathogenic (43). Nevertheless, the two *Rickettsia* taxa are closely related to other *Rickettsia* species that are recognized as human pathogens, specifically *Rickettsia japonica* and *Rickettsia heilongjiangensis* (46, 47). Thus, the epidemiology and pathological potential of *Rickettsia* strains such as *Rickettsia* sp. EEZA-CRETAV, in addition to *R. fournieri* and *Ca.* R. vini, should be further investigated. Likewise, the isolation of *Rickettsia* sp. EEZA-CRETAV is necessary to gain an insight into the epidemiological importance of this strain.

In addition to the *Rickettsia* taxon, this study has revealed for the first time different proteobacterial tick endosymbionts in *Argas* spp. from Spain, namely, *Coxiella*, *Rickettsiella* (Gamma-proteobacterium; Coxiellaceae) and *Francisella* (Gamma-proteobacterium; Francisellaceae) species. The detected species, commonly known as *Rickettsiella*-like, *Coxiella*-like, and *Francisella*-like, are intracellular obligatory endosymbionts important for tick survival. They play some role in B vitamins biosynthesis and their presence may interfere with the transmission (positively or negatively) of other microorganisms, including tick-borne pathogens (48). They are related to species responsible for important human diseases. For instance, *Coxiella burnetii* and *Francisella tularensis* cause Q fever and tularemia, respectively (49, 50). *Coxiella*-like species have been implicated in human and avian diseases (51, 52). Three different *Coxiella* strains have been successfully detected in this study. Of them, the *Coxiella* sp. from *A. reflexus* was homologous to the species previously detected in the same tick species, but the remaining two strains, designated as *Coxiella* sp. EEZA-CRETAV1–2, differ from the scarcely-known *Coxiella*-like strains of *Argas* species (14, 53–55). To date, *Rickettsiella* has been mainly reported in hard ticks (*Ixodes* spp. and *Haemaphysalis* spp.) and soft ticks belonging to the genus *Ornithodoros* (14). The presence of *Rickettsiella* in *Argas* ticks has been suggested for the bat tick *A. transgariepinus* from Namibia, but the available 16S rRNA sequences shared low identities with the published *Rickettsiella* sequences (17). In the present study, a *Rickettsiella* sp. similar to that of *O. normandi* from Tunisia (55) was detected in an *Argas* sp. EEZA-CRETAV2 specimen. Lastly, a strain closely related to *F. persicus* was found in an *Argas* sp. EEZA-CRETAV1 specimen. *Francisella persicus*, formerly *Wolbachia persica*, is an endosymbiont of *Argas arboreus* (previously referred to as *A. persicus*) (56). This bacterium has not been identified as an animal or human pathogen, but the analysis of its genome shows that this species conserves an important number of potentially functional virulence-associated genes, suggesting that it could be pathogenic to mammals (57).

Bacteria of Anaplasmataceae family, *Bartonella,* and *Borrelia* spp. have not been detected in the ticks analyzed. *Argas* spp. are recognized vectors of *B. anserine*, the agent of the avian

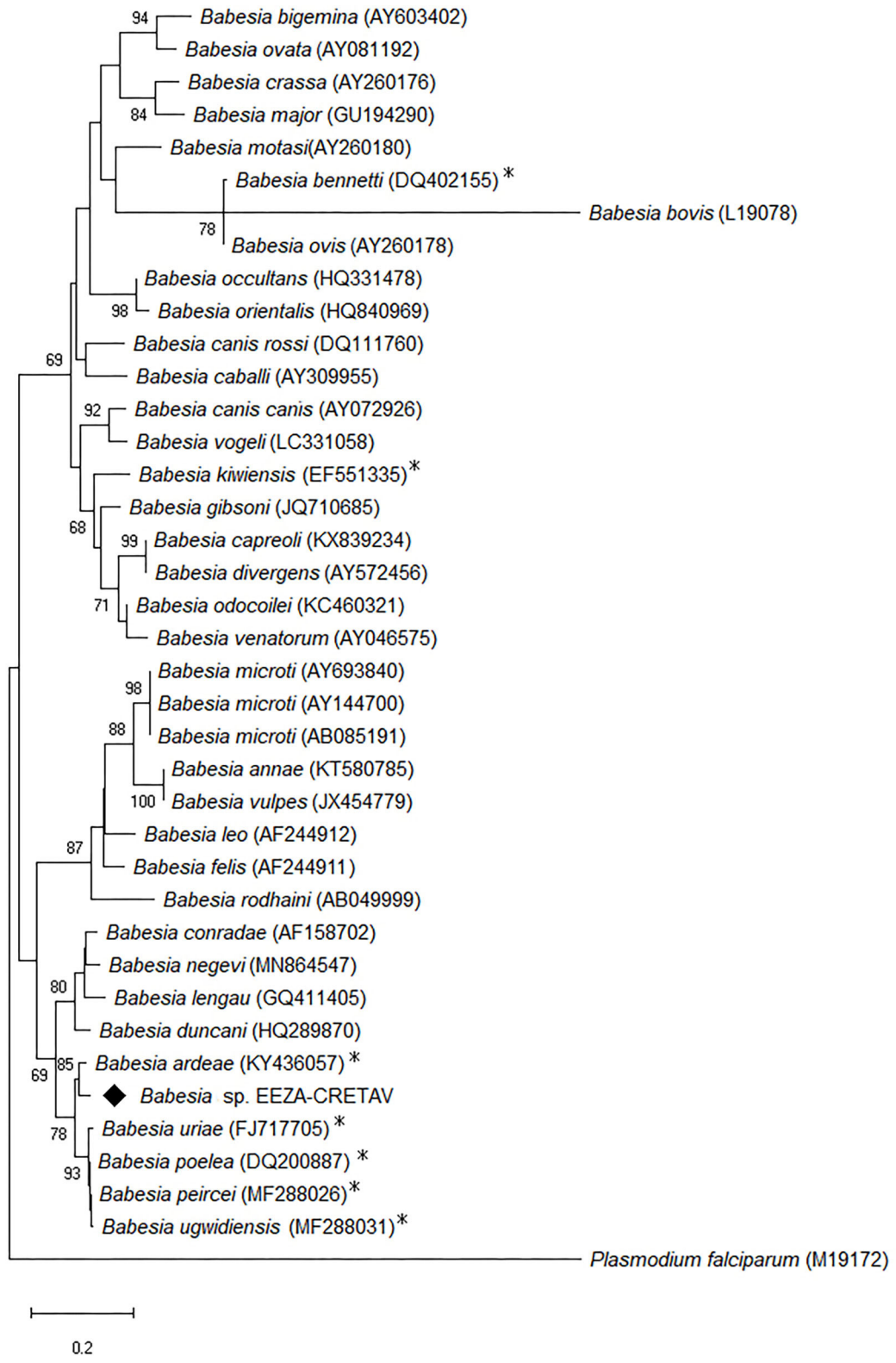

FIGURE 3 | Maximum likelihood trees of *Babesia* species based on 18S rRNA analysis. The evolutionary analysis was inferred using Tamura-Nei model + G model with Mega X. The analysis involved 39 nucleotide sequences and a total of 483 positions in the final dataset. The tree is drawn to scale, with branch lengths

(Continued)

FIGURE 3 | measured in the number of substitutions per site. Numbers (>65%) shown at the nodes correspond to bootstrapped percentages (for 500 repetitions). The GenBank accession number of the sequences used in this analysis is shown in brackets after *Babesia* taxon name. The species found in this study is marked with diamond and the species detected in birds with asterisk. Taxon names are followed by GenBank accession numbers and collection location where available. *Plasmodium falciparium* is used as outgroup.

TABLE 6 | GenBank accession numbers of the nucleotide sequences obtained in this study, differing from published sequences.

Organisms	Target gene	Accession no
Argas reflexus	16S rRNA	MW289075; MW289076
	12S rRNA	MW289084
	COI	MW288388; MW288389
Argas sp. EEZA-CRETAV1	16S rRNA	MW289069
	12S rRNA	MW289077
	COI	MW288380; MW288381
Argas sp. EEZA-CRETAV2	16S rRNA	MW289070; MW289071
	12S rRNA	MW289078; MW289079; MW289080
	COI	MW288382; MW288383; MW288384
Argas sp. EEZA-CRETAV3	16S rRNA	MW289072
	12S rRNA	MW289081; MW289082
	COI	MW288385
Argas sp. EEZA-CRETAV4	16S rRNA	MW289073
	12S rRNA	MW289083
	COI	MW288386
Argas sp. EEZA-CRETAV5	16S rRNA	MW289074
	COI	MW288387
Ricekttsia sp. EEZA-CRETAV	*ompA*	MW287618
	ompB	MW287619
	sca4	MW287620
	gltA	MW287621
	16S rRNA	MW296096
	17-kDa	MW287622
Coxiella sp. EEZA-CRETAV1	*rpoB*	MW287614
	groEL	MW287611
Coxiella sp. EEZA-CRETAV2	*rpoB*	MW287615
	groEL	MW287612
Coxiella sp. from *Argas reflexus*	*rpoB*	MW287616
Francisella sp. EEZA-CRETAV	*rpoB*	MW287617
Rickettsiella sp. EEZA-CRETAV	*groEL*	MW287613
Babesia sp. EEZA-CRETAV	18S rRNA	MW287597
	ITS1	MW287607
	ITS2	MW287606

spirochetosis, a worldwide distributed disease of veterinary importance that has not been reported from Spain (11, 40). The lack of virus detection in this study was unexpected, because diverse viruses are readily detected in *Argas* ticks (7). Scarce tick-borne viruses have been described from Spain and all but Meaban-like virus, a flavivirus found in *Ornithodoros maritimus* (38), are associated to ixodid ticks. Some of these viruses have a great relevance for human health, e.g., Crimean-Congo hemorrhagic fever virus, whose arrival in infected ticks has been suggested to take place through migratory birds (37, 58). This fact highlights the importance of studying viruses in soft ticks associated to birds in Spain, an important area in the migratory routes of many avian species, because what it is not sought, it is not found (59).

Two apicomplexan parasites have been found in this work, *Babesia* sp. and *A. bambarooniae*, though the latter species is not known as a tick-borne microorganism. In turn, the apicomplexan piroplasms *Babesia* (Aconoidasida; Babesiidae) are mainly vectored by ixodid ticks, though argasid ticks also were suggested as potential vectors (60). *Babesia* sp. EEZA-CRETAV has been amplified from an *Argas* sp. EEZA-CRETAV3 tick associated with rollers and the presence of the protozoan in the blood of rollers cannot be rejected. Sixteen *Babesia* species responsible for avian piroplasmosis, in addition to several strains that are not fully identified, are known (61). Of these, *Babesia frugilegica*, *Babesia shortti* and *Babesia benneti* have been reported from Spain (62–64). *Babesia* sp. EEZA-CRETAV is closely related to some of the scarcely genetically characterized *Babesia* species, mainly to *B. ardeae* (**Figure 3**). This species has been detected in Asia and its pathogenicity is unknown (61). This strain is also close to the human-pathogenic *B. ducani* that has been identified in North America, the United Kingdom and Australia (65). To our knowledge, *Babesia* spp. have not been identified in owls.

CONCLUSIONS

The sedentary lifestyle of soft ticks could imply a limited role of these ticks in the circulation of infectious agents (66). However, as indicated by this study, the high re-use of cavities within and between years by different bird species could importantly enhance the spread of microorganisms associated with soft nidicolous ticks, such as *Argas* ticks.

This study highlights the richness of nidicolous *Argas* ticks associated with cavity-nesting birds in a semi-desert area in Western Europe, and suggests that the diversity of this genus in Spain might be underestimated. Moreover, this work provides the first report of *Rickettsia* sp., *Coxiella* spp., *Rickettsiella* sp., *Francisella* sp. and *Babesia* sp., from soft ticks in Spain, and *A. bambarooniae* from Ixodida.

Further research should be carried out to confirm if the new genotypes of ticks and their microorganisms represent novel taxa and, additionally, to understand their role in the epidemiology of zoonoses using the One Health approach.

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

APa, RV, JO, and FV designed the initial study. JV and FV carried out the field work. APa performed the tick identification and tick processing. APa, APo, SS, and PS implemented the analysis of microorganisms. APa and FV wrote the first draft of the manuscript. All authors contributed to data interpretation and revisions.

FUNDING

RV was funded by the Research Grant Agency (VEGA) of the Ministry of Education, Science, Research and Sport of the Slovak Republic and Slovak Academy of Sciences (2/0023/20). FV received financial support from the projects CGL2014-55969 and PGC2018-097426-B-C22 (Spanish Ministry of Universities, Spanish State Research Agency, FEDER Program, European Union). JV was funded by a predoctoral grant (BES-2015-075951) of the Spanish Ministry of Science and Innovation.

ACKNOWLEDGMENTS

We would like to thank Agustín Estrada-Peña (University of Zaragoza) for helping with the taxonomy of *Argas* spp. We thank L. Bolonio and I. E. Cardiel (CEBIME, Conservación y Estudio de la Biodiversidad en Medios Esteparios) for providing tick samples. Teresa Martínez, Maite Amat, Miguel Ángel Calero, and Stanislav Kolencik helped with fieldwork. Junta de Andalucía kindly provided permits to sample birds and their nests. We thank Dr. Didier Raoult (Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, France), Dr. J. Stephen Dumler (The Johns Hopkins Hospital, USA), Dr. Houssam Attoui [UMR Virologie, National Institute for Agriculture, Food, and Environment (INRAE), France], Dr. Luis M. Hernández-Triana (Wildlife Zoonoses and Vector-borne Diseases Research Group, Animal and Plant Health Agency, United Kingdom), Dr. Joaquim Ruiz (Universidad Cientifica del Sur, Peru), Dr. Volker Fingerle (German National Reference Centre for *Borrelia*), and Dr. Matthias Niedrig (Centre for Biosafety, Robert Koch Institute, Germany) for providing positive controls.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.637837/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 May 2021
doi: 10.3389/fvets.2021.664731

Life Cycle and Genetic Identification of *Argas persicus* Infesting Domestic Fowl in Khyber Pakhtunkhwa, Pakistan

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Ticks transmit numerous pathogens to animals including humans; therefore, they are parasites of health concern. Soft ticks infesting domestic fowl in Pakistan are carriers of viruses and bacteria and cause unestimated economic losses in the poultry sector. The current study was intended to identify soft ticks infesting domestic fowl and understand their spatiotemporal distribution along 1 year. A sum of 7,219 soft ticks were collected from 608 domestic fowl in 58 infested shelters; 938 (12.9%) ticks were found on the host and 6,281 (87%) in the shelters. The collected ticks comprised 3,503 (48.52%) adults including 1,547 (21.42%) males and 1,956 (27.09%) females, 3,238 (44.85%) nymphs, and 478 (6.62%) larvae. The most prevalent life stages were adults, followed by nymphs and larvae. Overall tick prevalence considering all visited shelters was 38.66% (58/150). The highest tick prevalence was found in district Lakki Marwat (50.03%) followed by Peshawar (31.08%) and Chitral (18.88%) districts. All ticks were morpho-taxonomically identified as *Argas persicus*. To determine their life cycle, adult *A. persicus* were reared in the laboratory infesting domestic fowl (*Gallus gallus domesticus*). The life cycle was completed in 113–132 days (egg to egg) with a mean temperature of 33 ± 3°C and relative humidity of 65 ± 5%. Individual ticks were used for DNA extraction and subjected to polymerase chain reaction (PCR) using specific primers for the amplification of a partial fragment of mitochondrial cytochrome oxidase subunit I (*cox1*) and 16S ribosomal RNA (16S rRNA) genes. Obtained amplicons were compared using basic local alignment search tool (BLAST) to scan for homologous sequences. Phylogenetic trees showed *A. persicus* from Pakistan clustering with conspecific sequences reported from Australia, Chile, China, Kenya, and the United States. This is the first study aiming to reproduce the life cycle of *A. persicus* and genetically identify this tick in the region. Further studies are encouraged to investigate the pathogens associated with this soft tick species in Pakistan.

Keywords: soft ticks, life cycle, *Argas persicus*, domestic fowls, Pakistan

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: *05 February 2021*
Accepted: *09 March 2021*
Published: *19 May 2021*

Citation:
Zahid H, Muñoz-Leal S, Khan MQ, Alouffi AS, Labruna MB and Ali A (2021) Life Cycle and Genetic Identification of Argas persicus Infesting Domestic Fowl in Khyber Pakhtunkhwa, Pakistan. Front. Vet. Sci. 8:664731. doi: 10.3389/fvets.2021.664731

INTRODUCTION

Soft ticks in the genus *Argas* are parasites associated mostly with birds. Sixty-one species are currently described in the genus (1, 2), and at least four are parasites of fowl, namely, *Argas miniatus*, *Argas persicus*, *Argas radiatus*, and *Argas sanchezi* (3). *Argas persicus*, commonly referred as the fowl tick, is distributed chiefly in all continents (3). Wide analyses of the geographic distribution of *A. persicus* in the American Continent pointed out that this soft tick occurs in dry and subtropical environments and be absent in tropical latitudes (4).

Pakistan is immersed in one of the subtropical regions of the world. Suitable temperature and humidity conditions in the country facilitate the growth and development of ticks in domestic animals. The geography and climatic patterns of Pakistan covaries from high altitude and cold environments (Himalaya Mountains) to low and warm lands toward the sea. Soft ticks of the genus *Argas* in Pakistan are currently represented by four species only: *A. persicus*, *Argas reflexus*, *Argas abdussalami*, and *Argas rousetti* (1, 5–7). As in many other geographic regions of the world, *A. persicus* is a frequent fowl parasite in Pakistan (5, 6, 8).

Various environmental conditions have been reported showing an innocuous effect on tick diversity (9). In accordance with other subtropical regions of the world, the tick fauna of Pakistan concentrates within several regional climatic zones, especially in arid, sub-arid, and humid areas (5, 6, 10). Environmental conditions such as host availability, precipitation, temperature, and humidity shape the life cycle of a tick species (11, 12). As in other Argasids, when unfavorable environmental conditions prevail, *Argas* spp. can starve for several years sheltered in crevices or cracks (1). These ticks have the ability to reduce dehydration and enter diapause periods (13–15). Larvae are slow feeders generally and stay attached to their host for 5–10 days (13). Each life stage requires a successful and short blood uptake to molt (13). Nymphs feed several times and molt until reaching maturity as males or females (14, 16).

Pakistan has the 11th largest poultry industry in the world with a production of 1,163 million broilers annually. The poultry sector provides employment to over 1.5 million people, and investment is more than Rs 700 billion currently (Pakistan Economic Survey 2019–2020, Ministry of Finance, Government of Pakistan). Although large-scale poultry production grows in the country, domestic fowl are still abundant in cities and rural areas and bring important economic benefits to the population. Domestic fowl are typically raised either inside hen houses or freely, congregating at night, a fact that favors the maintenance of nidicolous *Argas* spp. (1). As blood sucking parasites, *Argas* spp. impose stress on their avian hosts, therefore affecting their health. Despite the economic importance, studies on ticks associated with fowl have been neglected in Pakistan. The present study aimed to assess the distribution, life cycle, and phylogenetic position of *Argas* ticks infesting domestic fowl along different regions of north western Pakistan.

MATERIALS AND METHODS

Study Sites

Tick collection was carried out during April 2018 to March 2019 in the region of Khyber Pakhtunkhwa (KP), specifically in the district of Peshawar (34°01′36.2″ N; 71°31′47.4″E, 331 m), Chitral (35°53′40.9″N; 71°41′31.1″E, 1,494 m), and Lakki Marwat (32.6135°N; 70.9012°E, 255 m). The Global Positioning System was used to collect the exact coordinates of each location, and a map was designed using ArcGIS v 10.3.1 (**Figure 1**).

The KP province is located in the north western region of Pakistan. Peshawar, the capital city, extends along the wide-ranging valley of Peshawar rivers. The winter begins in mid-November and ends in March, with a mean temperature ranging between 4 and 18.35°C. The summer starts in April and ends in October, with a mean temperature ranging from 25 to 40°C. Rainfall occurs both in winter and summer (climate-data.org). The selected areas for sampling in Peshawar district included Badaber villages, Jamrud, Mir Tayab Garhi, Peshawar, and Umar Payan. The largest district, Chitral, is situated in the north of KP province with an area of 14,850 km^2 and has a Mediterranean climate with warm summers and mild winters. The mean temperature during summer ranges between 22 and 32°C from April until October. The mean temperature during winter ranges from 0 to 11.4°C during November to March. Rainfall occurs throughout the year (climate-data.org). Areas selected for tick collection in district Chitral were Chitral, Chitral Gol National Park, Gahirat Gol, Kalash, and Singur. The district Lakki Marwat has desertic lands with abundant sand dunes and dry and hot weather. Summer starts in April and ends in late October, with the hottest month in June (30–45°C). A moderate winter starts in early November and continues until March, with temperatures ranging between 4 and 20°C. Rainfall is rare and mostly occurs in July and August (climate-data.org). The areas selected for sample collection in district Lakki Marwat included Lakki Marwat, Dara Tang, Pezu, Sarai Naurang, and Tajori.

Collection of Ticks, Prevalence, and Mean Infestation

A total of 150 shelters were visited twice per month along a year. In each of them, birds, wall cracks, ceilings, and floors were carefully examined in the search for soft ticks. The bark of trees in rural areas where the domestic fowl used to rest or shelter was examined as well. Ticks were collected alive in labeled sterile plastic bottles and manipulated using fine tweezers to avoid damaging the specimens. Collected ticks were transported to the Department of Zoology, Abdul Wali Khan University, Mardan, for identification and further analyses. The collected larvae and all nymphal instars were preserved in 100% ethanol.

The prevalence and mean infestation of ticks were calculated using the following formula:

$$\text{Shelter infestation prevalence (\%)} = \frac{\text{No. of infested shelters}}{\text{Total no. of shelters visited}} \times 100$$

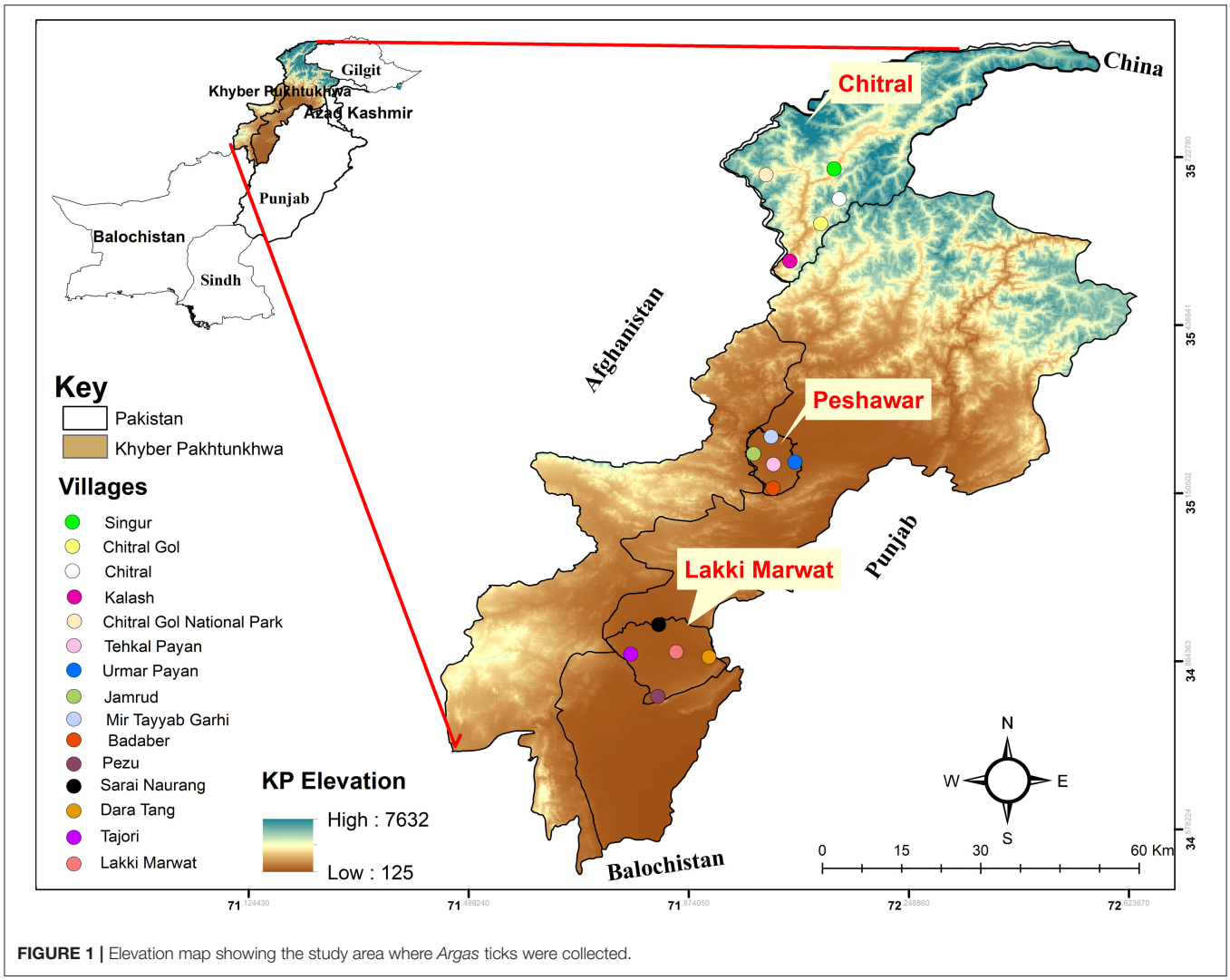

FIGURE 1 | Elevation map showing the study area where *Argas* ticks were collected.

$$\text{Host mean infestation} = \frac{\text{Total no. of ticks on host}}{\text{No. of infested host}}$$

Morphological and Molecular Identification of Ticks

Ticks were rinsed with distilled water and 70% ethanol for the removal of surface contamination. For nymphs and adults, external morphology was observed using a stereomicroscope (HT Stereozoom) and compared with available taxonomic keys (3, 14). Special attention was put to the anatomy of the idiosomal margins, since the integumental cell-like structures that this body region exhibits are important to separate species in the genus *Argas* (3). Identified engorged females were kept in Petri dishes at room temperature for life cycle observations.

Morphological diagnoses were confirmed by molecular tools. For that purpose, field collected specimens (one female, one male, one nymph, and two pools of 10 larvae per locality) were submitted to DNA extraction. Ticks were perforated with a sterile needle inside 1.5-ml tubes and heated at 50°C for ethanol evaporation. Genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer DNA extraction protocol. The integrity of DNA was confirmed by gel electrophoresis, and DNA concentration was quantified using a Nanodrop ND-100 (Thermo Fisher Scientific, Waltham, MA, United States). Samples were stored at −20°C.

PCR was performed to amplify two mitochondrial markers, a 606-base-pair (bp) fragment of the *cox1* gene and a 240-bp fragment of the 16S ribosomal RNA (rRNA) gene. In particular, for the 16S rRNA gene, we downloaded 82 homolog sequences from *Argas* spp. available in National Center for Biotechnology Information (NCBI). Based on an alignment of these sequences, primers 3′-TTTGGGACAAGA AGACCCTATGAA TTT-5′ (forward) and 3′-ACATCGAGG TCGCAATCAATTTTATC-5′ (reverse) were designed using highly conserved regions detected with Vector NTI v 11.5.3.

Primers GGAGGATTTGGAAATTGATTAGTTCC-5′ (forward) and 3′-ACTGTAA ATATATGATGAGCTCA-5′ (reverse) were employed to amplify *cox1* gene (17). PCR was performed in a 20-μl mix [1 μl of forward primer, 1 μl reverse primer, 2 μl of template DNA, 12 μl Dream*Taq* PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, United States), and 4 μl PCR grade water]. The PCR conditions were set as follows: initial denaturation temperature of 95°C for 5 min, followed by 35 cycles of 95°C denaturation for 30 s, 53°C (*cox1*) and 56°C (16S rRNA); annealing for 30 s, 72°C extension for 1 min; and final extension of 72°C for 5 min. PCR products were run on ethidium bromide-stained agarose gels and observed by UV trans-illumination (UVP BioDoc-It Imaging System, Upland, CA, USA).

Expected size amplicons were sequenced at Macrogen, Korea. The generated sequences were trimmed and assembled in SeqMan v 5.00 (DNAstar). A BLAST analysis was performed using the obtained consensuses (18). An alignment for each sequenced gene was constructed with ClustalW and edited in BioEdit alignment editor V 7.0.5 (19). Phylogenetic analyses were inferred by the maximum likelihood method for both genes using PhyML (20), with the General Time Reversible (GTR) model, five substitution rate categories, and 1,000 bootstrap replicates.

Life Cycle

A subgroup of five engorged females per collection site was separated to investigate the life cycle of *A. persicus*. Ticks were kept inside an incubator at 33 ± 3°C and 65 ± 5% relative humidity for survival and oviposition. Laid eggs were carefully transferred to 5-ml sterile plastic syringes and sealed with wet cotton to provide humidity. Hatched larvae, subsequent nymphal instars, and adults were feed on domestic fowl (*Gallus gallus domesticus*).

Statistical Analysis

All recorded observations such as collection data and life cycle were assembled and arranged in the spreadsheets of Microsoft Excel V 2013 for descriptive analysis [mean and standard deviation (SD)]. Chi-square test was used for chi-square difference (χ^2) using the Statistical Package for the Social Sciences (IBM SPSS, Version 21) considering 95% confidence interval (CI) and a significant $P < 0.05$.

Ethical Approval

The current study was approved by the advance studies and research board (Dir/A&R/AWKUM/2020.4871) of the Abdul Wali Khan University, Mardan. A written or oral consent was taken during collection from the owner of domestic fowl.

RESULTS

Collected Ticks

A total of 7,219 *Argas* ticks were collected from 608 domestic fowl in 58 infested shelters. Among these, 3,612 (50.03%) were collected in district Lakki Marwat, 2,244 (31.08%) in Peshawar, and 1,363 (18.88%) in Chitral. Overall tick prevalence considering all visited shelters was 38.66% (58/150). Different life stages were collected, including 478 (6.62%) larvae, 3,238 (44.85%) nymphs, 1,547 (21.42%) males, and 1,956 (27.09%) females (**Table 1**). A total of 938 (12.9%) ticks were found feeding on domestic fowl, while 6,281 (87%) were collected in the shelters and crevices. In particular, 373 larvae (78.03%) were found on domestic fowl, and 105 (21.96%) were collected wandering in the shelters. Postlarval stages were more abundant in shelters than on birds (**Table 2**). All nymphs and adult ticks were identified morphologically as *A. persicus* because of having <100 integumental cells around the body margin and by the

TABLE 1 | The abundance of different stages of collected *Argas persicus* ticks.

District	Total (%)	Larvae (%)	Nymph (%)	Male (%)	Female (%)	X^2	*P*-value
Chitral	1,363 (19)	87 (6)	746 (58)	204 (15)	326 (24)		
Peshawar	2,244 (31)	174 (6)	1,016 (45)	452 (20)	602 (27)	102.7	0.001
Lakki marwat	3,612 (50)	217 (8)	1,476 (40)	891 (24)	1,028 (28)		
Total	7,219 (100)	478 (7)	3,238 (45)	1,547 (21)	1,956 (27)		
Mean	2,406	159.3	1,079	515.7	652.0		
SD	1,133	66.23	369.1	347.9	353.7		
CI	0–324	162–199	0–138	0–153	0–522		

TABLE 2 | Number of collected *Argas persicus* ticks according to domestic fowl hosts (H) and their shelters (S).

District	Larvae H/S	Nymph H/S	Male H/S	Female H/S	Total H/S
Chitral	78/9	103/643	11/193	16/310	208/1,155
Peshawar	123/51	153/863	17/435	23/579	316/1,928
Lakki marwat	172/45	146/1,330	43/848	53/975	414/3,198
Total	373/105	402/2,836	71/1,476	92/1,864	938/6,281
Percentage	78.03/21.96	12.41/87.58	4.58/95.41	4.70/95.29	12.9/87

presence of a lateral line (**Figures 2A–C**) (3). Pools of larvae were identified by molecular tools.

Spatiotemporal Distribution of Ticks, Prevalence in Shelters, and Host Mean Infestation

The highest tick infestation was found during July, while the lowest tick infestation was observed in the month of January followed by February and December (**Table 3**, **Figure 3**). Considering the collection site, the highest prevalence was found in Lakki Marwat where a total of 3,612 ticks were collected from 269 domestic fowl followed by Peshawar where 2,244 ticks were collected from 197 domestic fowl, and the least prevalence was found in Chitral where 1,363 ticks were found on 142 domestic fowl. Nymphal instars (3,238; 45%) were most abundant, followed by adult females (1,956; 27%) and males (1,547; 21%). On the other hand, larval stages comprised 478 (7%) individuals in all three selected districts of KP (**Table 1**).

The hot climatic district Lakki Marwat, where the average temperature during summer ranges between 30 and 45°C, had the most infested shelters (26/50, 52%) followed by the moderate temperature (25–40°C) district Peshawar (18/50; 36%) and low temperature (22–32°C) district Chitral (14/50; 28%). On-host, tick infestation was calculated in each shelter, and the overall mean infestation on a single host was 11.87. The average tick infestation on domestic fowl in Lakki Marwat, Peshawar, and Chitral was 13.42, 11.39, and 9.59, respectively.

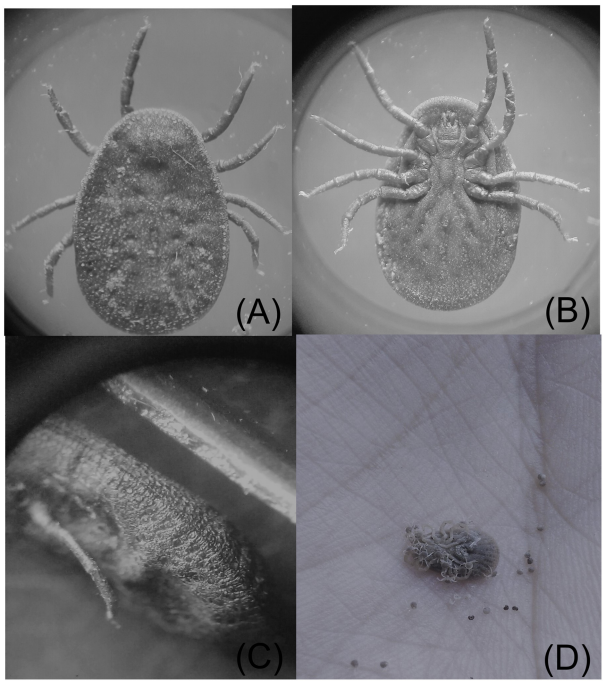

FIGURE 2 | (A) Dorsal, **(B)** ventral, and **(C)** lateral view of female *A. persicus*. **(D)** Maternal-like behavior in *A. persicus*.

Life Cycle

Egg Laying and Larval Hatching

We did not observe mating of *A. persicus* on the host body and was observed off-host. Female ticks were observed to feed on domestic fowl for 30–35 min and laid a batch of (20–30) rounded pale-yellow eggs after 12 ± 3 days. Successive feedings and ovipositions were observed up to six to seven times until the female died. After 2–5 days, the eggs became dark and dry and hatched after 15–20 days of incubation (**Table 4**). The body measurement of *A. persicus* was taken at each stage (**Table 5**). The emerged larvae were found to remain on the female's ventral surface (**Figure 2D**). After 5 ± 2 days, the larvae started questing for hosts and were allowed to feed on domestic fowl. Overall, the larvae remained attached mostly under the wings of the birds and for a period of 5 ± 1 days (**Table 4**).

Nymphal Instars

The larval stage was found to molt to eight-legged nymphs after 12 ± 3 days. We noted a total of five nymphal instars, each one feeding for 15–20 min. The specimens molted to the next nymphal instar in 12 ± 3 days after feeding.

Adults

Female ticks emerged from the fourth and fifth nymphal instars, while the third and fourth nymphal instars mostly molted to male ticks. We observed a preoviposition period of 12 ± 3 days in the incubator.

Genetic Identification and Phylogenetic Analysis

PCRs for *cox1* and 16S rRNA mitochondrial genes were positive in all samples, and one single haplotype for each gene was obtained. BLAST comparisons confirmed our morphological diagnosis since both *cox1* and 16S rRNA genes showed 98–100%

TABLE 3 | District wise spatial distribution of infested hosts (domestic fowl) and collected ticks (*Argas persicus*).

	No. of infested host	No. of ticks collected	No. of infested host	No. of ticks collected	No. of infested host	No. of ticks collected
Total (%)	197 (32.40%)	2,244 (31.08%)	142 (23.35%)	1,363 (18.88%)	269 (44.24%)	3,612 (50.03%)
Mean	39.4	448.8	28.4	272.6	53.8	722.4
SD	12.68	104.4	8.649	72.98	8.468	109.3
CI	23.6–55.1	319–578	17.66–39.14	182–363	43.2–64.3	586–858
P-value	0.0006		0.0011		0.0001	

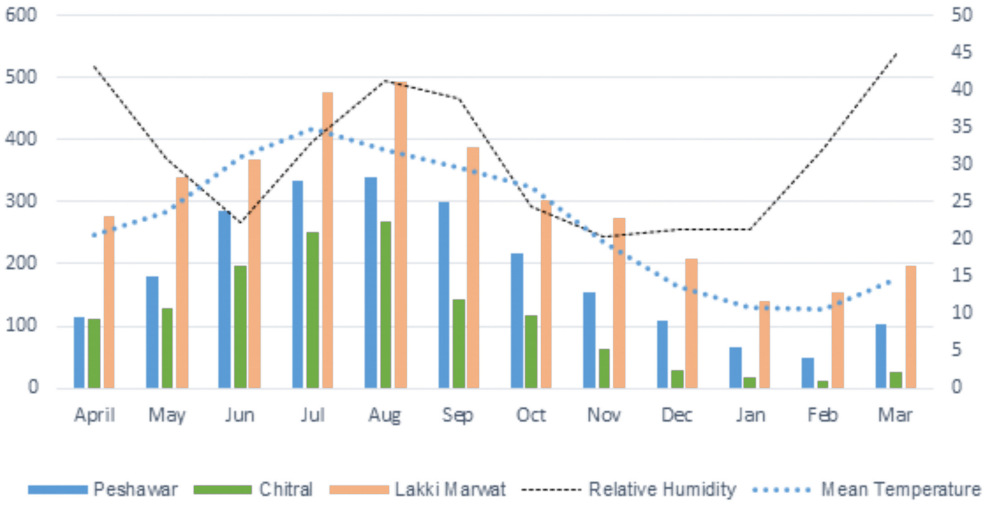

FIGURE 3 | Chart showing spatial distribution of *Argas* ticks in selected districts of KP.

TABLE 4 | Durations of life stages of *Argas persicus* infesting natural host (domestic fowl).

S#	Traits	Time duration of life stage (mean ± SD)	No. of examined ticks**
1	Pre-oviposition	12 ± 2.5 days	5
2	Eggs laying	2 ± 0.7 days	5
3	Eggs incubation	17 ± 1.3 days	–
4	Larvae free living	5 ± 1.6 days	86
5	Larvae attachment on host	5 ± 1.3 days	15
6	Larvae molting to nymph	12 ± 1.8 days	12
7	*Nymph feeding	22 ± 1.6 min	–
8	Nymph molting	12 ± 1.9 days	12
9	Adult feeding	33 ± 1.8 min	12
10	Complete life cycle	113–132 days (egg to egg)	–

*Nymphal stage includes a series of five nymphal instars.
**The number of ticks were same for three selected districts.

TABLE 5 | Body measurements of *Argas persicus*.

Ticks life stages*	Average weight in g	Size in mm (mean ± SD)
Male (UF)	0.003011	5.12 ± 0.12
Male (F)	0.010847	5.55 ± 0.55
Female (UF)	0.010833	6.25 ± 0.25
Female (F)	0.0325	8.12 ± 0.120
Female (FAO)	0.012375	8.12 ± 0.12
Egg (100×)	0.000099 (0.0099)	–
Larvae (F)	0.000133	1.001 ± 0.2
1st Nymph	0.0001	1.35 ± 0.15
2nd Nymph (UF)	0.0002	1.34 ± 0.08
2nd Nymph (F)	0.0005	2.05 ± 0.2
3rd Nymph (UF)	0.0008	2.02 ± -0.2
3rd Nymph (F)	0.0013	2.70 ± 0.07
4th Nymph (UF)	0.003	2.71 ± 0.06
4th Nymph (F)	0.004	3.72 ± 0.03
5th Nymph (UF)	0.007543	3.73 ± 0.02
5th Nymph (F)	0.014285	5.04 ± 0.03

F, fed; UF, unfed; FAO, fed after oviposition.
**The average of five tick's weight is given in the table.*

of identity with homologous sequences of *A. persicus* from other regions of the world. The phylogenetic analysis for *cox1* gene showed *A. persicus* from Pakistan clustering with conspecific sequences of China, Iran, Kazakhstan, Kenya, Romania, and the United States. In the 16S rRNA gene phylogenetic tree, the sequence from Pakistan was grouped with *A. persicus* from Australia, Chile, China, Kenya, and the United States (**Figures 4**, **5**). GenBank accession numbers for the sequences generated in this study are MW077849 and MT002847.

DISCUSSION

The majority of studies on ticks has been focused on the Ixodidae family (hard ticks), their associated pathogens, and risks for public and veterinary health (5, 6, 21–26). On the other hand, despite the economic importance of poultry, studies on soft ticks infesting domestic fowl have been neglected. In Pakistan, studies have been focused on the hard tick fauna, associated risk factors, morphological and molecular identification, seasonal variations, phylogenetic, and pathogens they carry; however, studies on soft ticks are limited (5, 6, 27). The present study reports the spatiotemporal distribution and molecular characterization of *A. persicus* infesting domestic fowl in various regions of KP, Pakistan, for the first time. The constructed phylogenetic tree revealed an evolutionary relationship of herein collected

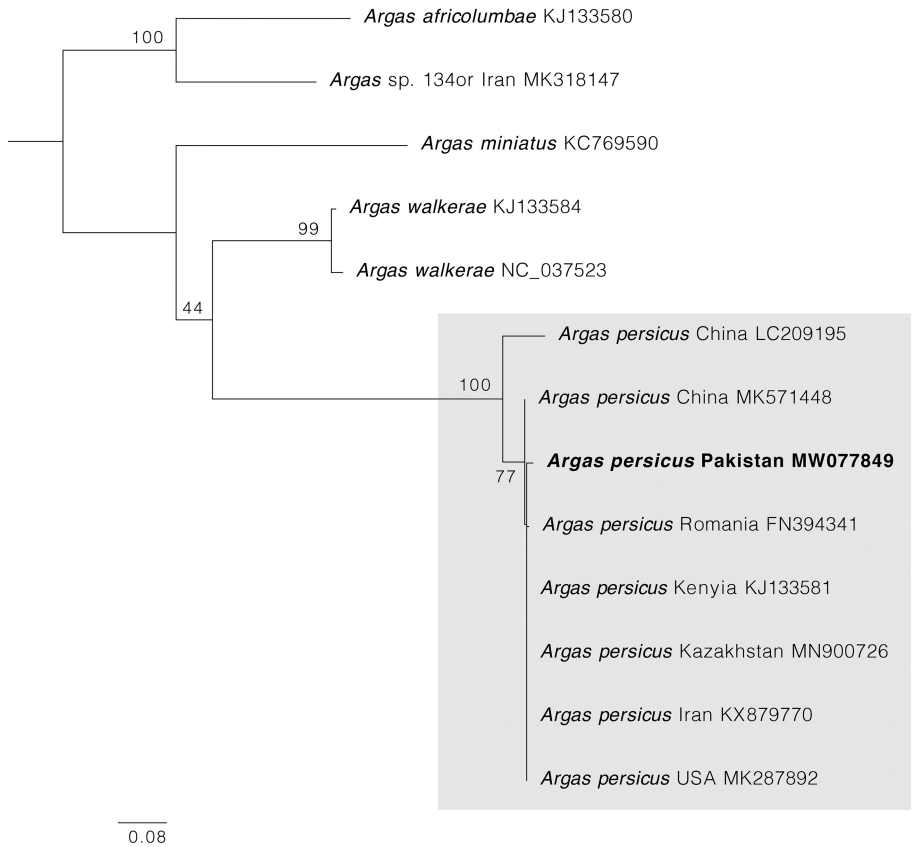

FIGURE 4 | Maximum likelihood tree constructed for the *cox1* sequence of *A. persicus* generated in this study. Species names are followed by country and accession numbers in parentheses. Bootstrapping values (1,000) are shown at each branch. The bar represents 0.08 substitutions per site. Sequence obtained in the present study is highlighted in bold.

A. persicus with *Argas* ticks from Romania, Australia, China, Chile, Iran, Kazakhstan, Kenya, and United States. Furthermore, the life cycle of *A. persicus* was carefully documented for the first time in the region under laboratory conditions using domestic fowl as hosts. Overall, the period in which *A. persicus* completes its life cycle was studied for the first time in the region.

A. persicus has adapted to parasitize fowl worldwide (28–31), and in the present study, we found that domestic fowl of the selected districts were highly infested by this soft tick. Extremely elevated and low temperatures in the northern regions of KP, especially in district Chitral, might serve as less-favorable environmental conditions for the survival of *A. persicus*, a fact that has been observed in other parts of the world for soft ticks (32). Tick survival and prevalence mostly depend on the environmental conditions such as temperature and humidity (11, 12). Districts with moderate climate such as Peshawar and western regions (Lakki Marwat) were found favorable for the infestation by *A. persicus*. It is well-known that high temperature and high humidity favor the development and persistence of various ticks, including *A. persicus*, in tropical and subtropical distributions (6, 30, 32, 33). The spatiotemporal distribution of *A. persicus* in different temperature regimes agrees with our previous report (6).

As the survival of the tick requires favorable temperature and humidity, the life span of the tick may differ from one region to another (11, 12). The life cycle of *A. persicus* was studied and documented in detail to obtain biological evidence for effective control strategies. Knowing the life cycle of a tick species is important because each life stage can vary in terms of pathogen transmission to the host (34–37). Larval stages are the most suitable life stage of a tick for the use of acaricides or vaccine (to date there is no available vaccine for the control of soft ticks) on the host because it remains attached for days on the host and can be easily targeted. In contrast, nymphal, and adult ticks feed for a short period of time and mostly remain off-host sheltering in the environment, a fact that precludes effective control measures.

Studies on the life cycle of *A. persicus* have been performed in populations of Egypt (16), and three nymphal instars were observed. Walker et al. (14) reported that, in general, there may be four nymphal instars in the life cycle of *A. persicus*. The findings of our study slightly disagree with previous reports in that five successive and prominent nymphal instars were observed before the adult stage. Differences in the number of

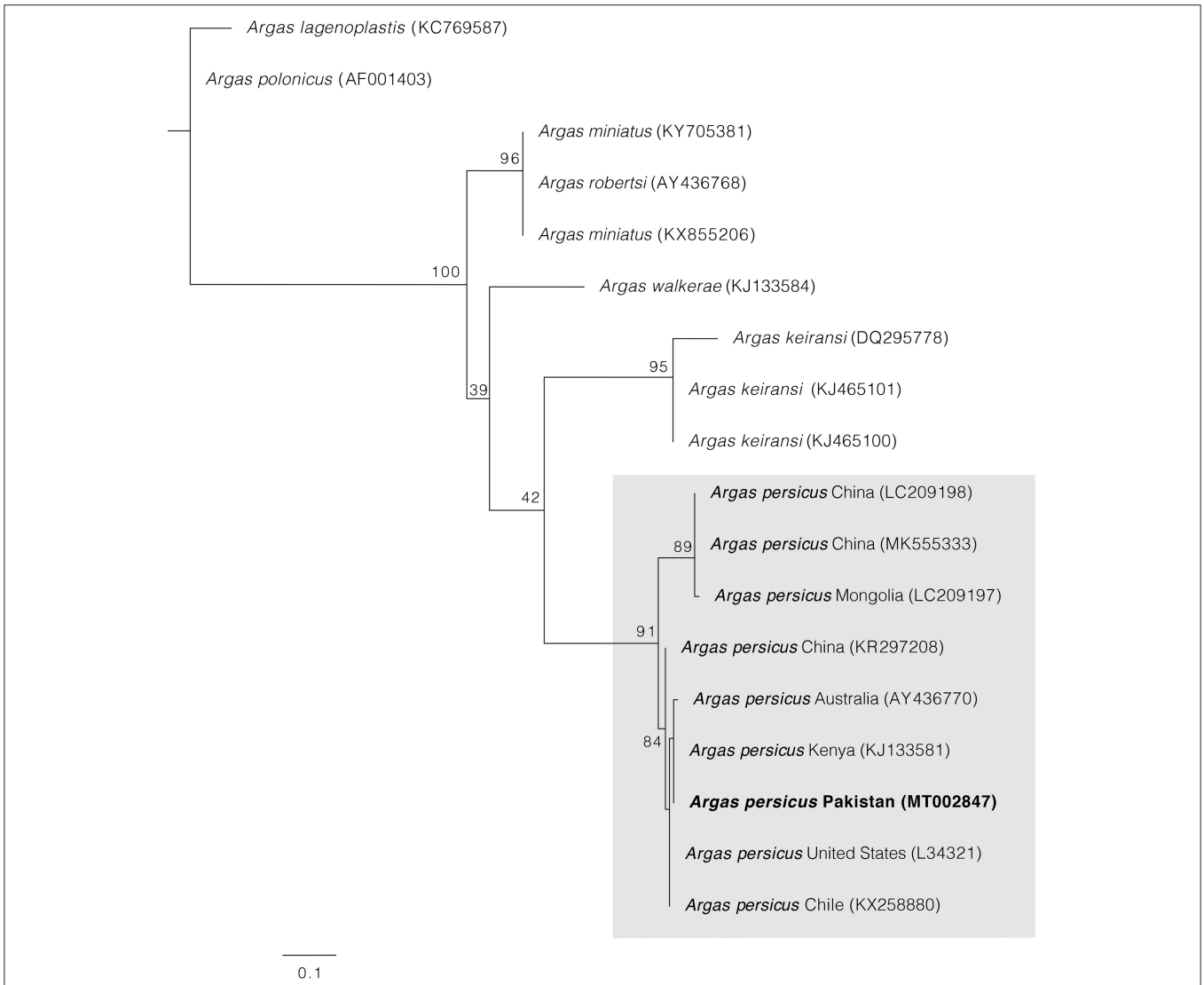

FIGURE 5 | Maximum likelihood tree constructed for the 16S sequence of *A. persicus* generate in this study. Species names are followed by country and accession numbers in parentheses. Bootstrapping values (1,000) are shown at each node. The bar represents 0.1 substitutions per site. Sequence obtained in the present study is highlighted in bold.

nymphal instars and life span observed in our study with respect to Walker et al. (14) and El-Kammah and Abdel-Wahab (16) may be due to the difference in host and environmental conditions.

We observed that the *A. persicus* adult females kept their larvae restricted to the ventral surface likely providing protection, a behavior that could be interpreted as maternal care. The same phenomenon has been previously observed in other soft ticks of the genus *Argas* and *Antricola*, such as *Argas striatus*, *Argas transgariepinus*, and *Antricola marginatus* (38, 39).

Many soft ticks are morphologically similar and lead to misidentification up to the species level based on external morphology; therefore, mistakes in their identification are not uncommon and have been described previously (40, 41). Genetic data are often required to accurately identify a given soft tick species. Indeed, mitochondrial genes such *cox1* and 16S rRNA have been utilized as markers for molecular identification of various tick species including soft ticks (42–44). Since the current systematics of soft ticks is still controversial, we opted to use the *cox1* and 16S rRNA to explore the phylogenetic relationships of *A. persicus* ticks from KP, Pakistan. The generated sequences showed 100% similarity to each other obtained from different regions in KP, Pakistan, and these generated sequences showed the closest similarities (98–100%) to the GenBank sequences deposited from various regions of the world. This fact indicates that the sequences for *the cox1* and 16S rRNA genes of *A. persicus* are highly conserved, even between vastly distanced populations. The findings of the present study are in agreement with previous reports, which suggest the use of *the cox1* and

16S rRNA genes as a suitable marker to identify *A. persicus* (4, 45–47). In the phylogenetic analyses, the generated sequence clustered in a separate subclade with the sequences deposited in GenBank for *A. persicus* from Australia, China, Chile, Kenya, and the United States. On the other hand, some close phylogenetic relationship was confirmed between different *Argas* species based on *cox1* and 16S rRNA, for instance, *Argas robertsi* from Australia and *Argas miniatus* from Brazil (47). These findings evidenced that there is a close phylogenetic relationship between *Argas* species from different geographic regions that deserves further attention. This may also be due to the lack of sufficient data deposited in NCBI from various regions for *Argas* ticks. Therefore, the addition of generated sequences during the present study is essential for drawing the evolutionary analysis of soft ticks.

CONCLUSION

The present study reported for the first time *A. persicus* ticks infesting domestic fowl in three districts, including the moderate temperature-region Peshawar, cold climatic-region Chitral, and hot climate-region Lakki Marwat, in KP, Pakistan. The ticks collected from the hosts were fewer in number compared to specimens found in the shelters. The life cycle of *A. persicus* in natural conditions was investigated for the first time in the region, and five nymphal instars were observed before the emergence of adults. The presence of *A. persicus* was confirmed at the molecular level by using *cox1* and 16S rRNA genes, and in phylogenetic trees, the generated sequences clustered with sequences from Australia, Chile, China, Kenya, and United States. Future studies are encouraged to investigate *A. persicus* as a potential reservoir for pathogens affecting the poultry industry and causing known and unknown infections and economic losses.

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) (MW077849 and MT002847) can be found in the article.

ETHICS STATEMENT

The current study was approved by the advance studies and research board (Dir/A&R/AWKUM/2020.4871) of the Abdul Wali Khan University Mardan. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

HZ and AA designed the study and acquired the budget. HZ, AA, and MK, collected the samples. AA, HZ, SM-L, ASA, MK, and ML performed the experiments and analyzed the results. All authors performed critical revision and approved the final manuscript.

FUNDING

The authors acknowledge the financial support provided by Pakistan Science Foundation and Higher Education Commission Pakistan.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 June 2021
doi: 10.3389/fvets.2021.684737

Argasid Ticks of Palearctic Bats: Distribution, Host Selection, and Zoonotic Importance

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The soft ticks (Ixodida: Argasidae) are ectoparasites of terrestrial vertebrates with a wide geographic distribution, occurring on all continents. These ticks are obligate blood-feeders, most of them show high degrees of host-specialization and several species in arid and tropical regions are important parasites of livestock and men. Species commonly occurring on domestic animals and man are generally well-known, with many studies focusing on their ecology, distribution or vectorial role. However, wildlife-specialist soft ticks are less studied. Nearly half of all soft tick species are bat specialists, with five species (*Carios vespertilionis, Chiropterargas boueti*, *Chiropterargas confusus*, *Reticulinasus salahi,* and *Secretargas transgariepinus*) occurring in the Western Palearctic. There is no comprehensive study on the distribution, hosts or pathogens in these soft ticks, although most species were shown to carry several viral, bacterial, or protozoan pathogens and also to occasionally infest humans. Based on a literature survey and 1,120 distinct georeferenced records, we present here the geographical range, host selection and vectorial potential for bat-specialist soft ticks occurring in the Western Palearctic (chiefly Europe, North Africa, and the Middle East). *Carios vespertilionis* shows the largest distribution range and was found on most host species, being ubiquitous wherever crevice-roosting bats occur. All the other species were located only in areas with Mediterranean climate, with *Ch. boueti, Chiropteraragas confusus*, and *R. salahi* are missing entirely from Europe. These three species have a host spectrum of bats roosting primarily in caves, while *S. transgariepinus* and *Ca. vespertilionis* is feeding primarily on crevice-roosting bat species. All but one of these soft tick species are known to feed on humans and may be vectors of important disease agents (*Rickettsia* spp., *Borrelia* spp., *Bartonella* spp., *Ehrlichia* spp., *Babesia* spp., several nairo-, and flaviviruses). As several crevice-roosting bat species show a continuous adaptation to human-altered areas, with certain species becoming common city-dwellers in the Western Palearctic, the study of bat specialist soft ticks is also important from an epidemiologic point of view.

Keywords: Chiroptera, host-specificity, Ixodoidea, soft ticks, zoonotic diseases

OPEN ACCESS

Edited by:
Sebastián Muñoz-Leal, University of Concepcion, Chile

Reviewed by:
José M. Venzal, Centro Universitario Regional Litoral Norte Salto, Universidad de la Republica, Uruguay
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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: *23 March 2021*
Accepted: *14 May 2021*
Published: *22 June 2021*

Citation:
Sándor AD, Mihalca AD, Domşa C, Péter Á and Hornok S (2021) Argasid Ticks of Palearctic Bats: Distribution, Host Selection, and Zoonotic Importance. Front. Vet. Sci. 8:684737. doi: 10.3389/fvets.2021.684737

INTRODUCTION

Ticks (Ixodoidea) are obligate blood-feeding arthropods, with a wide-spread occurrence and ∼935 species known as parasites of terrestrial vertebrates (1, 2). The group has an ancient origin, with their first appearance suggested for the Cretaceous [65–146 mya, (3)], and widespread diversification and dispersal occurring during the Tertiary some 5 to 65 mya ago (4). Taxonomically, modern ticks are divided into three families (Argasidae, Ixodidae, and Nuttalliellidae) with the two most speciose being the hard ticks (Ixodidae) with 742 recognized species (2), followed by soft ticks (Argasidae) with 193 species, as listed in the last comprehensive checklist of this later group (1). The Nuttalliellidae consist of a single species (*Nuttalliella namaqua*), and is considered to be the most ancient among the three tick families, showing several intermediate characters specific for the other two (5).

Argasidae includes two subfamilies, Argasinae and Ornithodorinae, both with several genera, and subgenera, with differing numbers according to different authors (1, 6–10). They have a world-wide distribution, with most species being distributed in the tropics and dry regions of the globe (6). Argasid ticks show diverse adaptation to using their hosts. Most members of the family are characterized by a single, prolonged larval blood feeding and multiple, short blood feeding events of subsequent developmental stages on several host individuals, however other adaptations (e.g., no larval feeding or lack of blood-feeding in adults, etc.) were recorded in certain species (11). By doing so, these ticks are capable of taking up pathogens (viral, bacterial, or protozoan) and transfering them to other hosts, thus they have important vectorial role (6). Most of soft ticks inhabit holes and crevices and have access to hosts only occasionally, hence they developed extreme adaptations to prolonged fasting and short feeding bouts whenever hosts are available (12). Their vectorial capacity for several important zoonotic diseases is well-known, including human relapsing fever (its causative agent transmitted by *Ornithodoros* spp.), tick-borne relapsing fevers (caused by several *Borrelia* spp. transmitted mainly by *Ornithodoros* and *Argas* spp.) or African swine fever (vectored by *Ornithodoros moubata*, *Ornithodoros porcinus*, *Ornithodoros erraticus*, or *Ornithodoros savignyi*) causing severe economic losses (6, 13).

Soft ticks have a special relationship with bats (Mammalia: Chiroptera). Bats are widely distributed, show high species diversity (being the second largest order of mammals) and several adaptations, which make them ideal host candidates for tick parasitism (14). Their morphological adaptations for flight hinders the range of their behavioral responses to reduce tick burden (e.g., their highly specialized limbs are inadequate for proper grooming), most species are social, spending their resting periods in dense groups and they are highly attached to their specific roosting sites, of which most are either underground (caves) or crevices in rocks or trees—excellent hiding places for soft ticks (11). Thus, several soft tick species-groups evolved specific associations with bat hosts. For example, all the known 17 species of the Nearctic soft tick genus *Antricola* (and *Parantricola*) are exclusive parasites of bats (15), together with all species belonging to the subgenus *Carios*, *Chiropterargas*, *Nothoaspis*, and *Reticulinasus,* and several other species from the genera *Alectorobius* and *Ornithodoros* (**Supplementary Material** and references therein). While most of these soft tick species are tropical in their distribution, there are at least five species which regularly occur on bats in the Western Palearctic. These species are *Carios vespertilionis*, *Chiropterargas boueti*, *Chiropteraragas confusus*, *Reticulinasus salahi*, and *Secretargas transgariepinus*. All these parasitize bats mainly roosting either inside caves (*Ch. boueti*, *Chiropteraragas confusus*, and *R. salahi*) or crevices (*Ca. vespertilionis* and *S. transgariepinus*).

Our knowledge on the distribution and ecology of bat-specialist soft tick species is scanty, as most of the literature only lists occurrence records or describe specific case reports, without a systematic review on their range, status and importance. Here, we collated the published records on these five soft tick species in the Western Palearctic, looking for data on their geographical distribution, host-parasite relationships and vectorial importance and also raising awareness on future challenges posed by some of these species on human health. In the wake of recent climate change events and urbanization trends in bats' distribution, we also intended to look for the abiotic (climate linked) and biotic (host distribution linked) factors regulating the distribution of bat specialist soft ticks in the Western Palearctic.

MATERIALS AND METHODS

Database Creation

Our methodology followed a three-step algorithm. First a database search was performed, using keywords as: "soft ticks," "bats," "Argasidae," and "Western Palearctic," "*Argas boueti*," "*Argas confusus*," "*Argas transgariepinus*," "*Argas vespertilionis*," and "*Ornithodoros salahi*" in the following databases: Web of Science, Zoological Record, Google Scholar, and Global Biodiversity Information Facility (www.gbif.org). Subsequently, copies of the original publications were obtained and the references cited in these works were traced. This process was repeated until no new references were found. In the third step we extracted each individual host-tick record from the references, noting the location, date, host and parasite species, development stage (for ticks) and pathogen (if) mentioned. These records were introduced into a database and individually georeferenced to create distribution maps.

Distribution Maps

For the maps, we overlaid the different hosts' range with the presence data for each tick species. Each host range was set with transparency, so the more ranges overlap, the more intense the range color is—a proxy for multiple host species presence. For host ranges of main bat host species we used the freely available shape files from the website of the International Union for Conservation of Nature (IUCN) Red List (16). IUCN ranges were used previously primarily for conservation biology of bats (17) or other mammals (18), but also for establishing the relationships between bats, insect ectoparasites and their vectored pathogens (19). In the following step, we intersected the ranges with the contour of the Western Palearctic. Western

TABLE 1 | Bat-specialist ticks recorded in the Western Palearctic.

	Free stages	Collected from host	Total number of host species	Number of primary host species	Number of secondary hosts	Non-bat host species	Unknown/Undefined host	Total
Chiropterargas boueti	2	16	14	14	0	1	1	19
Chiropterargas confusus	1	13	9	4	3	1	1	15
Secretargas transgariepinus	5	43	12	4	8	1	8	56
Carios vespertilionis	55	812	42	6	36	3	145	1,012
Reticulinasus salahi	2	15	4	1	2	1	1	18
TOTAL	65	899	44			3	156	1,120

Number of records with known hosts, free stages, and host-types.

TABLE 2 | Primary and secondary bat host species of soft ticks (Argasidae) in the Western Palearctic.

Tick species	Primary host species	Secondary host species	Non-bat hosts
Chiropterargas boueti	*Asellia tridens*, *Nycteris thebaica*, *Otonycteris hemprichii*, *Pipistrellus kuhlii*, *Pipistrellus christii*, *Rhinolophus clivosus*, *Rhinolophus mehelyi*, *Rhinopoma cystops*, *Rhinopoma microphyllum*, *Rousettus aegyptiacus*, *Tadarida aegyptiaca*, *Tadarida teniotis*, *Taphozous nudiventris*, *Taphozous perforatus*	–	*Homo sapiens*
Chiropterargas confusus	*Asellia tridens*, *Nycteris thebaica*, *Otonycteris hemprichii*, *Pipistrellus kuhlii*, *Rhinolophus ferrumequinum*, *Rhinopoma cystops*, *Tadarida aegyptiaca*, *Taphozous nudiventris*, *Taphozous perforatus*	–	*Allactaga euphratica*
Secretargas transgariepinus	*Eptesicus serotinus*, *Eptesicus isabellinus*, *Plecotus austriacus*, *Hypsugo savii*	*Myotis emarginatus*, *Myotis myotis*, *Myotis mystacinus*, *Otonycteris hemprichii*, *Pipistrellus nathusii*, *Plecotus christii*, *Rhinolophus ferrumequinum*, *Rhinopoma cystops*	*Homo sapiens*
Carios vespertilionis	*Eptesicus serotinus*, *Myotis mystacinus*, *Nyctalus noctula*, *Pipistrellus kuhlii*, *Pipistrellus nathusii*, *Pipistrellus pipistrellus*, *Vespertilio murinus*	*Asellia tridens*, *Barbastella barbastellus*, *Eptesicus isabellinus*, *Eptesicus nilssoni*, *Hypsugo savii*, *Miniopterus pallidus*, *Miniopterus schreibersii*, *Myotis alcathoe*, *Myotis bechsteinii*, *Myotis blythii*, *Myotis brandtii*, *Myotis dasycneme*, *Myotis daubentonii*, *Myotis emarginatus*, *Myotis myotis*, *Myotis nattereri*, *Nyctalus lasiopterus*, *Nyctalus leisleri*, *Otonycteris hemprichii*, *Pipistrellus maderensis*, *Pipistrellus pygmaeus*, *Plecotus auritus*, *Plecotus austriacus*, *Plecotus christii*, *Plecotus gaisleri*, *Rhinolophus ferrumequinum*, *Rhinolophus mehelyi*, *Rhinopoma cystops*, *Rousettus aegyptiacus*, *Tadarida teniotis*, *Taphozous nudiventris*, *Rhinolophus blasii*	*Homo sapiens*, *Canis familiaris*, *Picus viridis*
Reticulinasus salahi	*Rousettus aegyptiacus*	*Eptesicus serotinus*, *Taphozous perforatus*	*Homo sapiens*

Palearctic contour was delimited following the borders previously published (20, 21).

Host-Parasite Relationships

Using the database we mapped each host-parasite relationship and delimited the primary/accidental hosts. For deciding primary/accidental hosts of any soft tick species we used an arbitrary rule. Any bat species which held more than 5.0% of any specific soft tick's record is considered a primary host of the respective tick species, while hosts with <5.0 % of all cumulative records of a particular tick are considered accidental hosts, following a system previously proposed for bat-bat fly associations (22, 23).

RESULTS

The complete database contains altogether 1,151 entries (4,856 individual ticks), collected from 899 hosts (4,378 ticks), together

TABLE 3 | List of bat species (Chiroptera) and their role as primary and secondary soft tick (Argasidae) hosts in the Western Palearctic (*N*, number of hosts with ticks).

Bat species	*N*	Primary soft tick species	Secondary soft tick species
Asellia tridens	3	*Chiropterargas boueti*, *Chiropterargas confusus*	*Carios vespertilionis*
Barbastella barbastellus	6	–	*Carios vespertilionis*
Eptesicus isabellinus	8	*Secretargas transgariepinus*	*Carios vespertilionis*
Eptesicus nilssoni	13	–	*Carios vespertilionis*
Eptesicus serotinus	54	*Secretargas transgariepinus*, *Carios vespertilionis*	*Reticulinasus salahi*
Hypsugo savii	14	*Secretargas transgariepinus*	*Carios vespertilionis*
Miniopterus pallidus	1	–	*Carios vespertilionis*
Miniopterus schreibersii	3	–	*Carios vespertilionis*
Myotis alcathoe	4	–	*Carios vespertilionis*
Myotis bechsteinii	1	–	*Carios vespertilionis*
Myotis blythii	1	–	*Carios vespertilionis*
Myotis brandtii	11	–	*Carios vespertilionis*
Myotis dasycneme	17	–	*Carios vespertilionis*
Myotis daubentonii	3	–	*Carios vespertilionis*
Myotis emarginatus	4	–	*Carios vespertilionis*
Myotis myotis	12	–	*Carios vespertilionis*
Myotis mystacinus	34	*Carios vespertilionis*	–
Myotis nattereri	7	–	*Carios vespertilionis*
Nyctalus lasiopterus	4	–	*Carios vespertilionis*
Nyctalus leisleri	14	–	*Carios vespertilionis*
Nyctalus noctula	47	*Carios vespertilionis*	–
Nycteris thebaica	2	*Chiropterargas boueti*, *Chiropterargas confusus*	–
Otonycteris hemprichii	5	*Chiropterargas boueti*, *Chiropterargas confusus*	*Secretargas transgariepinus*, *Carios vespertilionis*
Pipistrellus kuhlii	34	*Chiropterargas boueti*, *Chiropterargas confusus*, *Carios vespertilionis*	–
Pipistrellus maderensis	8	–	*Carios vespertilionis*
Pipistrellus nathusii	52	*Carios vespertilionis*	–
Pipistrellus pipistrellus	297	*Carios vespertilionis*	–
Pipistrellus pygmaeus	26	–	*Carios vespertilionis*
Plecotus auritus	24	–	*Carios vespertilionis*
Plecotus austriacus	21	*Secretargas transgariepinus*	*Carios vespertilionis*
Plecotus christii	3	–	*Secretargas transgariepinus*, *Carios vespertilionis*
Plecotus gaisleri	2	–	*Carios vespertilionis*
Rhinolophus clivosus	1	*Chiropterargas boueti*	*Carios vespertilionis*
Rhinolophus ferrumequinum	7	*Chiropterargas confusus*	*Carios vespertilionis*
Rhinolophus mehelyi	2	*Chiropterargas boueti*	*Carios vespertilionis*
Rhinopoma cystops	6	*Chiropterargas boueti*, *Chiropterargas confusus*	–
Rhinopoma microphyllum	1	*Chiropterargas boueti*	–
Rousettus aegyptiacus	11	*Chiropterargas boueti*, *Reticulinasus salahi*	*Carios vespertilionis*
Tadarida aegyptiaca	3	*Chiropterargas boueti*, *Chiropterargas confusus*	*Carios vespertilionis*
Tadarida teniotis	3	*Chiropterargas boueti*	*Carios vespertilionis*
Taphozous nudiventris	7	*Chiropterargas boueti*, *Chiropterargas confusus*	*Carios vespertilionis*
Taphozous perforatus	3	*Chiropterargas boueti*, *Chiropterargas confusus*	*Reticulinasus salahi*
Vespertilio murinus	56	*Carios vespertilionis*	–

with a total of 65 cases of free ticks (involving 313 individuals), while collection circumstances were unknown for 156 cases (n = 165 ticks, only tick species and geographic location known). Altogether 44 bat species were recorded to host soft ticks, with most records noted for *Ca. vespertilionis* (**Table 1**). For a number of 16 cases the records mention only generic Chiroptera, while seven cases were assigned either to *Myotis* spp., *Pipistrellus* spp., or *Plecotus* spp. For 19 cases (1.9% of all records) the host is known, but it is not a bat species: 16 cases refer to humans, while one case each refer to a bird (*Picus viridis*), to a dog (*Canis familiaris*), while one to a rodent (*Allactaga euphratica*). Host species are listed in **Tables 2**, **3**. *Carios vespertilionis* had the most diverse host spectrum, with altogether 42 different host species (6 primary and 36 secondary hosts), *Ch. boueti* had the most

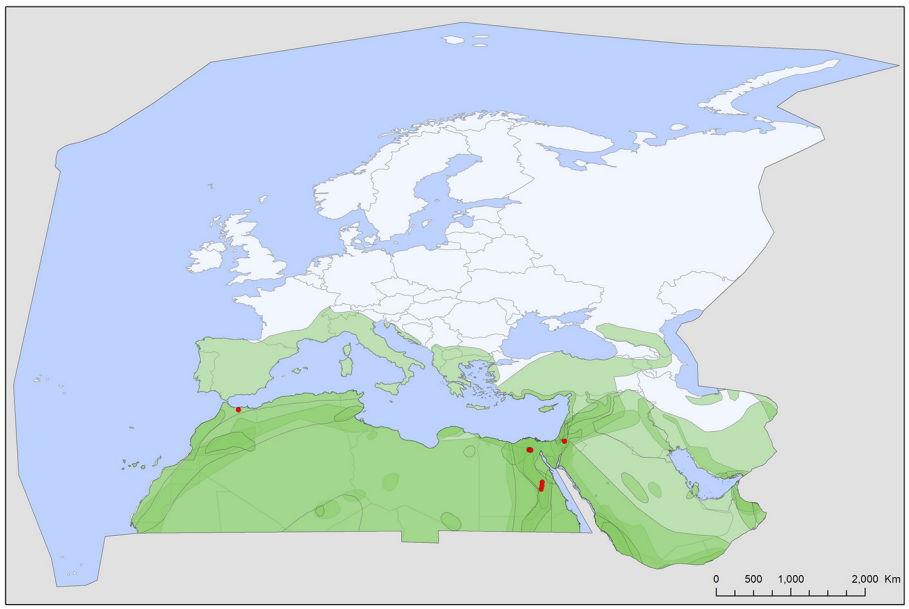

FIGURE 1 | Geographic distribution of *Chiropterargas boueti* records in the Western Palearctic, overlayed to the geographic ranges for the 14 bat species studied as main hosts (**Table 2**) of this tick. Transparent layers were mapped on top of one another to highlight regions with dense range overlap. Some species have additional range overlap in Africa and Central and South Asia.

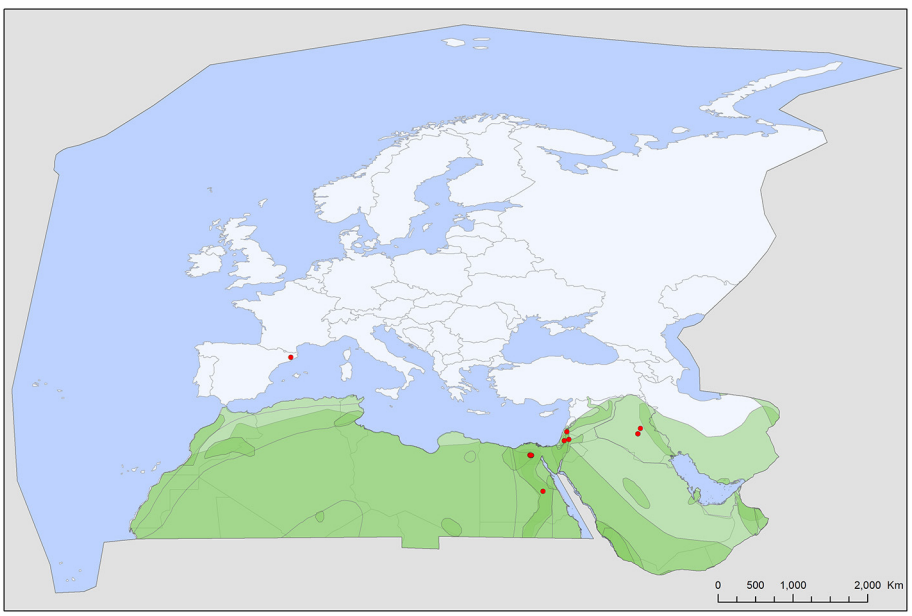

FIGURE 2 | Geographic distribution of *Chiropterargas confusus* records in the Western Palearctic, overlayed to the geographic ranges for the nine bat species studied as main hosts (**Table 2**) of this tick. Transparent layers were mapped on top of one another to highlight regions with dense range overlap. Some species have additional range overlap in Africa and Central and South Asia.

primary hosts (14), while *R. salahi* had a single primary host holding 87.7% of all records. Most ticks were recorded on crevice-dwelling bat species (76.6%), although for three species (*Ch. boueti, Chiropteraragas confusus*, and *R. salahi*) most primary bat hosts are cave-dwelling ones (24).

Most tick records refer to subadult stages (only larvae being recorded on hosts, 93.13% of all ticks collected), with adults (males $n = 25$, females $n = 67$) and nymphs ($n = 221$) being collected from the environment or known bat roosts. Significantly more *Ca. vespertilionis* (mean intensity: 5.99 CI:

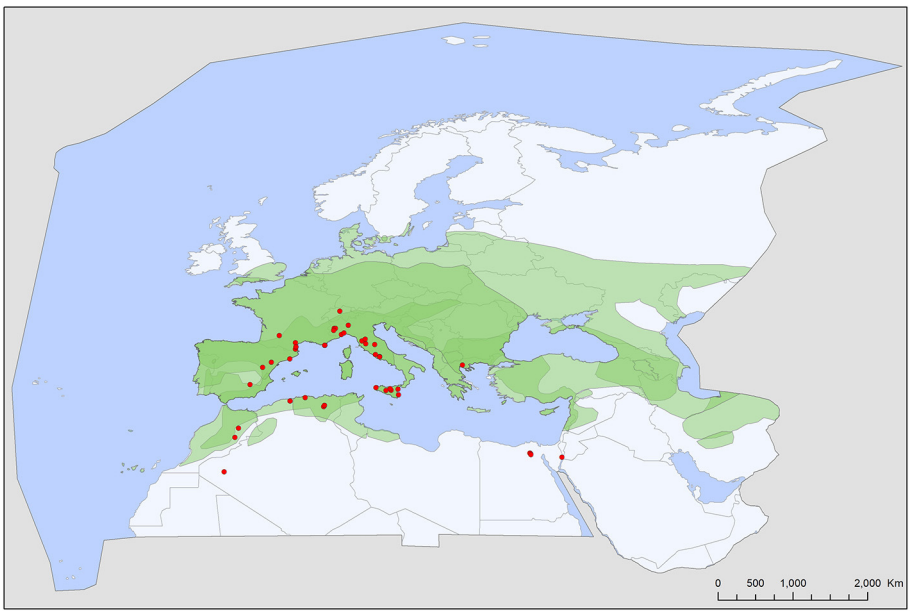

FIGURE 3 | Geographic distribution of *Secretargas transgariepinus* records in the Western Palearctic, overlayed to the geographic ranges for the four bat species studied as main hosts (**Table 2**) of this tick. Transparent layers were mapped on top of one another to highlight regions with dense range overlap. Some species have additional range overlap in Africa and Central and South Asia.

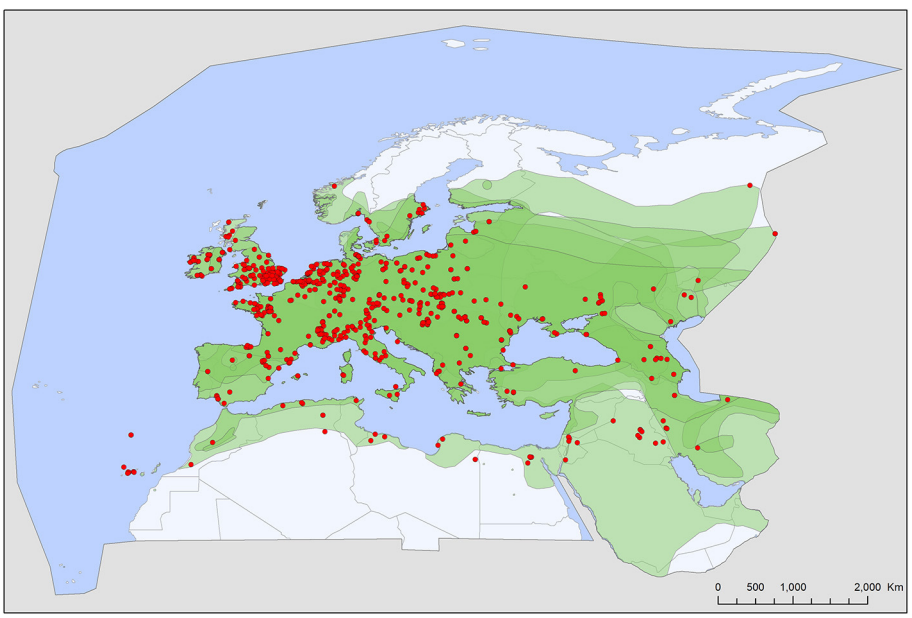

FIGURE 4 | Geographic distribution of *Carios vespertilionis* records in the Western Palearctic, overlayed to the geographic ranges for the seven bat species studied as main hosts (**Table 2**) of this tick. Transparent layers were mapped on top of one another to highlight regions with dense range overlap. Some species have additional range overlap in Africa and Central and South Asia.

1.9–18.3) were collected from members of the genus *Pipistrellus* than from any other host species ($x^2 = 21.0216$, $p < 0.001$).

Soft tick records showed a wide geographic distribution, covering most of the Western Palearctic, with significant differences between the extents of individual ranges. All five soft tick species show overlapping ranges in North Africa, most species (4/5) had a primarily Mediterranean range, with *Ch. boueti*, *Chiropteraragas confusus*, and *R.*

TABLE 4 | List of bacterial, protozoan, and viral pathogens identified in bat specialist soft ticks.

Pathogen group	Pathogen species	Reference
Secretargas transgariepinus		
Bacteria	*Rickettsia hoogstraalii*	(25, 26)
Viruses	Keterah (KTRO, nairoviruses)	(27)
Carios vespertilionis		
Bacteria	*Coxiella burnetii*	(28, 29)
	Ehrlichia sp. Av	(30)
	Ehrlichia sp. AvBat	(31)
	Rickettsia africae-like	(25)
	Rickettsia helvetica	(25)
	Rickettsia lusitaniae	(25)
	Rickettsia raoultii	(32)
	Rickettsia rickettsii	(32)
	Rickettsia sp. Av22	(25)
	Rickettsia sp. AvBat	(31)
	Rickettsia spp. (SFG group)	(30)
	Bartonella sp. Ia23	(25)
	Bartonella sp. Iv76	(25)
	Bartonella spp.	(19)
	Borrelia afzelli	(33)
	Borrelia burgdorferi s.l.	(34)
	Borrelia spp.	(35)
	Borrelia sp. CPB1 ("Relapsing Fever Group")	(31)
	Borrelia spp. ("Relapsing Fever Group")	(33)
Piroplasmida	*Babesia vesperuginis*	(30, 36–38)
	Babesia venatorum	(30)
Viruses	Issyk-Kul virus (IKV, nairoviruses)	(39–41)
	Keterah (KTRO, nairoviruses)	(27)
	Soft tick bunyavirus (STBV, nairoviruses)	(42)
	Sokuluk (SOKV, flaviviruses)	(41)
	Tick-borne encephalitis virus, (TBEV, flaviviruses)	(43)

salahi being exclusively found in North Africa and the Middle East (**Figures 1**, **2**, **5**). *Carios vespertilionis* and *S. transgariepinus* are distributed also in Europe (**Figures 3**, **4**). Most records of soft ticks came from bats caught in (or in immediate vicinity of) man-made structures (buildings, ruins, and underground channels: 66%), with 13.6% being collected from caves. The rest were collected from bats caught in diverse habitats (roost unknown) while hosts were in active flight.

Several viral, bacterial, and piroplasmid pathogens were identified in two soft tick species of bats. The most common groups were bacteria (*Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, and *Rickettsia* spp.), but also five different viruses (belonging to flaviviruses and nairoviruses), as well two piroplasmids (*Babesia* spp.) were identified in soft ticks of bats (**Table 4**).

DISCUSSION

A total of five different soft tick species (Acari: Argasidae: *Ca. vespertilionis*, *Ch. boueti*, *Ch. confusus*, *R. salahi*, and *S. transgariepinus*) were recorded to be specialized to bats of the Western Palearctic. These ticks were found on 44 different bat species, showing diverse host-pattern (**Figure 6**). Most records came from a single tick species (*Ca. vespertilionis*, 88.7% of all records, **Table 1**), which not only has the highest number of host species, but also the widest distribution, covering the whole region of the Western Palearctic (**Figure 4**). Argasid ticks of bats primarily parasitize crevice dwelling host species, although there are three tick species (*Ch. boueti*, *Chiropteraragas confusus*, and *R. salahi*), for which most of the primary hosts are cave-dwelling bats. Soft tick occurrences showed a wide geographical distribution, covering most of the Western Palearctic. However, significant differences were found between the extent of individual ranges, with the range of three species being limited to North Africa and the Middle East. While overlapping areas are small, there is a region (northeastern part of Egypt and Israel) where all five species occur (**Figures 1**–**5**).

There is a considerable overlap between primary hosts among the different soft tick species. One bat species (*Pipistrellus kuhlii*) is the primary host for three different tick species, while further nine bat species regularly harbor two different argasid species (**Table 2**; **Figure 6**). Most tick species show a distribution that considerably overlaps with the range of their primary bat host (**Figures 1**, **2**, **4**, **5**), with two notable exceptions. *Secretargas transgariepinus* shows a reduced range in comparison to its primary hosts' range, with several records in NE Africa, where primary hosts registered in the Western Palearctic do not occur (**Figure 3**). Records in this area came from bats exclusively distributed in Africa (*Rhinopoma* spp., *Taphozous* spp.), suggesting that on the African continent other primary hosts may occur. This species is well-known to regularly occur on bats performing large scale migrations like *Pipistrellus* spp. (24), hence several northern records may suggest accidental overshoots of argasid larvae collected from a bat in active migration (44). Another notable exception is the sole record of *R. salahi* in the Iberian Peninsula (**Figure 5**), far from the main range of its sole primary host, *Rousettus aegyptius*.

Specific Accounts

Chiropterargas boueti is a very poorly known species. Most information on this species was published in the original description (45), as well in its redescription (46). It has a wide distribution, primarily on the African continent, reaching Central and South Africa, with scattered records in Central Asia and the Middle East (47, 48). It is primarily a tick of cave dwelling tropical bats, with primary host species being *Rhinopoma* spp., with an extralimital occurrence in the Western Palearctic (**Figure 1**). Its ecology and vectorial capacity is unknown, while it is known to attack humans (46).

Chiropterargas confusus has a similar occurrence to the previous species, with which it shares most of its primary host species and also the occurrence records in the Western Palearctic (**Figure 2**). Its ecology and distribution are poorly known, with

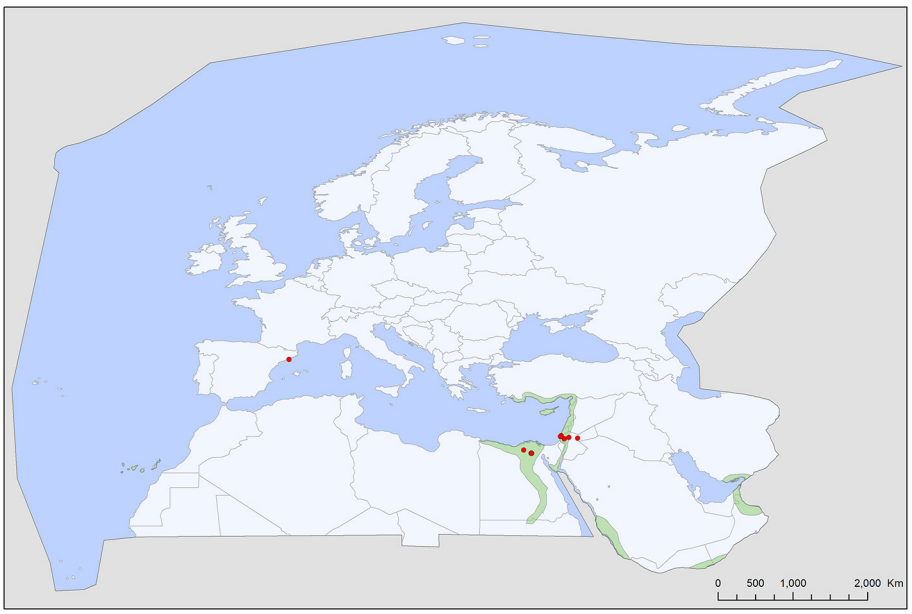

FIGURE 5 | Geographic distribution of *Reticulinasus salahi* records in the Western Palearctic, overlayed to the geographic ranges for of *Rousettus aegyptiacus*, the sole primary host of this tick. *R. aegyptiacus* range extends into to the tropical and subtropical regions of Africa and Asia.

only a handful of records listed in Northern, Eastern and Southern Africa and Central Asia (46, 48, 49). In the Western Palearctic, this species has a narrow range, with records in NE Africa and the Middle East. There is no published information on its vectorial role. There is a putative record of its occurrence on a non-bat host (50), suggesting its suitability as a more generalist tick species.

Secretargas transgariepinus has a primarily tropical African distribution, with scattered records in North Africa and the Mediterranean region of Europe (51). It is primarily a parasite of crevice-dwelling bats, commonly occurring on *Eptesicus* spp., *Hypsugo savii*, and *Plecotus* spp. in the region (**Tables 2**, **3**). The distribution of this argasid tick shows limited overlap with the range of its primary bat hosts in Europe, probably because its occurrence is limited by climatic factors (**Figure 3**). There is no clear seasonality in its records (**Figure 7**), and the apparent peak activity likely reflects an observation bias. The species is known for maternal care (52) and is a suspected vector (**Table 4**) for the Keterah virus (KTRO, nairoviruses) and spotted fever-causing bacteria of the genus *Rickettsia* (25–27). The species is regularly recorded on humans, with several cases known from Egypt and Italy (53).

Carios vespertilionis is the most common soft tick species of bats in the Western Palearctic (**Table 1**; **Figure 4**). It has the largest geographic distribution among bat ticks worldwide (54), with extensive morphological and genetic diversity along its wide range (36, 55). Its distribution mirrors the geographic range of the primary host species and it is the only soft tick species which may occur at the northernmost latitudes, wherever bats are present (**Figure 4**). It is also the species which has the highest number of records and known host species (**Tables 1**, **2**). The species primarily occurs on crevice dwelling species (26 out of the 42 recorded host species, **Table 2**), with a particular affinity toward *Pipistrellus* spp., members of which usually host high number of individual ticks. These ticks may exert behavioral or even pathological impacts on their hosts (56), especially if they occur in high numbers (57, 58). While only larvae were recorded on hosts, roost sites (especially artificial ones) are important locations for adults, too (56, 59). This species was recorded in each month (**Figure 8**), and the seasonal distribution of records shows a summer peak. However, we suggest that this is mainly related to the timing of bat-research efforts in the field, rather than to a true activity peak of the ticks. *Carios vespertilionis* was recorded in multiple instances on humans (53, 60) and also on other vertebrates (**Table 2**) (61, 62). This species is known vector of several bacterial, protozoan and viral pathogens (**Table 4** and references therein).

Reticulinasus salahi is the host specialist tick of the Egyptian fruit bat, *Rousettus aegyptiacus* (63). It occurs in the Western Palearctic only where its primary host is present (north-east corner of Africa and the Middle East, but missing from Cyprus, **Figure 5**). It's single European record came from an accidental host (64). There is no information published on its vectorial capacity, although several cases are known when humans were infested by this argasid species (53, 63).

Apart of the species listed above, a few accidental records refer to several other Palearctic soft tick species that may also accidentally infest bats, as exemplified by two bird-specialists (*Argas reflexus* and *Ornithodoros coniceps*) and a rodent specialist (*Ornithodoros tholozani*) (65–68).

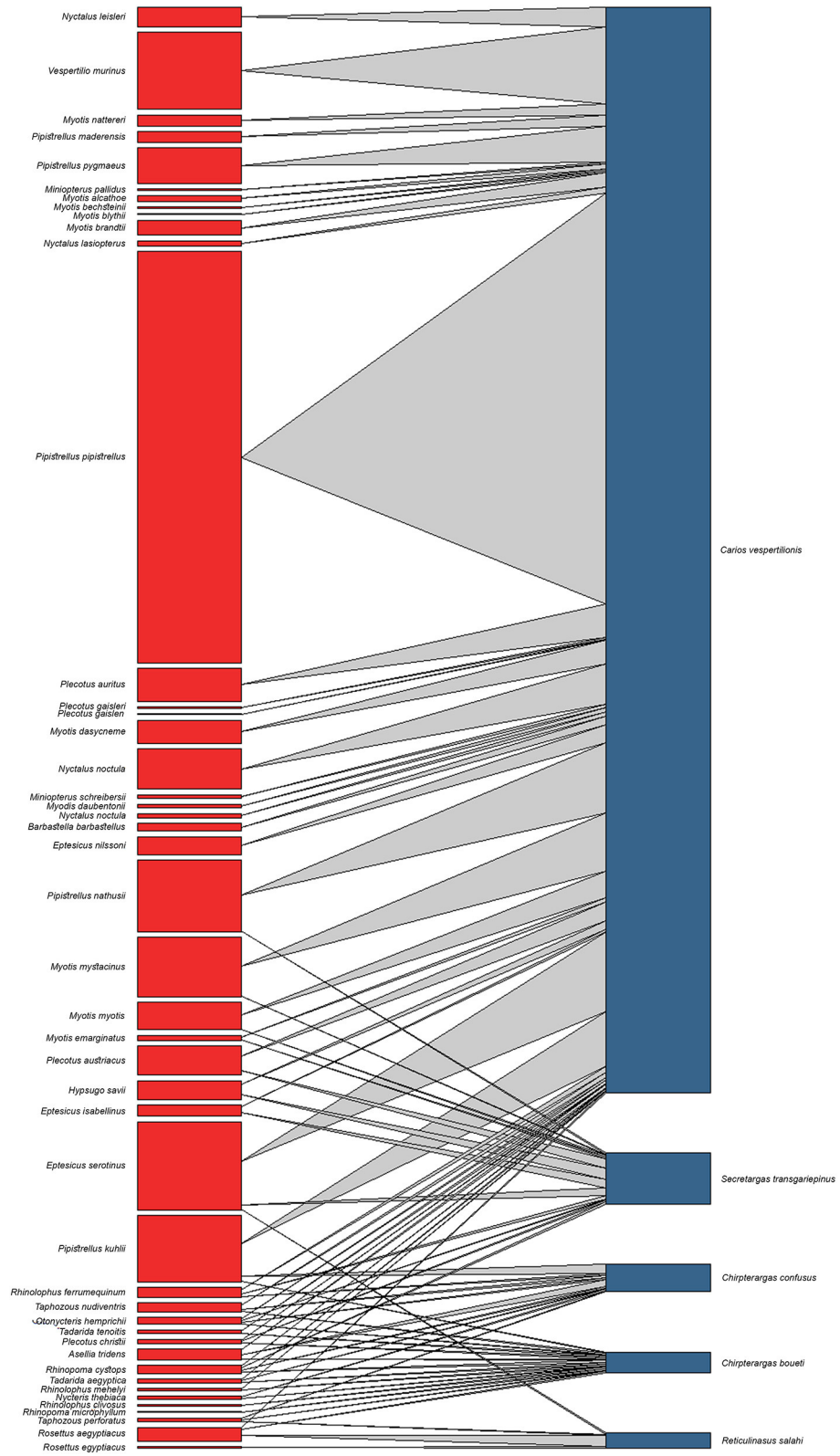

FIGURE 6 | Quantitative interaction web based on bat specialist Argasidae ticks and their respective bat hosts. Links between nodes represent the sum of individual tick occurrences for a given bat species.

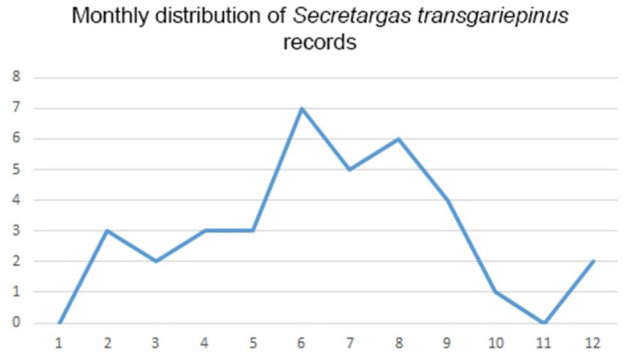

FIGURE 7 | Monthly distribution of collection dates for *Secretargas transgariepinus* ticks.

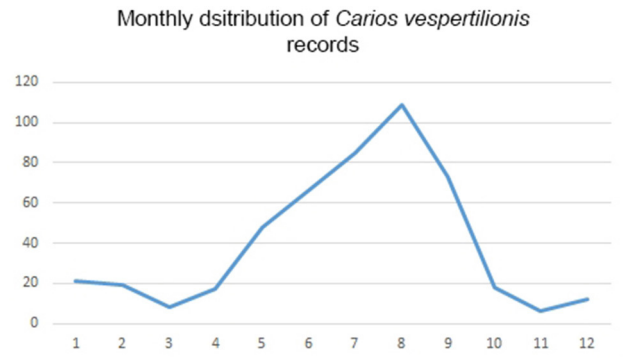

FIGURE 8 | Monthly distribution of collection dates for *Carios vespertilionis* ticks.

Two out of the five bat-specialist soft ticks recorded in the Western Palearctic have a wide range. These species (*Ca. vespertilionis* and *S. transgariepinus*) are parasites of crevice-dwelling species in the Western Palearctic and both have a wide palette of primary and accidental host species (**Table 1**; **Figure 6**). Their host species are small to middle sized insectivorous bats, which do not depend on the accessibility of large underground roost sites and regularly roost is small groups, actively seeking anthropogenic shelters (24). As these bat species (chiefly *Pipistrellus* spp., the group of small *Myotis*, *Nyctalus noctula*, *Plecotus* spp. and *Eptesicus* spp.) are feeding mainly on flying small moths and dipterans (24), they easily can find food and shelter even in the most urbanized areas of the region. Hence, it is not a surprise that these species show increase both in their range and populations. In addition, they are among the few bat species which became true urban dwellers (69). Especially large urban settings offer to these species not only hunting areas (70) and roost sites in the active period, but also suitable hibernating areas. During the last decades it has become an increasing trend for several such bat species to use large buildings (e.g., multistorey office buildings and block of flats) for autumn congregations or wintering sites in major cities (71). This tendency increased not only the number of these bats inside highly urbanized areas (72), but also the contacts with humans (73, 74). These bat species regularly harbor soft ticks (while their roosts offer habitat for adult ticks), and both *Ca. vespertilionis* and *S. transgariepinus* are known to be competent vectors for a series of viral, bacterial and protozoan pathogens (**Table 4**), some of which are zoonotic. While *S. transgariepinus* is currently a rare species in the Western Palearctic, whose range is seemingly limited by climatic factors, increasing temperatures in the near future may favor further range extension for this species, especially as its hosts will possibly have broader distribution. If these trends will continue in the near future, the increasing presence of bats and their soft ticks may pose a new epidemiologic challenge in highly urbanized areas.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AS, AM, and SH designed the study and acquired the budget. AS and ÁP screened the reference publications and built the database. CD analyzed the data and created the maps. AS wrote the manuscript. All authors performed critical revision and approved the final manuscript.

FUNDING

This research was supported by the by NKFIH-132794 (to SH and AS). ÁP was supported by the Collegium Talentum Programme of Hungary and by the NTP-NFTÖ-20 grant. While working on this project, AS was supported by the János Bolyai Research Scholarship of Hungarian Academy of Science and the ÚNKP 19-4-ÁTE-10 New National Excellence Program of the MIT.

ACKNOWLEDGMENTS

We thank for help in gathering literature for Tamara Szentiványi and Mohamed L. Bendjeddou. The content of this manuscript has been published in part, as a chapter in the dissertation of ÁP.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.684737/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 23 June 2021
doi: 10.3389/fvets.2021.704399

Ornithodoros puertoricensis (Ixodida: Argasidae) Associated With Domestic Fowl in Rural Dwellings From Córdoba Department, Caribbean Colombia

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OPEN ACCESS

Edited by:
Damer Blake,
Royal Veterinary College (RVC), United Kingdom

Reviewed by:
Sergio Eduardo Bermúdez, Gorgas Memorial Institute of Health Studies, Panama
Jose Reck, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, Brazil

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Specialty section:
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

Received: *02 May 2021*
Accepted: *25 May 2021*
Published: *23 June 2021*

Citation:
López Y, Robayo-Sánchez LN, Muñoz-Leal S, Aleman A, Arroyave E, Ramírez-Hernández A, Cortés-Vecino JA, Mattar S and Faccini-Martínez ÁA (2021) Ornithodoros puertoricensis (Ixodida: Argasidae) Associated With Domestic Fowl in Rural Dwellings From Córdoba Department, Caribbean Colombia. Front. Vet. Sci. 8:704399. doi: 10.3389/fvets.2021.704399

Ticks of genus *Ornithodoros* are nidicolous parasites associated with a wide array of vertebrates. In humans, their bites cause hypersensitivity reactions and are capable to transmit pathogens of health concern. In the department of Córdoba, Caribbean region of Colombia, the first report of an *Ornithodoros* soft tick was made in 1980 by Betancourt, who described the collection of *Ornithodoros talaje* in human dwellings. Nevertheless, current the records of *O. talaje* made in South America have been questioned and likely correspond to misidentifications with morphologically similar species. Between October and December of 2020, we visited rural areas of four localities from three municipalities within the department of Córdoba: Cuero Curtido and Severá (municipality of Cereté), El Espanto (municipality of Planeta Rica), and Arroyo Negro (municipality of San Carlos). Search for soft ticks was performed in 46 human domiciles and peridomiciliary areas. We searched in areas frequented by domestic animals, inspecting cracks in the walls and fowl nests. Infestation by soft ticks was found in 13% (6/46) of visited houses. Overall, 215 ticks were collected (26 larvae, 144 nymphs and 45 adults) from nests of domestic birds or in the adjacent walls. Larvae, nymphs and adults were morphologically identified as *Ornithodoros puertoricensis*. Molecular identification of ticks was confirmed by sequencing the tick mitochondrial 16S gene of adults, pools of nymphs and larvae. Pairwise comparisons showed a 99% of identity with *O. puertoricensis* from Panama. This study reports for the first time *O. puertoricensis* associated with domestic fowl in rural dwellings in Colombia, and expands the geographical distribution of this tick species toward the Córdoba department. Importantly, local people described exposure to tick bites while sleeping in infested houses; therefore, the transmission of soft tick-borne pathogens is now of concern in the region.

Keywords: soft ticks, parasites, fowl nests, domiciliary infestation, *Ornithodoros*, Colombia

INTRODUCTION

Ticks of the genus *Ornithodoros* are nidicolous arthropods that parasite a wide range of vertebrates, such as reptiles, birds and mammals, including humans (1). The saliva of *Ornithodoros* ticks can cause toxicosis (hypersensitivity and immunological response ranging from mild dermal lesions to systemic disease) (2) and transmit pathogenic agents, such as tick-borne relapsing fever (TBRF) group borreliae, to humans and domestic animals (3). Particularly in South America, *Ornithodoros brasiliensis*, *Ornithodoros fonsecai*, *Ornithodoros mimon*, *Ornithodoros rietcorreai*, *Ornithodoros rioplatensis*, *Ornithodoros rostratus*, and *Ornithodoros spheniscus* cause toxicosis (4–10). Moreover, human cases of TBRF were described during the first decade of the 20th century mainly in Colombia and Venezuela, and *Ornithodoros rudis* was involved as the vector (11–13). Interestingly, in the last two decades, studies have molecularly identified putative novel species of relapsing fever group borreliae in countries without reported cases, such as Chile and Brazil (14, 15).

In Colombia, while the study of TBRF vanished decades ago, 10 species of *Ornithodoros* are known to occur, namely *Ornithodoros azteci*, *Ornithodoros furcosus*, *Ornithodoros hasei*, *Ornithodoros marinkellei*, *Ornithodoros marmosae*, *Ornithodoros peropteryx*, *Ornithodoros puertoricensis*, *Ornithodoros rossi*, *Ornithodoros talaje,* and *Ornithodoros yumatensis* (16). Noteworthy, *O. rudis*, *O. furcosus*, and *O. puertoricensis* have been reported infesting human dwellings in the country (17–19). Adults of some Neotropical soft ticks are morphologically similar, and early reports of these three species was subject of confusion in Central and South America (16, 17). Therefore, old reports of *Ornithodoros* spp. made in Colombia need confirmation.

Ornithodoros puertoricensis was reported in Colombia for the first time in Ayacucho, in Cesar department, Caribbean region (20). Several years later, Betancourt (21) made the sole record of an *Ornithodoros* soft tick in the department of Córdoba (also in the Caribbean region). Specifically, the specimens were collected inside human dwellings at San Carlos municipality. At that time, the ticks were sent to the University of California and identified as *O. talaje* (21). Currently, the records of *O. talaje* made in South America have been questioned (16, 22) and likely correspond to misidentifications with morphologically similar species of the group. Therefore, the species reported by Betancourt (21) remains to be confirmed.

Fowl are common host for soft ticks of genus *Argas* (23) and there is evidence that *Ornithodoros* spp. ticks do parasitize domestic birds in Central America (20). Although 10 species of *Ornithodoros* occur in Colombia, fowl have never been implicated as hosts.

We carried out a prospective study in order to confirm the presence and identity of neglected *Ornithodoros* in rural dwellings from the department of Córdoba, including the San Carlos municipality. Our results demonstrate that soft ticks do occur in that region of Colombia and infest fowl nests.

MATERIALS AND METHODS

Geographic Area and Sampling Sites

Between October and December of 2020, we visited rural areas of four localities in three municipalities within the department of Córdoba, Colombia: Cuero Curtido ("locality 1") and Severá ("locality 3") (N 08°55'53" –W 75°57'31" and N 08° 90'77" –W 75°87'54", respectively) in the municipality of Cereté; El Espanto ("locality 2") (N 08° 31'46" –W 75°39'51") in the municipality of Planeta Rica; and Arroyo Negro ("locality 4") (N 08°42 '46" –W 75°40'21") in the municipality of San Carlos (**Figure 1**).

The three municipalities share similar environmental conditions (12–87 m of altitude, temperature between 25 and 28°C, and average relative humidity of 81%) (24–26). Municipality of Cereté has a population of 105,815 inhabitants (26), Planeta Rica of 64,205 inhabitants (24), and San Carlos of 23,532 inhabitants (25). The four visited localities were selected by convenience criteria, considering the easiness of access, and the researchers' safety regarding public order conditions in the area.

Searches for *Ornithodoros* ticks were performed in 46 human domiciles and peridomiciliary areas (eight houses in locality 1, three in locality 2, eight in locality 3, and twenty-seven in locality 4). The houses were constructed with wood, "bahareque" (clay or mud with sticks or canes), and "guadua" (woody bamboo) in their walls, reinforced with cardboard. All had dirt floors and palm leaves as roofs. The presence of domestic animals such as dogs, cats, pigs, chickens, turkeys, and ducks was common in all investigated houses, and the animals circulated freely inside the houses and in the peridomiciliary area. We examined domestic animals' resting areas, chicken coops, and nests of chickens, ducks and turkeys. Around these areas, cracks in the walls and nest debris were inspected. Inspections inside the inhabitants' rooms were not allowed for privacy reasons in most of the houses.

Tick Collection and Identification

While larvae, nymphs, adults and exuviae were collected in 70% ethanol, eggs were maintained alive, transported to the laboratory, and kept in darkness inside an incubator (25°C, 80% relative humidity). Hatched larvae were mounted onto slides with Hoyer's medium and examined by light microscopy for morphological identification (27). A subset of adults was prepared for scanning electron microscopic examinations for greater definition of morphological characteristics. Larvae were morphologically identified following taxonomical keys (28). Morphological identification of adults and nymphs was done with original descriptions of *Ornithodoros* spp. (27, 29).

Molecular and Phylogenetic Analyses

Morphological identification of ticks was complemented by molecular analysis. For this purpose, DNA extraction (QIAGEN DNeasy Blood & Tissue kit) was performed on individual adults, and larvae and nymphs of each locality were pooled (up to 10 larvae, up to 4 nymphs). Successful extractions were confirmed by PCR targeting the tick mitochondrial 16S gene for each sample

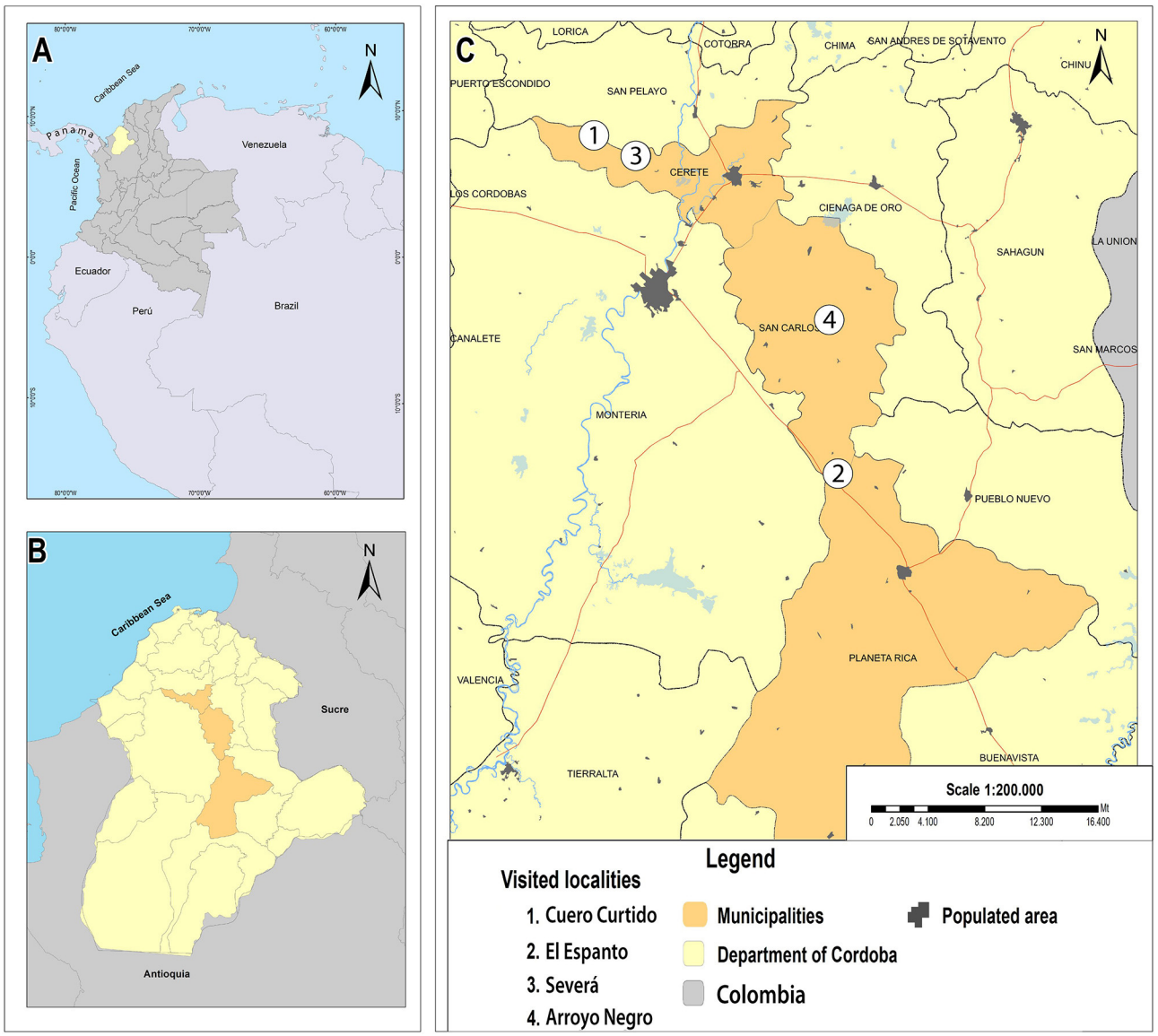

FIGURE 1 | Map showing the collection locations. **(A)** Map of South America showing the location of Córdoba department within Colombia. **(B)** Map of Córdoba department showing the investigated municipalities. **(C)** Studied localities within the Cereté, San Carlos, and Planeta Rica municipalities.

with primers described elsewhere (30). Amplicons of expected size were Sanger-sequenced at the Molecular Genomics Core of the University of Texas Medical Branch (Galveston, TX). Obtained sequences were assembled with Geneious (31) and the consensuses compared with sequences available in GenBank using BLASTn (32).

An alignment with 33 sequences of Argasidae retrieved from GenBank was constructed in MAFFT (33). A phylogenetic analysis using the approximately maximum likelihood method was implemented in FastTree 2 (34), selecting the GTR model and five rates categories of sites. *Ornithodoros brasiliensis* (GU198363) and *Ornithodoros rostratus* (DQ295780) sequences rooted the tree.

RESULTS

Tick Collection

Infestation by *Ornithodoros* ticks was found in 12% (1/8) of the visited houses in locality 1, in 33% (1/3) of locality 2, in 25% (2/8) of locality 3, and 7% (2/27) of locality 4. Overall, 215 ticks were collected (26 larvae, 144 nymphs, 13 females, and 32 males) (**Table 1**). All were found in fowl nests and on adjacent walls. Briefly, 48 ticks were collected in locality 1, of which eight were adults (males) and 40 were nymphs, all collected in a hen's nest in the kitchen of a house with a dirt floor and cardboard-reinforced-wooden walls (**Figures 2C,D**). In locality 2 we found exuviae during inspection of the substrate (soil/sand) of a chicken nest

TABLE 1 | *Ornithodoros* spp. collected in rural dwellings from Córdoba department, Colombia.

Municipality	Locality	Collection area	Total of collected specimens	Number of ticks submitted to DNA extraction	Individual/pool DNA extraction
Cereté	1 (Cuero Curtido)	Hen's nest	8♂, 40N	8♂	Adults individually
Cereté	3 (Severá)	Chicken nests	21♂, 6♀, 94N	3♂, 4♀, 10N	Adults individually/3 pools (one of 2N; two of 4N)
San Carlos	4 (Arroyo Negro)	Bahareque walls	1♂, 3♀, 10N	1♂, 3♀, 10N	Adults individually/6 pools (three of 1N; two of 2N, one of 3N)
Planeta Rica	2 (El Espanto)	Bahareque wall	2♂, 4♀, 26L	2♂, 3♀, 10L	Adults individually/1 pool of 10L
Total			215	54	34

♂, *Males;* ♀, *Females; N, nymphs; L, Larvae.*

FIGURE 2 | Dwellings where soft ticks were collected in the Córdoba department, Colombia. **(A)** Chickens lying adjacent to an external bahareque wall in locality 4 (Arroyo Negro); **(B)** Bahareque construction; note the crevices on the wall and the bird nest (arrow) in locality 2 (El Espanto); **(C)** chicken nest adjacent to the wall inside a dwelling in locality 1 (Cuero Curtido); **(D,F)** *Ornithodoros* ticks (arrow) collected in fowl nest debris in locality 1 (Cuero Curtido) and locality 3 (Severá), respectively; **(E)** *Ornithodoros* ticks (arrow) collected in between bahareque debris from a dwellings wall in locality 2 (El Espanto).

adjacent to an external wall of a warehouse made of bahareque (**Figure 2B**). In this wall we collected 32 ticks (four females, two males, 26 larvae and a group of eggs) (**Figure 2E**). Interestingly, the owners of the warehouse recognized the ticks, named them as "Pitos," and also referred to bites of these arthropods while sleeping. In locality 3 a total of 121 ticks [27 adults (six females, 21 males) and 94 nymphs] were collected in chicken nests made of dried-banana-leaves, found in a warehouse and a kitchen, respectively, of two houses with guadua walls, dirt floor, and palm roof (**Figure 2F**). In locality 4, 14 ticks [four adults (three females and one male) and 10 nymphs] were collected in bahareque walls adjacent to chicken nests, in a warehouse and a room of two houses, respectively (**Figure 2A**).

Remarkably, domiciles where we found ticks were comparatively rudimental and precarious; had poor hygiene, and sanitary conditions; dirt in the most of their floors, and no record of recent pest chemical control.

Morphological and Genetic Identification of Specimens

Based on the examination of 10 slide mounted specimens, larvae were identified morphologically as *O. puertoricensis* because of the following combination of traits: dorsal plate pyriform; 17–18 pairs of dorsal setae (seven anterolateral, 6–7 posterolateral and four central); hypostome pointed, with dentition formula 3/3 in middle length (**Figures 3A–C**).

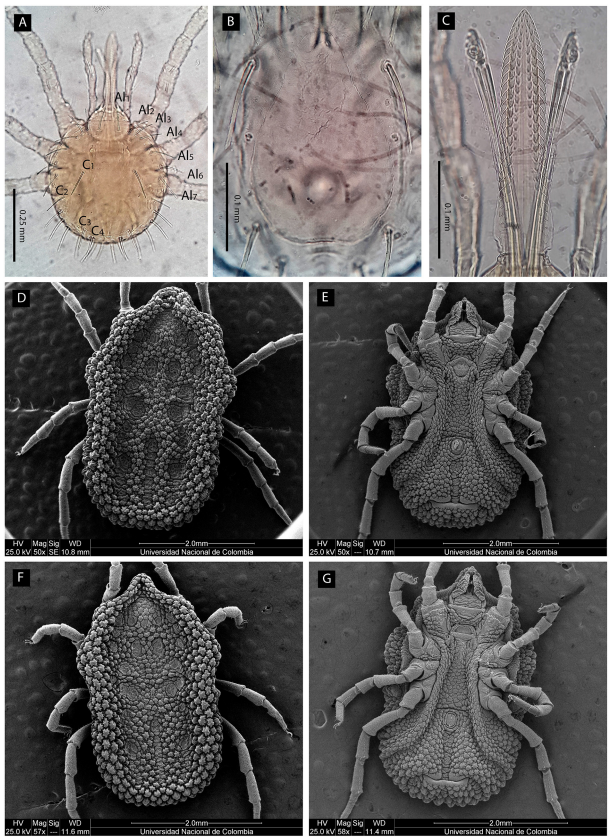

FIGURE 3 | Optical and scanning electron microscopic micrographs of *O. puertoricensis* collected in Córdoba department, Colombia. Larva: **(A)** dorsal view, **(B)** dorsal plate, and **(C)** hypostome. Female: **(D)** dorsal and **(E)** ventral views. Male: **(F)** dorsal and **(G)** ventral views. Al, anterolateral setae; C, central setae.

Nymphs and adults were identified morphologically as the same species by the combination of the following characters: presence of cheeks, conical mammillae, anteromedian disk present, posteromedian file of disks merging with median disk (**Figures 3D–G**). Examined larvae and adults were deposited in the "Colección Parasitológica Veterinaria Julio Mario Rodríguez Peña" at the Universidad Nacional de Colombia (UNAL: CPV-UN: 2021ACAR001–003). Genetic identification of ticks was performed individually for 14 males, 10 females, nine pools of nymphs and one pool of larvae collected in the four localities (**Table 1**). Two haplotypes with two polymorphisms consisting of adenine-guanine transitions were retrieved (99.5% of identity between them). Both sequences were 99.2–99.7% identical to *O. puertoricensis* from Panama available in GenBank (KX685710) (35). Haplotype I was found in 28 pools of ticks (14 males, nine females and five pools of nymphs) from the four localities, while haplotype II was only found in six pools of ticks (one female, four pools of nymphs and one pool of larvae) from two of the localities (El Espanto and Arroyo Negro). Tick mitochondrial 16S rDNA sequences of *O. puertoricensis* generated in this study were deposited in GenBank under accession numbers MZ005589 and MZ005590.

The phylogenetic analysis determined that *O. puertoricensis* from the Córdoba department in Colombia are closely related to a homologous species from Panama. A previous sequence of *O. puertoricensis* from Haiti (AF113932) branches basally to the Colombian and Panamanian ticks. Collectively, the sequences of *O. puertoricensis* cluster as a sister group to *Ornithodoros cerradoensis* (**Figure 4**). This topology is consistent with previous phylogenies including the *O. talaje* group (27).

DISCUSSION

This study reports for the first time *O. puertoricensis* infesting domestic fowl nests in rural dwellings in Colombia, and expands the geographical range of this tick species to the Córdoba department. However, *O. puertoricensis* is no stranger, since it has been reported in the northwestern region of the country. The first record of *O. puertoricensis* in Colombia was published by Fairchild in 1966, referring to adult specimens collected in the Ayacucho municipality, in Cesar department (20). Later, in 2009, Paternina et al. described the finding of larvae infesting dogs in the Sucre department (36), and recently, larvae and females were described infesting synanthropic rodents (i.e., *Mus*, *Rattus*) and human dwellings in Urabá region, in the Antioquia department (19, 37, 38). In addition, Butler and Gibbs (39) stated that *O. puertoricensis* occurs in the Colombian pacific region as well.

Ornithodoros puertoricensis was described based on larvae collected on rats (*Rattus* spp.) in Puerto Rico (40). Moreover, amphibians, reptiles and mammals host larvae of *O. puertoricensis* in Jamaica (*Herpestes javanicus*, *Nectomys* sp., and *Proechimys* sp.), Panama (*Rattus* sp., *Sylvilagus brasiliensis*, *Eyra barbara*, *Dasyprocta punctata*, *Felis silvestris catus*, *Rhinella marina*, *Varanus dumerilii*, *Python regius*, *Python bivittatus*, and *Homo sapiens*), Trinidad (*Proechimys trinitatus* and *Nectomys squamipes*), Nicaragua (*Dasyprocta punctata* and *Didelphis marsupialis*), Venezuela (*Proechimys guyannensis*, *Proechimys semispinosus*, *Dasyprocta fuliginosa*, *Sigmodon alstoni*, *Zygodontomys brevicauda*, *Sylvilagus floridanus*, *Tamandua tetradactyla*, *Conepatus semistriatus*, *Monodelphis brevicaudata*, *Marmosa robinsoni*, *Artibeus lituratus*, *Iguana* sp., and unidentified lizard), and Puerto Rico (*Felis silvestris catus*) (20, 35, 41–46). In particular, the sole record of *O. puertoricensis* associated with birds comes from Mexico, where larvae were collected on *Speotyto cunicularia* (29). Meanwhile, Dunn in 1931 collected larvae and adults of *O. talaje* on chickens and chicken coops in the Panama City market (20). Nevertheless, records of *O. talaje* in Panama prior to 1947 are questionable because of morphological confusion with *O. puertoricensis* (20, 40). Dunn's descriptions are similar to our findings in that they suggest that *Ornithodoros* soft ticks do associate with domestic fowl. Even though in the present study we did not collect larvae feeding on animals, it is likely fowl could maintain the biological cycle of *O. puertoricensis* in the visited localities, since eggs, larvae, nymphs and adults were taken from their nests. Thus, our results suggest

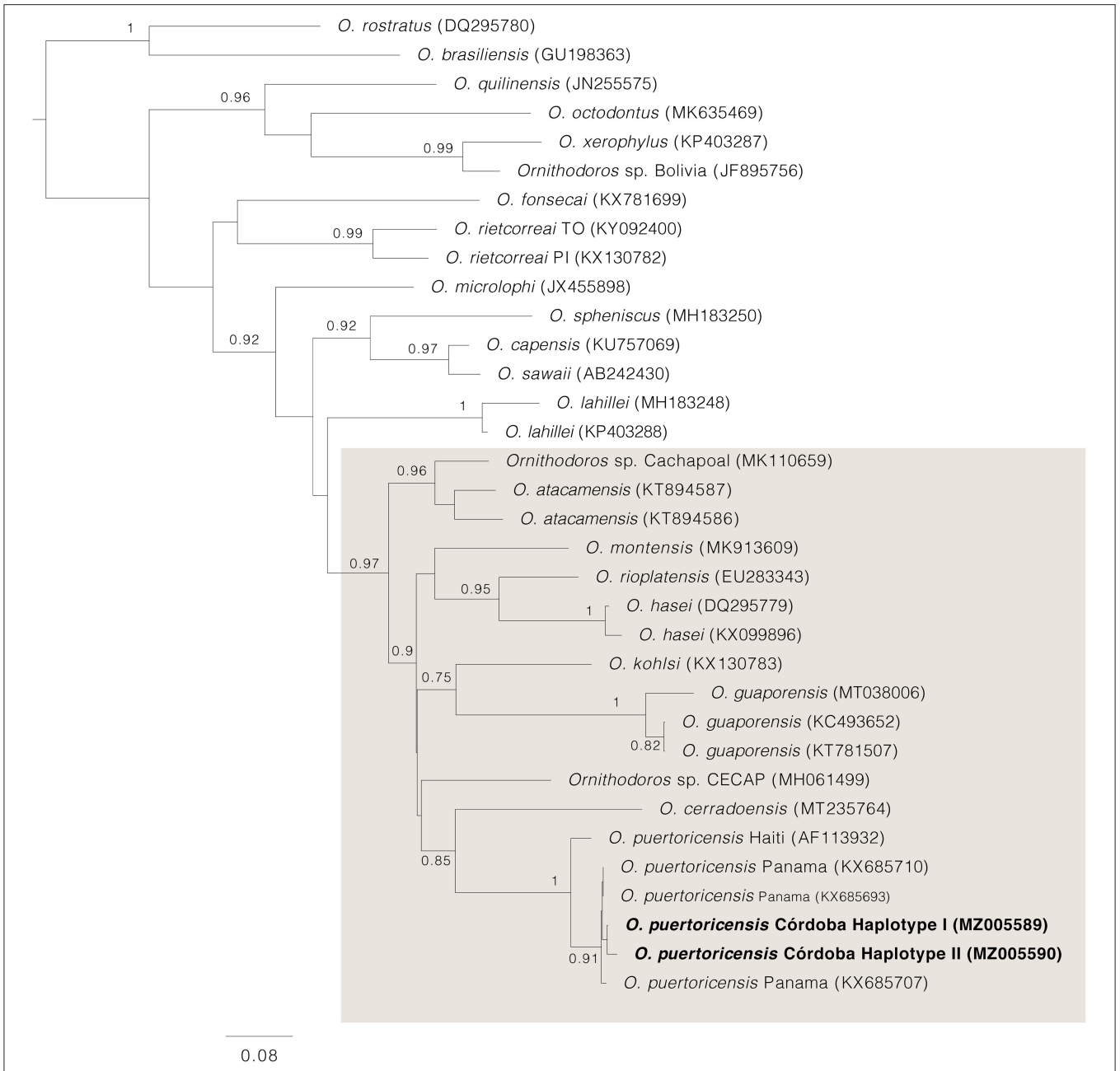

FIGURE 4 | Approximately maximum likelihood phylogenetic tree for a subset of *Ornithodoros* spp. Support values >0.75 are shown above or below main branches. The position of *O. puertoricensis* collected at Córdoba department is highlighted in bold. The *O. talaje* group is boxed in gray.

that the previous report of *O. talaje* made by Betancourt (21) at the same locations probably corresponds to *O. puertoricensis*.

Ornithodoros puertoricensis do infest human dwellings in Panama and Colombia (19, 35) and mild toxicosis was reported after their bites (35). Inside houses, *O. puertoricensis* seems to parasitize humans during the night (35). Indeed, during our investigations, the inhabitants of infested houses recognized the ticks and reported nocturnal parasitism as well. Interestingly, the common name for *O. puertoricensis* in the region (i.e., "Pito"), is the same one assigned to triatomine bugs, vectors of Chagas disease in Colombia (47). To acknowledge this coincidence in common names is valuable from an epidemiological point of view and in further inquiries looking for soft ticks infesting domiciliary environments in the region. Finally, evidence that *O. puertoricensis* transmits human pathogens is currently lacking. However, the role as a vector should not be ruled out, since other *Ornithodoros* ticks are reservoirs of TBRF spirochetes of human health concern.

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 at: https://www.ncbi.nlm.nih.gov/genbank/, MZ005589; https://www.ncbi.nlm.nih.gov/genbank/, MZ005590.

AUTHOR CONTRIBUTIONS

YL, SM-L, SM, and ÁF-M designed the initial study. YL, AA, and ÁF-M carried out the field work. YL, SM-L, LR-S, AR-H, and JC-V performed the tick identification and tick processing. YL, SM-L, EA, and ÁF-M implemented the molecular analysis. YL, SM-L, and ÁF-M wrote the first draft of the manuscript. All authors contributed to data interpretation and revisions.

FUNDING

ÁF-M was supported by a Fogarty fellowship under Grant No. 2D43TW010331.

ACKNOWLEDGMENTS

We thank Dr. David H. Walker for providing constructive reviews of the manuscript. We are grateful to Yeimi López for preparing the maps of **Figure 1**.

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ORIGINAL RESEARCH
published: 06 August 2021
doi: 10.3389/fphys.2021.725635

Transcriptomic Analysis of Salivary Glands of *Ornithodoros brasiliensis* Aragão, 1923, the Agent of a Neotropical Tick-Toxicosis Syndrome in Humans

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: 15 June 2021
Accepted: 12 July 2021
Published: 06 August 2021

Citation:
Reck J, Webster A, Dall'Agnol B, Pienaar R, de Castro MH, Featherston J and Mans BJ (2021) Transcriptomic Analysis of Salivary Glands of Ornithodoros brasiliensis Aragão, 1923, the Agent of a Neotropical Tick-Toxicosis Syndrome in Humans. Front. Physiol. 12:725635. doi: 10.3389/fphys.2021.725635

Tick salivary glands produce and secrete a variety of compounds that modulate host responses and ensure a successful blood meal. Despite great progress made in the identification of ticks salivary compounds in recent years, there is still a paucity of information concerning salivary molecules of Neotropical argasid ticks. Among this group of ticks, considering the number of human cases of parasitism, including severe syndromes and hospitalization, *Ornithodoros brasiliensis* can be considered one of the major Neotropical argasid species with impact in public health. Here, we describe the transcriptome analysis of *O. brasiliensis* salivary glands (ObSG). The transcriptome yielded ~14,957 putative contigs. A total of 368 contigs were attributed to secreted proteins (SP), which represent approximately 2.5% of transcripts but ~53% expression coverage transcripts per million. Lipocalins are the major protein family among the most expressed SP, accounting for ~16% of the secretory transcripts and 51% of secretory protein abundance. The most expressed transcript is an ortholog of TSGP4 (tick salivary gland protein 4), a lipocalin first identified in *Ornithodoros kalahariensis* that functions as a leukotriene C_4 scavenger. A total of 55 lipocalin transcripts were identified in ObSG. Other transcripts potentially involved in tick-host interaction included as: basic/acid tail secretory proteins (second most abundant expressed group), serine protease inhibitors (including Kunitz inhibitors), 5' nucleotidases (tick apyrases), phospholipase A_2, 7 disulfide bond domain, cystatins, and tick antimicrobial peptides. Another abundant group of proteins in ObSG is metalloproteases. Analysis of these major protein groups suggests that several duplication events after speciation were responsible for the abundance of redundant compounds in tick salivary glands. A full mitochondrial genome could be assembled from the transcriptome data and confirmed the close genetic identity of the tick strain sampled in the current study, to a tick strain previously implicated in tick toxicoses. This study

provides novel information on the molecular composition of ObSG, a Brazilian endemic tick associated with several human cases of parasitism. These results could be helpful in the understanding of clinical findings observed in bitten patients, and also, could provide more information on the evolution of Neotropical argasids.

Keywords: RNA, next-generation sequencing, argasid, nymph, secretory proteins

INTRODUCTION

Ticks (Ixodida) are composed of three families, the hard (Ixodidae), soft (Argasidae), and monotypic Nuttalliellidae (Guglielmone et al., 2010). The Ixodida is obligate blood-feeding ecto-parasites that secrete a cocktail of bioactive salivary gland-derived components during feeding, to counteract the vertebrate host's defense mechanisms, such as blood-clotting, platelet aggregation, and inflammation (Mans, 2019). Ixodids in all life stages feed for prolonged periods that may last for days to weeks during which hundreds to thousands of salivary gland proteins are differentially secreted, presumably to evade the host's changing immune responses, but also to adapt to a changing feeding environment due to the host's healing responses (Francischetti et al., 2009). Argasids show much more diverse feeding behavior in different life stages. In the larval stages, some species molt to nymphs without feeding, other species feed rapidly within minutes, while some species feed for prolonged periods of days-weeks resembling ixodids. In the nymphal stages, instars from some species do not feed, but molt to the next developmental stage, while females from some species also do not feed, although the majority of species requires a blood meal for successful oviposition (Hoogstraal, 1985).

In the case of *Ornithodoros brasiliensis* Aragão, 1923, the larvae molt to nymphs without feeding, while nymphal stages fed within 25–35 min similar to adults (Ramirez et al., 2016). Bites by this tick species result in toxicoses most probably due to injection of salivary gland components into the feeding site (Reck et al., 2011, 2013a, 2014; Dall'Agnol et al., 2019). Toxicoses by the "mouro tick" exhibit as erythemic and swollen lesions, hyperemia of oral/ocular mucosa pruritus, and tachypnea and slow wound-healing processes, and in recent years, cases of human hospitalization after tick bite have been reported (Reck et al., 2011, 2013a,b, 2014; Dall'Agnol et al., 2019). It has been shown that salivary gland homogenates can inhibit wound healing and endothelial cell proliferation *in vitro* (Reck et al., 2013b). To date, little is known about the salivary gland protein composition of this tick species that would help to explain these effects or symptoms.

Salivary gland transcriptome sequencing is a useful tool to generate catalogs of salivary gland-derived transcripts and has been important in the description of salivary gland protein sequence diversity (Mans, 2020). As such, characterization of the salivary gland transcriptome of *O. brasiliensis* would create an important resource to elucidate the molecular mechanisms behind mouro tick toxicoses. Argasid salivary gland transcriptomes have been characterized in detail using conventional cDNA library Sanger sequencing for *Antricola delacruzi* Estrada-Peña, Barros-Battesti, and Venzal, 2004 (Ribeiro et al., 2012), *Argas monolakensis* Schwan, Corwin, and Brown, 1992 (Mans et al., 2008a), *Ornithodoros coriaceus* Koch, 1844 (Francischetti et al., 2008a), and *Ornithodoros parkeri* Cooley, 1936 (Francischetti et al., 2008b). Argasid salivary gland transcriptomes have also been characterized using next-generation sequencing and assembly strategies for *Ornithodoros moubata* (Murray, 1877) (Pérez-Sánchez et al., 2021), *Ornithodoros erraticus* (Lucas, 1849) (Oleaga et al., 2021), and *Ornithodoros rostratus* Aragão, 1911 (Araujo et al., 2019). The salivary gland transcriptome of *O. brasiliensis* has not been described yet. The aim of the current study was to sequence the nymphal salivary gland transcriptome of *O. brasiliensis* ticks using next-generation sequencing.

MATERIALS AND METHODS

Tick Collection and RNA Extraction

Nymphs of *O. brasiliensis* were collected in the field from a site previously implicated in tick parasitism of travelers in Brazil (Dall'Agnol et al., 2019) and maintained unfed in the laboratory for ~2 months before dissection. Salivary glands were dissected from 10 nymphs and placed in RNA later before storing at −70°C. Total RNA was extracted using the RNeasy Protect Mini Kit (QIAGEN Group). Briefly, glands were suspended in 500 µl RLT buffer and disrupted by 10X passage through an 18G needle followed by 10X passage using a 24G needle. Residual genomic DNA was removed with DNase I digestion. Total RNA quantification was performed using the Qubit fluorimeter 2.0 (Life Technologies, Carlsbad, CA).

Library Construction and Next-Generation Sequencing

For library preparation, 1.0 µg purified total RNA was used with the TruSeq stranded mRNA sample preparation kit (Illumina, San Diego, CA). Poly-A mRNA was isolated, fragmented (for 3 min), and converted to double-stranded cDNA, adapters ligated, and PCR amplified for 12 cycles. Amplified bands were size selected from 450 to 1,200 bp. Bands were excised, purified, and sequenced using the Illumina MiSeq system (300 bp×300 bp). Raw sequence reads were submitted to GenBank under BioProject PRJNA719007 with small read archive accession number: SRR14139641.

Transcriptome Assembly

Raw Illumina reads were quality trimmed (0.001 quality limit) and TruSeq adapters removed using CLC Genomics Workbench.

Reads were imported as single or paired end reads. The paired end reads were merged to produce a merged dataset (Merged), while the single reads were used as unpaired (Single) and to produce a single-merged dataset (SM). Duplicates were also removed from these datasets to produce three duplicate removed datasets (Mddup, Sddup, and SMddup; Pienaar et al., 2021). These datasets were used to assemble the transcriptome using Trinity v2.4.0 and CLC Genomics Workbench v 20.0. Trinity was used with default parameter settings of kmer size 25. For CLC Genomics Workbench, kmer sizes were used in step sizes of 5 starting at 15 up to 60 and an additional assembly using kmer 64 (11 assemblies) with assembly parameters: mismatch cost-2, insertion cost-3, deletion cost-3, length fraction-0.9, similarity-0.9, minimum contig length-240, kmer size-variable, and bubble size-automatic. Given the different dataset structures used, a total of 72 assemblies were produced.

Extraction of the Mitochondrial Genome and Phylogenetic Analysis

The mitochondrial genome was identified in the assemblies by BLASTN analysis (Altschul et al., 1993), using the previously published mitochondrial genome for *O. brasiliensis* (Burger et al., 2014). The mitochondrial genome was annotated using the MITOS server to identify tRNA genes (Bernt et al., 2013). Protein coding and rRNA genes were identified using BLAST analysis (Altschul et al., 1993). The translated COI, CYTB, ND1, ND2, and ND4 proteins were used for phylogenetic analysis as previously described (Mans et al., 2015, 2019, 2021).

Extraction of CDS and Quality Assessment

Open reading frames (ORFs) were extracted using a Perl-script and chimeric and duplicate sequences removed by clustering at 90% identity using CD-HIT (Li and Godzik, 2006). The single dataset was mapped against the clustered ORFs using CLC Genomics Workbench and ORFs with TPM>1 (transcripts per million) were selected. BLASTX analysis against the ACARI database reduced the dataset further by selecting ORFs with E-values below 0.004 for further analysis. The transcriptome quality was measured for accuracy, completeness, contiguity, and chimerism using the Benchmarking Universal Single-Copy Orthologs (BUSCO; Simao et al., 2015). The final set of ORFs was submitted to GenBank under BioProject PRJNA719007.

Bioinformatic Analysis of the Transcriptome

To identify potential secretory peptides translated ORFs were submitted to SignalP (Petersen et al., 2011), while TMHMM and Phobius (Krogh et al., 2001; Kall et al., 2004) were used to identify membrane proteins. Potential housekeeping and secretory proteins were identified by BLASTP analysis against an ACARI database annotated using the KEGG database and GhostKOALA (Kanehisa et al., 2016a,b), the TSFAM database (Ribeiro and Mans, 2020), and an in-house annotation of secretory protein families (de Castro et al., 2016). To identify functional orthologs of proteins with experimentally verified functions, BLASTP analysis of secretory proteins was performed against the NCBI non-redundant database before phylogenetic analysis was performed to confirm clustering in functional clades with high bootstrap support. Protein families were aligned using MAFFT (Katoh and Standley, 2013), and maximum-likelihood analysis performed using IQ-Tree2 v 1.6.12 (Minh et al., 2020) with a standard 1,000,000 bootstraps.

RESULTS AND DISCUSSION

Transcriptome Assembly

A total of 58,490,632 paired reads were generated that yielded 2,285,451 merged reads (Merged) after quality trimming and merging and 10,505,566 single reads (Single) after quality trimming, resulting in a combined 12,791,017 reads (SM). Removal of duplicate reads resulted in 1,301,316 merged reads (Mddup), 6,841,083 single reads (Sddup), and combined 8,142,399 reads (SMddup). Assembly resulted in 100,397 contigs after clustering with CDHIT that was further reduced to 44,052 contigs with TPM>1. This resulted in 16,908 contigs with E-values >0.004 and 14,957 contigs after manual curation. BUSCO analysis indicated 95.0% completeness with 92.6% as single genes, 2.4% as duplicated, 2.7% fragmented, and 2.3% missing from a set of 1,066 conserved genes. This compares well with other tick transcriptomes sequenced thus far (**Figure 1A**). Comparison to two other soft tick salivary gland transcriptomes (*O. rostratus* and *Ornithodoros turicata* which belong to the Neotropic and Nearctic *Pavlovskyella*), for which protein sequence data are available in the public repositories, indicates that *O. brasiliensis* generally has longer contigs although it also has higher numbers of short contigs, which may indicate that some contigs may be truncated (**Figure 1B**). Reciprocal best hit analysis indicated that *O. brasiliensis* shares 7,441 orthologs in total with these transcriptomes (**Figure 1C**). This can be considered a minimum, since this is limited by the numbers of contigs submitted for the other transcriptomes, i.e., *O. rostratus* (n = 6,602) and *O. turicata* (n = 7,544). As such, the percentage of orthologs shared for each transcriptome is 89% of *O. rostratus* and 82% of *O. turicata*. These measures were taken to indicate a well-represented high quality transcriptome. BLASTP analysis of ACARI database using the transcriptome retrieved as highest number of hits, proteins from related soft ticks, such as *O. rostratus*, *O. turicata*, *O. erraticus*, and *O. moubata* (**Figure 2**). This may be expected but is also a good measure of transcriptome quality.

Mitochondrial Genome Analysis

Previously, *O. brasiliensis* ticks shown to cause toxicoses and for which a mitochondrial genome was sequenced were collected at a site distant from the current study (Reck et al., 2013a; Burger et al., 2014). Since the collection sites were approximately 60 km apart, the question was raised regarding the genetic relationship of the ticks collected in this study and that of the mitochondrial genome previously sequenced for this species. The full-length mitochondrial genome was assembled in the transcriptome and was 99% identical to the previously sequenced genome and was

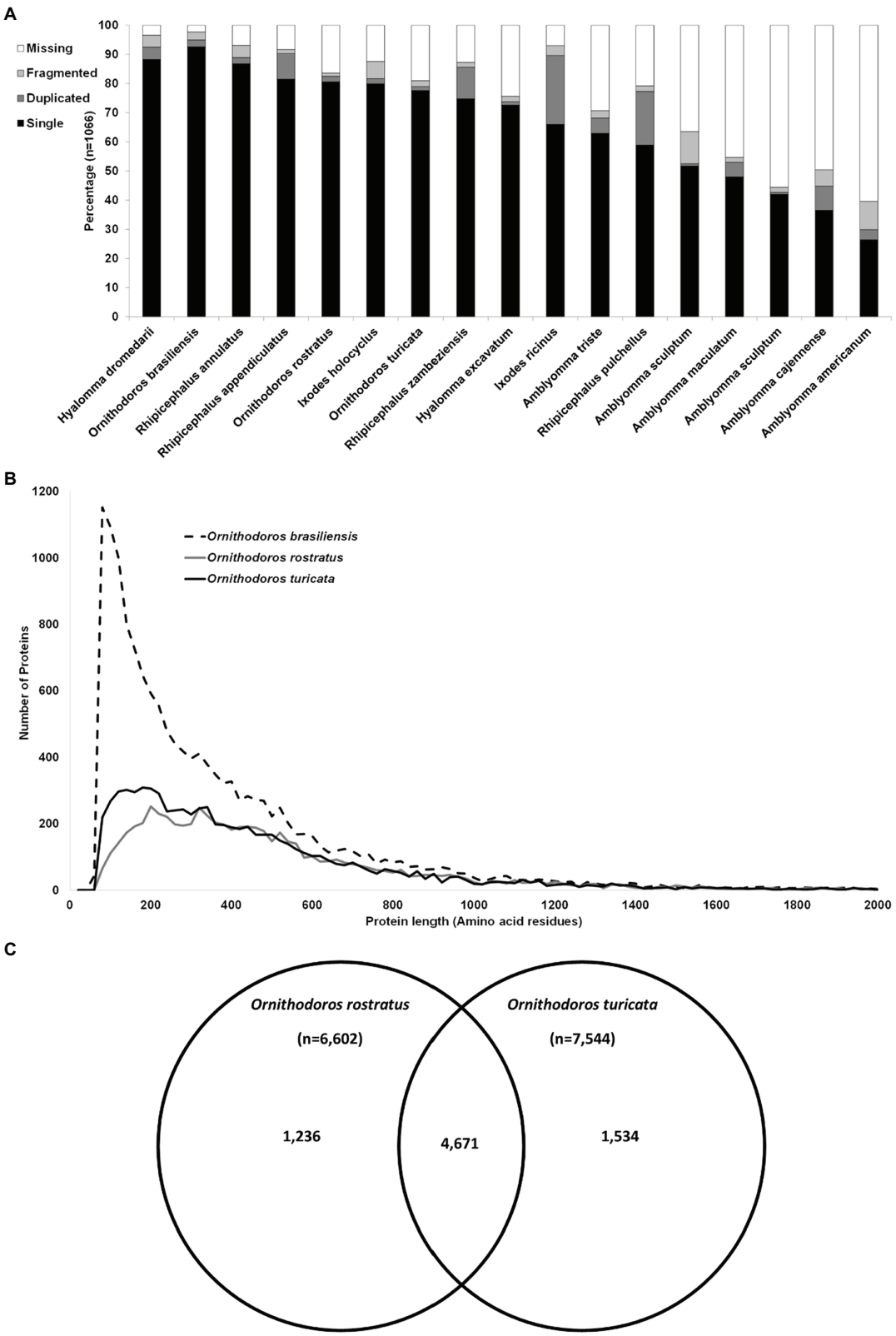

FIGURE 1 | Quality assessment of the transcriptome. **(A)** Comparison of the BUSCO analysis of all published salivary gland transcriptomes and *Ornithodoros brasiliensis*. The species are indicated on the horizontal axis, while the percentage of single complete, duplicated complete, missing, and fragmented is indicated on

(continued)

FIGURE 1 | the vertical axis. Transcriptomes used in the comparison are as: *H. dromedarii* (Bensaoud et al., 2018), *R. annulatus* (Antunes et al., 2019), *R. appendiculatus* (de Castro et al., 2016), *O. rostratus* (Araujo et al., 2019), *I. holocyclus* (Rodriguez-Valle et al., 2018), *O. turicata* (Bourret et al., 2019), *R. zambeziensis* (de Castro et al., 2017), *H. excavatum* (Ribeiro et al., 2017), *I. ricinus* (Schwarz et al., 2013, 2014), *A. triste* (Garcia et al., 2014), *R. pulchellus* (Tan et al., 2015), *A. sculptum* (Moreira et al., 2017), *A. maculatum* (Karim et al., 2011), *A. sculptum* (Esteves et al., 2017), *A. cajennense* (Garcia et al., 2014), and *A. americanum* (Karim and Ribeiro, 2015). **(B)** Comparison of protein sequence length in amino acid residues presented up to 2,000 residues against the number of proteins. Proteins were binned in windows of 20 based on protein length. **(C)** Reciprocal best hit analysis of the transcriptome of *O. brasiliensis* (*n* = 14,957) against two closely related Neotropic and Nearctic tick species. Indicated are orthologs shared uniquely between species pairs or shared between all three species.

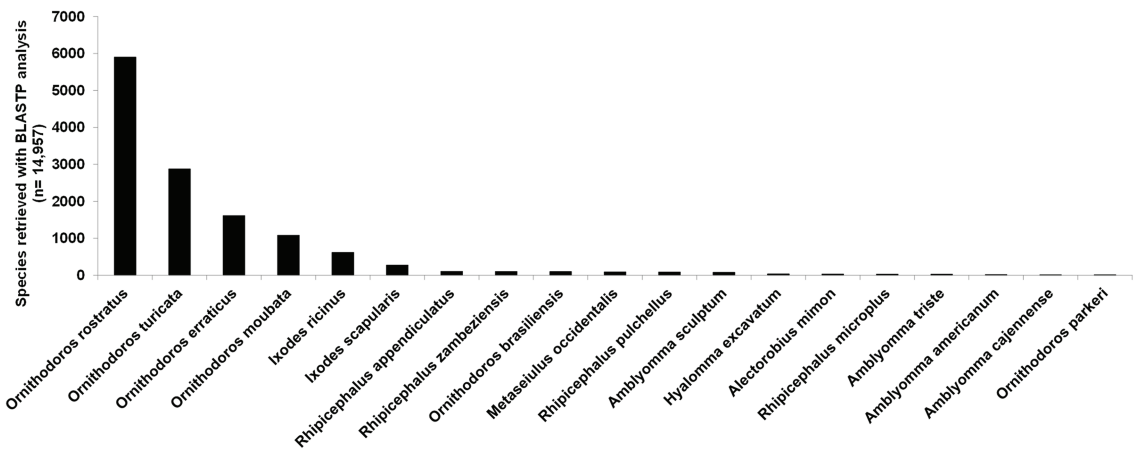

FIGURE 2 | BLASTP analysis of 14,957 contigs against the ACARI database. Species with more than 10 hits are indicated.

deposited under accession number MW864544. Phylogenetic analysis indicated that both genomes cluster together with high support in a clade shared with *O. rostratus* in what has been described as the Neotropic *Pavlovskyella* clade (**Figure 3**). The sister-clade with high bootstrap support is that of the Afrotropic *Ornithodoros* sensu stricto group suggesting as previously indicated that these clades probably diverged during continental breakup of Gondwanaland ~127 MYA (Mans et al., 2019). This places the transcriptome within a phylogenetic context for comparative analysis and suggests that the transcriptomes of Afrotropic *Ornithodoros* and Neotropic *Pavlovskyella* should share extensive similarities with regard to orthologs and function. In addition, full-length 18S and 28S rRNA sequences were retrieved from the study and were deposited under GenBank accession numbers MW857182 and MW877711, respectively. These commonly used phylogenetic markers can therefore also be retrieved from transcriptome sequencing projects.

Composition of the Transcriptome

The transcriptome can be divided into housekeeping (*n* = 12,471), secretory (*n* = 368), and unknown categories (*n* = 2,118; **Figure 4A**). Housekeeping proteins are defined as all proteins not part of the secretory class that is involved in general cellular or organismal housekeeping functions, while secretory proteins are defined as those with secretory peptides, considered to be secreted into the feeding site during feeding, while unknown proteins refer to proteins with significant BLASTP hits in the database, but which has no annotation. These classifications have been used in all tick transcriptome studies dating back to Ribeiro et al. (2006). Housekeeping proteins account for ~83.3% of the transcriptome, secretory proteins for ~2.5%, and unknown proteins for ~14.1%. Reads mapped back to the transcriptome indicate that housekeeping proteins account for ~42% of the coverage, secretory proteins for ~53%, and unknowns for ~5%. This would suggest that secretory proteins are present at higher concentrations in the salivary glands relative to the housekeeping proteins. To corroborate this, the first 39 proteins with highest coverage are secretory proteins and comprise 43% of the total TPM coverage (80% of the secretory protein contribution; **Figure 4B**). The high abundance of secretory proteins was previously observed in soft tick salivary glands and may be explained by the fact that soft ticks synthesize secretory proteins and store them in large secretory granules that occupy most of the space in the salivary gland cells until secretion (Mans and Neitz, 2004; Mans et al., 2004). Abundance of secretory transcripts was also observed in conventional Sanger sequenced argasid salivary gland transcriptomes, as well as in those sequenced with next-generation sequencing technologies (Francischetti et al., 2008a,b; Mans et al., 2008a; Araujo et al., 2019).

Housekeeping Proteins

The housekeeping proteins can be divided into various functional classes as classified by the KEGG database (**Figure 5**). These include proteins involved in metabolism, protein synthesis (transcription and translation), protein folding, sorting, degradation and excretion (FSDE), environmental sensing processes, such as signal transduction systems, cellular processes, such as cell growth, death, and motility, and organismal processes, such as the circulatory,

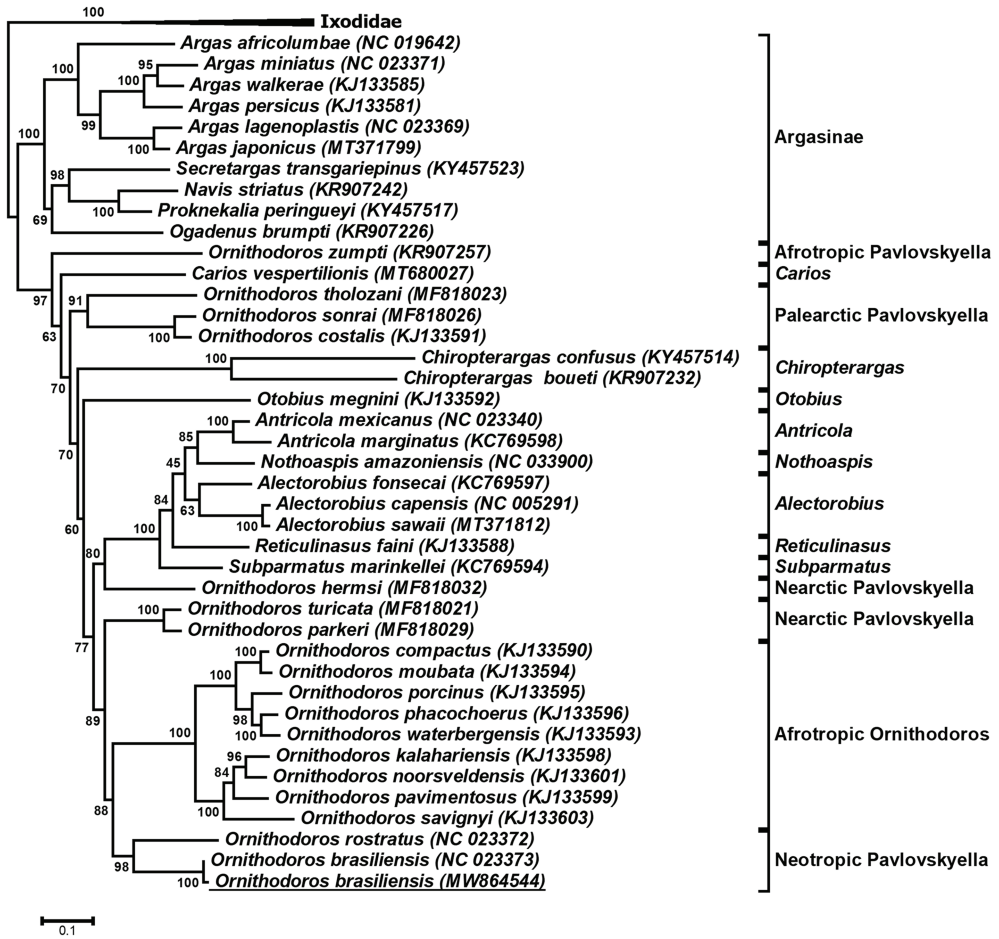

FIGURE 3 | Phylogenetic analysis of argasid mitochondrial genes. Indicated is a maximum-likelihood tree of the protein coding genes COI, CYTB, ND1, ND2, and ND4 of the mitochondrial genome. Bootstrap values for 1,000,000 bootstraps are indicated. The tree was rooted with the Ixodidae. The mitochondrial genome obtained in the current study is underlined.

developmental, digestive, endocrine, excretory, immune, nervous, and sensory systems. Signal transduction processes are notably enriched with regard to number of proteins. The most abundant housekeeping classes include those involved in protein synthesis (transcription and translation), FSDE (**Figure 6A**), that cumulatively account for 54% of TPM coverage for housekeeping proteins. This may be expected for an organ, such as salivary glands whose primary function is the synthesis, folding, and sorting of secretory proteins.

Some housekeeping functions with specific reference to tick biology may be highlighted. Of ~270 proteins identified in vertebrates to function as part of the protein secretory pathway (Gutierrez et al., 2020), 216 orthologs could be identified with confidence (**Supplementary Table 1**). The reason for many of those not identified may be due to multiple isoforms in the vertebrate secretory system which may be only represented by single proteins in ticks. This suggests that a large portion of the secretory system is present in the current transcriptome and is also conserved in ticks. This may be expected since the secretory pathway may be considered one of the Lineages of Life processes conserved in all Metazoa (Mans et al., 2016). Similarly, several orthologs of proteins previously implicated in neuronal control of salivary gland secretion in ticks (Šimo et al., 2014) were identified (**Supplementary Table 2**). This included hormones, such as bursicon, crustacean hyperglycemic hormone, eclosion hormone, elevenin, insulin, and orcokinin, as well as hormone receptors, such as allatostatin receptor, calcitonin gene-related peptide type 1 receptor, corazonin receptor 1, dopamine receptor 1, dopamine receptor 2-like, dopamine D2-like receptor, elevenin receptor 2, insulin-like growth factor 1 receptor, tachykinin-like peptides receptor, and periviscerokinin/Cap2b receptor.

It was previously indicated that the heme biosynthesis pathway is incomplete in ticks with ixodids lacking hemA-hemE, but possess hemF-hemH (Braz et al., 1999; Mans et al., 2016; Perner et al., 2016). Argasids possessed hemB and hemF-hemH (Mans et al., 2016). BLASTP analysis using hemA-hemH for *Metaseiulus occidentalis* indicated orthologs for hemB, hemF, and hemG, consistent with previous observations for argasids.

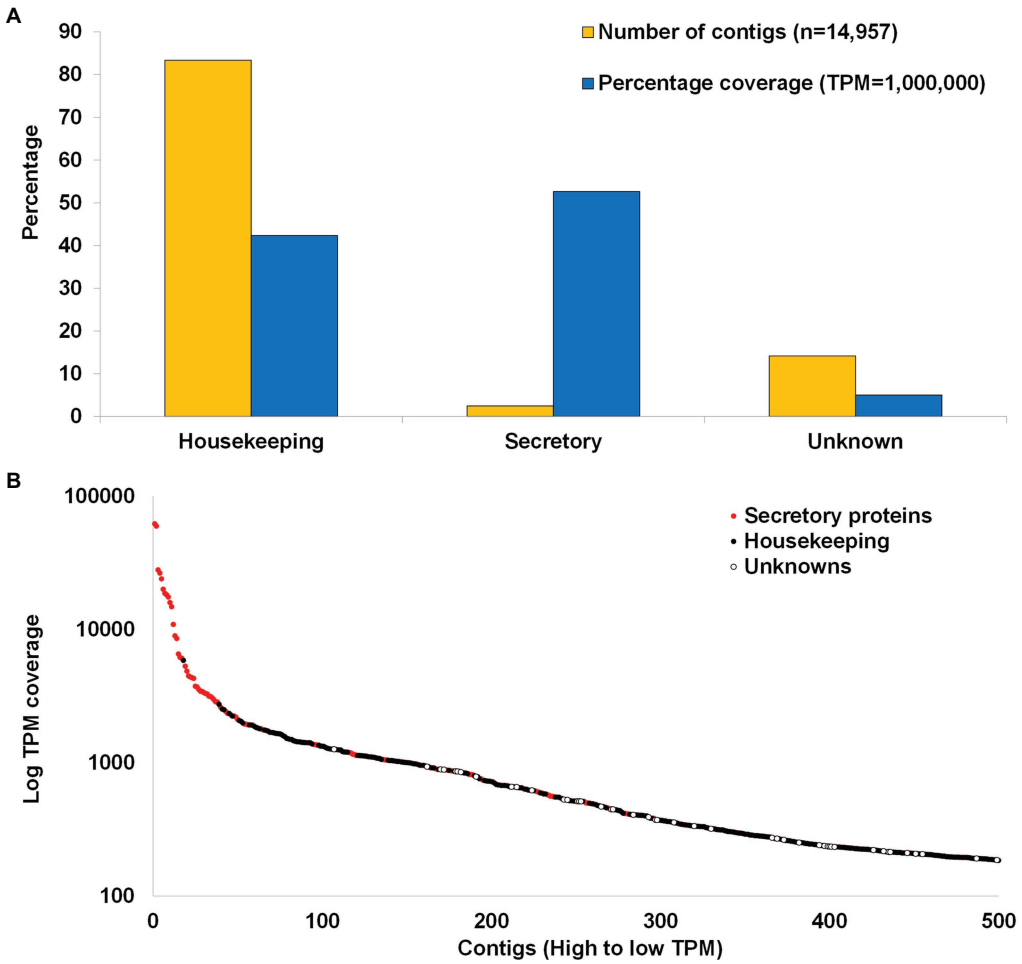

FIGURE 4 | Summary of transcriptome. **(A)** Comparison of the housekeeping, secretory, and unknown classes of contigs with regard to number of contigs contained in each class and the cumulative coverage of each class. **(B)** The first 500 contigs with the highest TPM coverage. Red dots indicate secretory proteins and black dots indicate housekeeping while white dots indicate unknown proteins.

Secretory Proteins

Secretory protein families identified in the transcriptome included the majority of protein families generally found in tick salivary glands (**Figures 6B, 7**). The most abundant protein families in terms of sequence coverage (TPM) were the lipocalin, basic tail secretory family (BTSP), 7 disulfide bond domain (7DB), Kunitz-BPTI domains, 7 cysteine domain, and reprolysin, comprising ~98% of the total (**Figure 6B**). The lipocalin, basic tail, reprolysin, and Kunitz-BPTI families were also the most abundant in terms of the number of family members (**Figure 7**). This has previously been observed in soft tick salivary gland transcriptomes (Francischetti et al., 2008a,b; Mans et al., 2008a; Araujo et al., 2019). In addition, a number of peptides with secretory signals but no BLASTP hits were also identified as unknown proteins.

Potential Proteins Functional at the Tick-Host Interface

Approximately 120 protein functions involved at the tick-host interface has been experimentally validated (Mans et al., 2019). BLASTP analysis using these proteins found 73 potential orthologs for 13 functions (**Table 1**). This includes the 5' nucleotidase family member apyrase that inhibits platelet aggregation by hydrolyzing ADP (Ribeiro et al., 1991; Mans et al., 1998; Stutzer et al., 2009).

For the lipocalin family, a number of orthologs were found for proteins with known function, including leukotriene B_4 (LTB_4) scavengers (Mans and Ribeiro, 2008a). The LTB_4 scavengers did not have the motifs conserved for complement C5 inhibition or thromboxane A_2 scavenging and probably do not possess these functions (Nunn et al., 2005; Mans and Ribeiro, 2008a). Orthologs were also found for the biogenic amine scavengers and these orthologs possessed the biogenic amine-binding motif of the lower-binding site, while some also possessed the conserved amino acid residues involved in the upper-binding site of TSGP1 (Mans et al., 2008b). These orthologs therefore likely bind both histamine and serotonin. Orthologs for leukotriene C_4 scavengers were also found (Mans and Ribeiro, 2008b).

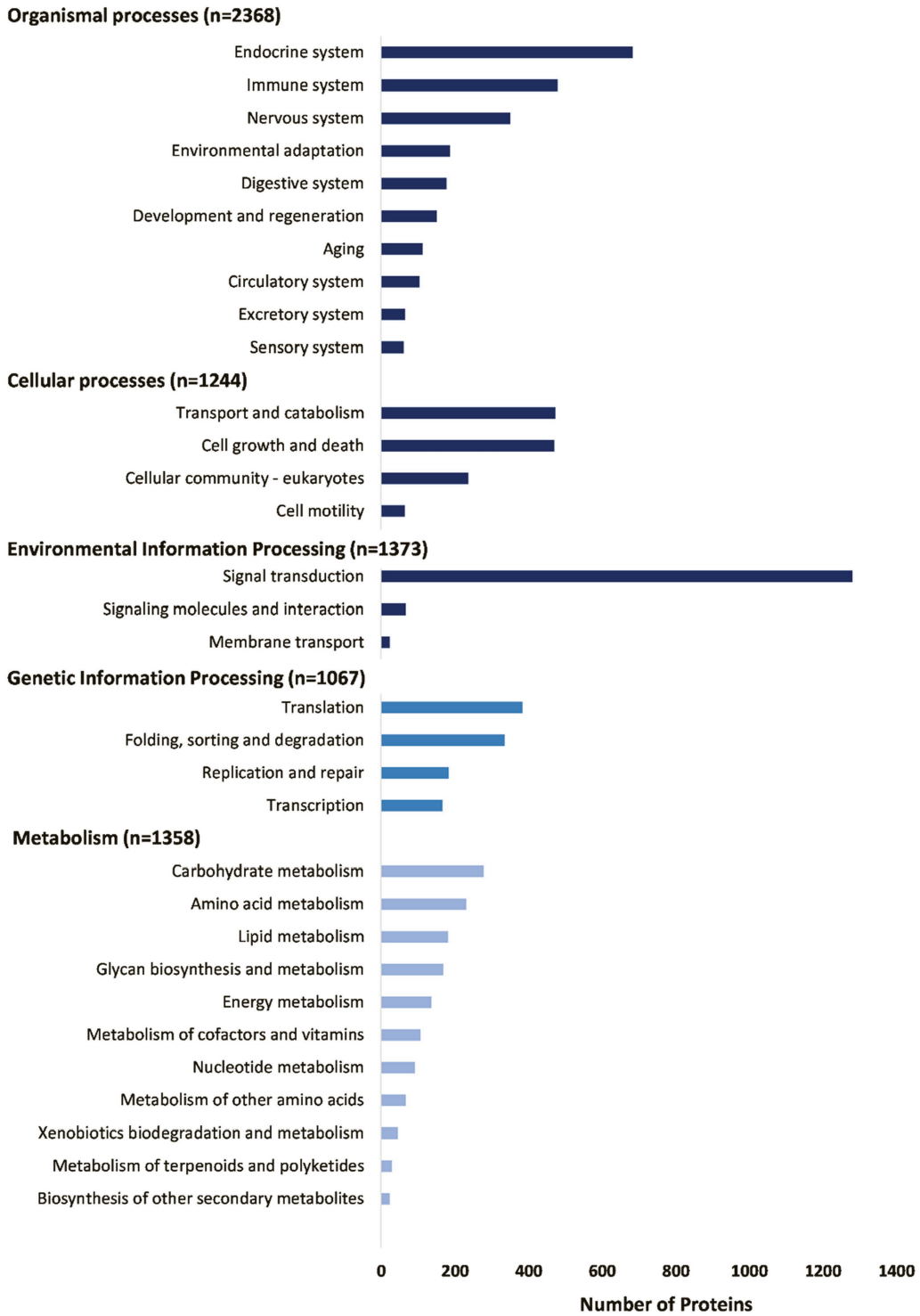

FIGURE 5 | Summary of housekeeping proteins as annotated by the GhostKOALA search of the KEGG database.

In addition, an ortholog for the lipocalin (savicalin) was found. Savicalin was implicated in antimicrobial activity (Cheng et al., 2010). Other potential secretory antimicrobial orthologs present are defensins, microplusins, and trypsin inhibitor-like domains (Nakajima et al., 2001; Fogaça et al., 2004, 2006; Sasaki et al., 2008).

Four orthologs for adrenomedullin (vasodilation) were found in the transcriptome. Adrenomedullin was possibly acquired by soft ticks from the genus *Ornithodoros* by horizontal gene transfer from mammals and has thus far been found in *O. coriaceus*, *O. moubata*, *O. parkeri*, and

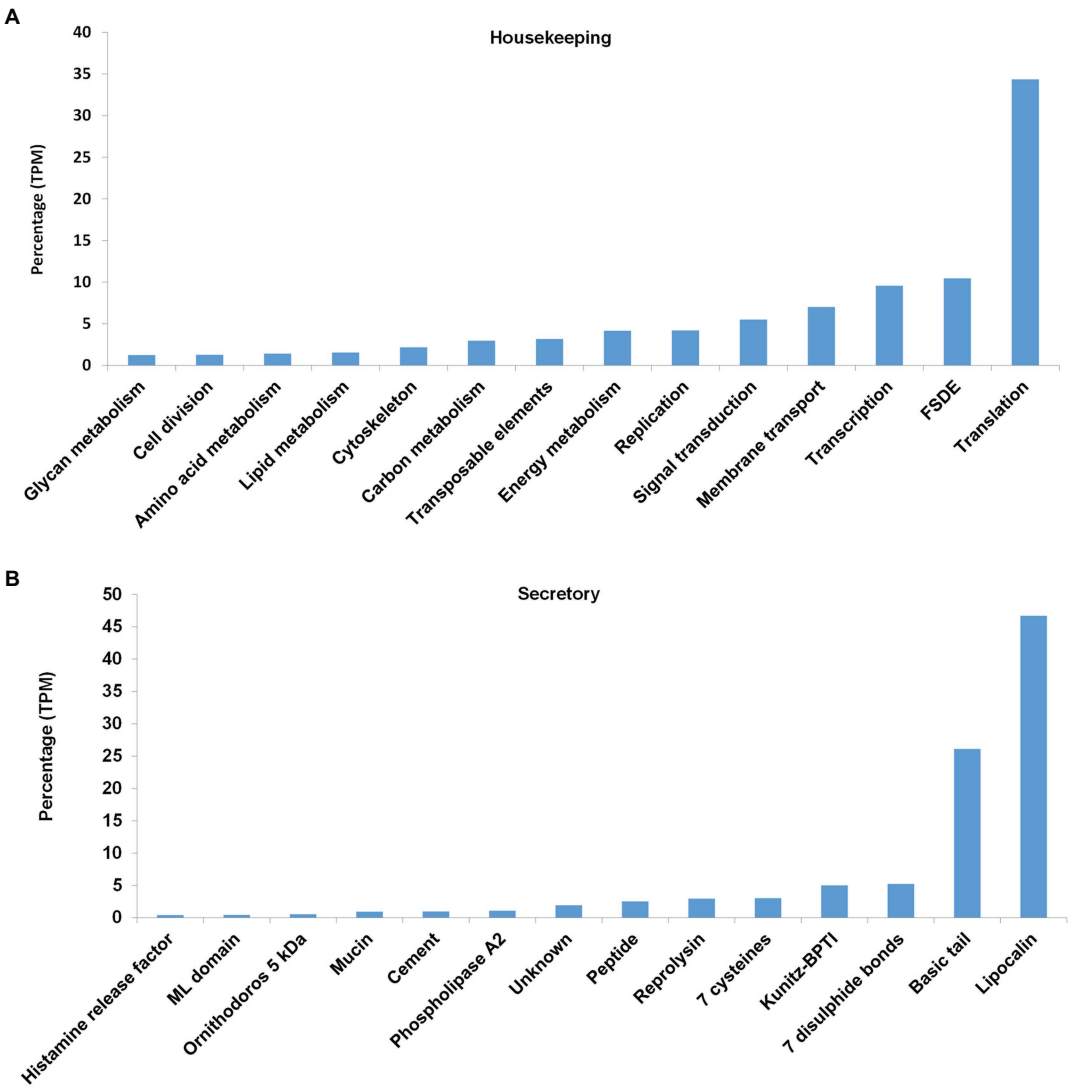

FIGURE 6 | Abundance of housekeeping and secretory proteins expressed as percentage of total coverage (TPM). **(A)** For the housekeeping proteins, the classes with >1% total coverage (~90% of total coverage) are indicated. FSDE: folding, sorting, degradation, and excretion. **(B)** For the secretory proteins, the classes with >0.2% total coverage (~98% of total coverage) are indicated.

O. rostratus (Iwanaga et al., 2014; Araujo et al., 2019). It would therefore seem as if the horizontal gene transfer occurred in the ancestral lineage to the *Pavlovskyella* and *Ornithodoros* (**Figure 3**). The presence of adrenomedullin in *O. brasiliensis* is therefore not surprising.

For members of the Kunitz-BPTI family, three orthologs of savignygrin were found. Single domain orthologs to the savignygrins were found that possessed the integrin RGD motif on the substrate binding presenting loop of the BPTI fold (Mans et al., 2002a). These inhibitors target the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ and inhibit platelet aggregation. Orthologs have also been found in *A. monolakensis* suggesting that these inhibitors were evolved in the ancestral argasid lineage (Mans et al., 2008c). A double-domain Kunitz-BPTI protein (Obras12157) with the same integrin RGD recognition motif located in the second Kunitz domain was also found. Such double Kunitz domain proteins were also observed in the transcriptomes of *O. coriaceus* and *O. parkeri* (Francischetti et al., 2008a,b). To date, no experimental evidence exists that these target integrin $\alpha_{IIb}\beta_3$, but it is likely that they target an integrin. The savignygrins are the Kunitz-BPTI proteins with the highest coverage for this family, as may be expected for an inhibitor that targets highly abundant platelet receptors in the host (Mans, 2019). Of interest is that no orthologs were found for the thrombin inhibitors like monobin, ornithodorin, or savignin (van de Locht et al., 1996; Nienaber et al., 1999; Mans et al., 2002b, 2008c), or the fXa inhibitors (Waxman et al., 1990; Gaspar et al., 1996; Joubert et al., 1998). This was also observed for Nearctic tick species, such as *O. coriaceus* and *O. parkeri* (Francischetti et al., 2008a,b). It was suggested

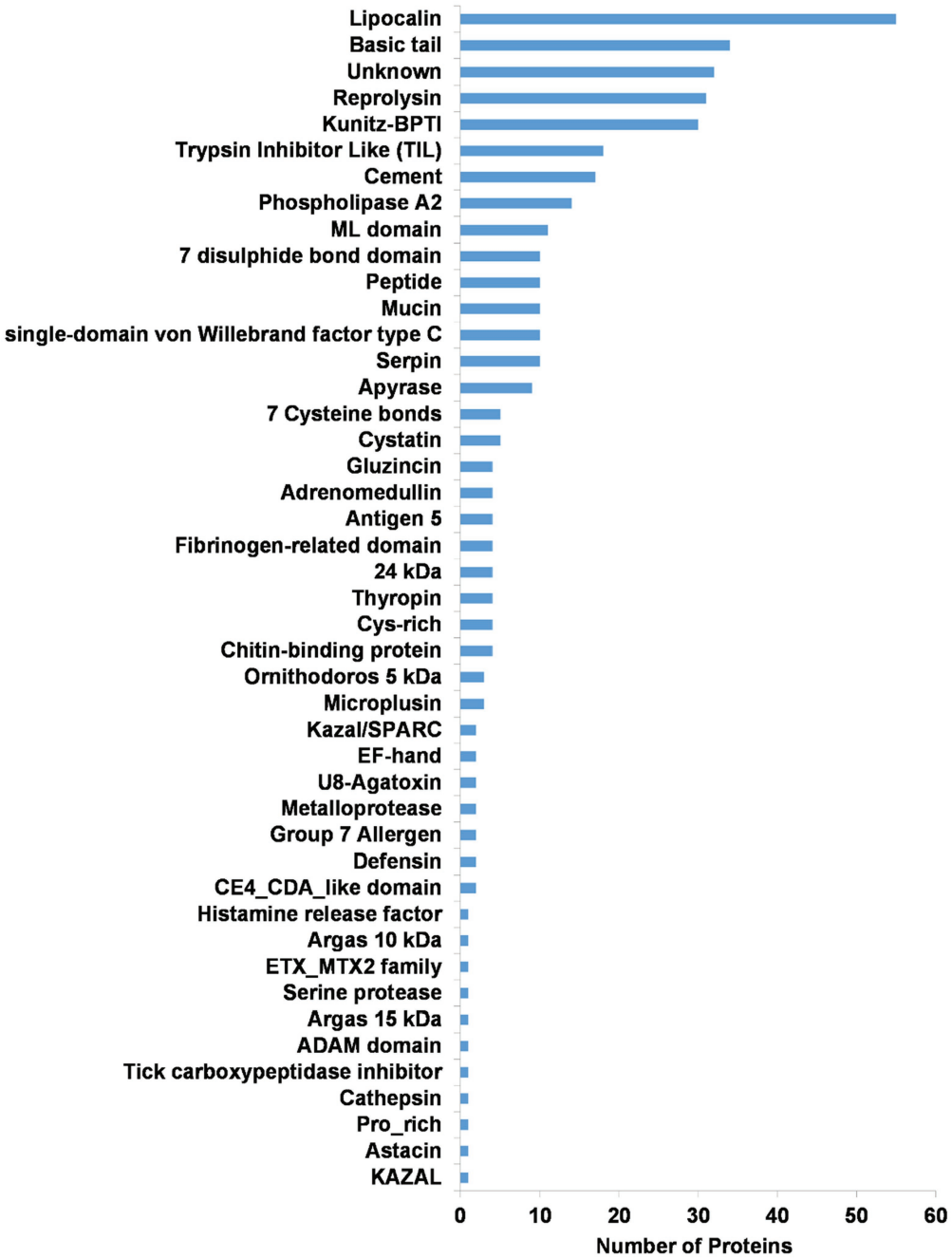

FIGURE 7 | Summary of the secretory protein families found in the salivary gland transcriptome of *O. brasiliensis*. Indicated are numbers of proteins for each class.

that fXa inhibitors evolved exclusively within the genus *Ornithodoros* while thrombin inhibitors evolved in the ancestral argasid lineage (Mans et al., 2008c). Absence of these inhibitors in other Ornithodorinae lineages may indicate gene losses probably associated with host preferences. Possible orthologs to longistatin may be involved in blood coagulation modulation by activating plasminogen and in the immune system by targeting the receptor for advanced glycation end products (Anisuzzaman et al., 2011, 2014). Another ortholog associated with fibrinolysis is carboxypeptidase inhibitor that targets plasma carboxypeptidase B (thrombin-activatable fibrinolysis inhibitor) leading to fibrinolysis (Arolas et al., 2005). As such, a number of inhibitors that targets the blood coagulation cascade are present.

Orthologs of cystatin-2 from *O. moubata* were also detected. These cystatins inhibit cathepsin L and S allowing for modulation of the inflammatory responses in the host (Grunclová et al., 2006; Salát et al., 2010). Orthologs of serpins were also found that may inhibit elastase and cathepsin G acting as immunomodulators and platelet aggregation inhibitors (Prevot et al., 2006; Chmelar et al., 2011). It should be noted

TABLE 1 | Orthologs for proteins with experimentally confirmed functions.

Protein	Function/target	Protein family	Orthologs in transcriptome
Apyrase	ATP/ADP hydrolysis	5' Nucleotidase	Obras3069, Obras3096, Obras3118
Moubatin	Leukotriene B_4 binding	Lipocalin	Obras9831, Obras10708, Obras11692, Obras11697
TSGP1	Biogenic amine binding	Lipocalin	Obras8892, Obras9626, Obras11694, Obras11530, Obras11349, Obras10580, Obras10207
TSGP4	Leukotriene C_4 binding	Lipocalin	Obras8357, Obras9228, Obras9233, Obras9535, Obras9761, Obras10025, Obras10029, Obras10067, Obras10069, Obras10481, Obras11033, Obras12035
Savicalin	Antimicrobial	Lipocalin	Obras10004
Defensin	Antimicrobial	Defensin	Obras14234, Obras33514
Microplusin	Antimicrobial	Microplusin	Obras16239, Obras16257, Obras20108
TIL domain	Antimicrobial	TIL domain	Obras537, Obras9191, Obras9385, Obras10342, Obras12982, Obras17500, Obras17566, Obras18660, Obras19202, Obras19481, Obras20420, Obras20432, Obras20659, Obras20998, Obras22358, Obras23412, Obras24115, Obras32341
Adrenomedullin	Vasodilation	Adrenomedullin	Obras13747, Obras14144, Obras18381, Obras19199
Savignygrin	Integrin $\alpha_{IIb}\beta_3$	Kunitz-BPTI	Obras11352, Obras16076, Obras19477
Cystatin-2	Cathepsin B/C/H/L/S	Cystatin	Obras12401, Obras14164, Obras18092
Longistatin	Plasminogen	EF-hand	Obras12168, Obras12393
Serpin	Elastase/cathepsin G	Serpin	Obras4634, Obras5463, Obras5157, Obras5522, Obras5610, Obras5731, Obras5670, Obras5698, Obras6172, Obras6365
Carboxypeptidase inhibitor	Carboxypeptidase B	Inhibitor_I68	Obras19216

that the orthologs to which functions could be ascribed comprise ~19% of all the secretory proteins found in the transcriptome, suggesting that numerous undescribed functions still exist for this tick species.

Potential Proteins Involved in Tick Toxicoses of *O. brasiliensis*

The toxicoses and bite of *O. brasiliensis* are accompanied by pruritis, local edema and erythema, pain, and blisters, while histopathology of the feeding site indicates extensive subcutaneous edema and hemorrhage (Reck et al., 2013a, 2014). Possibly, salivary proteins that may contribute to this clinical outcome would include the clotting and platelet aggregation inhibitors that would induce hemorrhage. On the other hand, the LTB_4 and LTC_4 scavengers may inhibit edema and erythema (Mans and Ribeiro, 2008b). Additional contributors to edema and hemorrhage may be the secretory proteolytic enzymes found in the transcriptome that includes a cathepsin, two serine proteases, and 38 metalloproteases (astacin, gluzincin, and reprolysin). These may also impact in wound-healing processes leading to prolonged recovery times.

Evolutionary Perspectives on the Salivary Gland Transcriptome of *O. brasiliensis*

The salivary gland transcriptome of *O. brasiliensis* shows a number of evolutionary features previously found for argasids. This includes the presence of the major salivary gland secretory protein families conserved in all ticks (Mans et al., 2008a), as depicted in **Figure 7**. A number of orthologs for functions thus far conserved in argasids were also identified and include biogenic amine, leukotriene B_4, and leukotriene C_4 scavengers, as well as apyrase, savignygrin, and defensins (Mans and Ribeiro, 2008b). These functions are thus far conserved in all argasid species that feed on blood (Mans et al., 2016) and underscore their important role in blood-feeding. The only argasid salivary gland transcriptome sequenced to date that did not show these conserved features of protein families and conserved functions, were the salivary gland transcriptome for adult *A. delacruzi* (Ribeiro et al., 2012), which lacked all conserved features. This tick does not feed on a host in the adult phase and it is likely that this difference is due to differential expression in different life stages, and that these conserved features will be found in its blood-feeding stages. Since *Antricola* groups well within the Ornithodorinae, it may be expected that their lack of conserved protein families and functions in non-feeding phases is a derived trait. Conversely, the conserved protein families and functions as observed in *O. brasiliensis* and other argasids, indicate evolution of these traits in the last common ancestor to the Argasidae, or even the Ixodida (Mans et al., 2016).

CONCLUSION

The current study reported 14,957 unique transcripts for the nymphal salivary gland transcriptome of *O. brasiliensis* considered to be of high quality. The transcriptome is enriched with secretory proteins with high abundance that belongs to well-characterized secretory protein families. Several orthologs could be identified of experimentally verified functions, while the data indicated that numerous functions remain to be discovered. Some of the secretory proteins identified in the transcriptome could also be linked to the clinical disease syndrome caused by *O. brasiliensis* and opens new avenues to identify the proteins involved in toxicoses.

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 at https://www.ncbi.nlm.nih.gov/genbank/, SRR14139641.

AUTHOR CONTRIBUTIONS

JR conceived the study, supplied the material, analyzed the data, and wrote the primary manuscript. AW supplied the material, analyzed the data, and critically reviewed the manuscript. BD supplied the material and critically reviewed the manuscript. RP, MC, and JF performed the experiments, analyzed the data, and critically reviewed the manuscript. BM conceived the study, performed the experiments, analyzed the data, and wrote the primary manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work is based on the research supported wholly/in part by the National Research Foundation of South Africa (grant numbers: 76499, 108998, 98862, and 118540) and the Economic Competitive Support Programme (30/01/V010).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fphys.2021.725635/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 26 July 2022
DOI 10.3389/fvets.2022.865664

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

RECEIVED 30 January 2022
ACCEPTED 27 June 2022
PUBLISHED 26 July 2022

CITATION
Chen Z and Liu J (2022) A review of argasid ticks and associated pathogens of China. *Front. Vet. Sci.* 9:865664. doi: 10.3389/fvets.2022.865664

A review of argasid ticks and associated pathogens of China

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It has been recorded 221 species of soft ticks in the world. However, the classification system of Argasidae is still unclear with nearly two-third controversial species in genus level. Therefore, comprehensive research is still necessary. In 2016, Wen and Chen overviewed the valid species of soft ticks in China for the first time. Up to now, the soft tick fauna of China remains poorly known. Although several studies have been undertaken, the information regarding soft ticks and associated diseases are fragmentary. To facilitate the future study of this group, the scattered information on soft ticks of China is herein synthesized. Toward the end of 2021, 15 valid species of argasid ticks have been reported, of these, 9 species (60%) including *Argas beijingensis*, *A. japonicus*, *A. persicus*, *A. sinensis*, *A. vespertilionis*, *A. vulgaris*, *Ornithodoros lahorensis*, *O. tartakovskyi,* and *O. papillipes* have been recorded biting humans. *Argas persicus* is the most common species, and its borne pathogens are widely investigated, while most other argasid ticks are not sufficiently studied in China. Here, we summarize detailed information regarding hosts, geographical distribution, molecular data, and vector roles of argasid ticks in China.

KEYWORDS

Argasidae, host and distribution, molecular characters, tick-borne pathogens, China

Introduction

Ticks are obligate hematophagous ectoparasites of a wide variety of mammals, birds, reptiles, and amphibians. They cause direct injuries by blood-sucking and are important vectors of a large variety of human, domestic, and wild-animal pathogens, including viruses, bacteria, and protozoans, which can damage to livestock production and human health (1). Tick species can be grouped into three current families (Argasidae, Ixodidae, and Nuttalliellidae) and one extinct family (Deinocrotonidae) (1, 2). Argasidae is second to Ixodidae with regard to the number of species. However, there is widespread disagreement concerning the taxonomy above the species level (i.e., subgenus and genus) in this family, with nearly two-third controversial species (3). According to various schools of scientific thought, the following five classification systems have been proposed for Argasidae: The American school of acarologists (4, 5), the French school (6, 7), the Soviet school (8–10), the cladistic scheme of Klompen and Oliver (11), and, most recently, a molecular system of classification by Mans and colleagues (12). The classification systems of American and Soviet schools are based on unique morphological characters, which are determined by the degree of phenetic differentiation, without reflecting the evolutionary history. The classification

system of cladistic school is based on morphology and biology, and was first proposed from a phylogenetic perspective. The French school only proposes a simple list of taxonomic rank, in which the taxa are not supported by morphological or biological characters. Burger et al. (13) first tested the genus–level classification of soft ticks by using mitochondrial genome and nuclear rRNA sequences. Their analyses strongly supported a clade of neotropical species within the subfamily Ornithodorinae, which included species from two genera, *Antricola* and *Nothoaspis*, and two subgenera, *Ornithodoros* (*Alectorobius*) and *Ornithodoros* (*Subparmatus*). Additionally, their analysis strongly supported a clade called *Ornithodoros sensu stricto* consisting of *O. savignyi* and four other *Ornithodoros* species (*O. brasiliensis*, *O. moubata*, *O. porcinus*, and *O. rostratus*) (13). Mans et al. (12) first proposed a molecular classification system for soft ticks based on the mitochondrial genome and nuclear sequence data. This classification system corresponds broadly with that of Klompen and Oliver (11), in which *Carios* and *Chiropterargas* were included in the subfamily Ornithodorinae, and *Alveonasus* in the subfamily *Argasinae*. There were also modifications made to several genera and subgenera. For example, the taxonomic status of *Ogadenus*, *Secretargas*, *Proknekalia*, *Alveonasus*, and *Chiropterargas* suggested as subgenera by Klompen and Oliver (11) were all promoted to the genus level by Mans et al. (12). Additionally, Mans et al. (12) established a new genus, *Navis*. This molecular classification system has essential reference significance. Later, Mans et al. (14) modified this classification scheme after analyzing the phylogenetic status of the bat tick *Argas vespertilionis* (Latreille) (*Carios vespertilionis*), and suggested that the subfamily Argasinae should be divided into six genera: *Alveonasus*, *Argas*, *Navis*, *Ogadenus*, *Proknekalia*, and *Secretargas*. The subfamily Ornithodorinae contains nine genera: *Alectorobius*, *Antricola*, *Carios*, *Chiropterargas*, *Nothoaspis*, *Ornithodoros*, *Otobius*, *Reticulinasus*, and *Subparmatus* (12, 14). This represents significant progress in the systematic classification of soft ticks. However, the further studies involving more controversial species and species from understudied regions should be conducted.

China is a country whose argasid fauna is poorly known (only 15 species reported thus far) (15, 16). In China, studies on ticks prior to the 1960s are scarce and not systematically documented. According to Li (17), the earliest research on soft ticks can be traced back to 1929, when Faust found an *Argas* sp. on domestic dogs in China. Later, Feng Lanzhou began to study the development of *Borrelia duttoni* in *O. moubata* collected abroad. In 1951, Feng and Huang collected *A. persicus* (Oken) from Shanxi province (17). Since then, research on soft ticks in China has been gradually developing, and includes case reports, morphological descriptions, biological characters, pathogens, and studies on the protein composition and karyotype characters of ticks (18–50). Wen and Chen (15) reviewed valid species of soft ticks for the first time (15). Toward the end of 2015, they listed valid argasid names of the world and China, and proposed a Chinese scientific term for each valid species and genus. Chen and Yang (51) published a monograph named "Systematics and taxonomy of Ixodida," in which argasid ticks from China were systematically redescribed. Over the past two decades, we have witnessed the emergence and re-emergence of tick-borne diseases. However, systematic surveys of soft ticks and associated pathogens still lack in China. Here, we reviewed literature on soft ticks published in Chinese, English, Russian, and Japanese to provide a detailed summary of argasid ticks and associated pathogens in China.

Argasid ticks and associated pathogen in fauna of China

As previously described, the classification system for soft ticks requires improvement. With further studies on more controversial species and the application of integrated methods, the taxonomic status of some tick species or groups is likely to change in the future. To prevent confusion in species names caused by constant changes, we followed Guglielmone et al. (3) and temporarily adopted the genus-level classification of Argasidae proposed by Hoogstraal (5) throughout this article. With regard to the nomenclature of the tick hosts, we place the genus name after the common name, except for the hosts identified to species level by authors. Some host species might have been misidentified; however, to avoid missing information, we quote the original name reported in the literature.

Currently, the argasid tick fauna of China consists of 15 species from two genera, *Argas* (10 species) and *Ornithodoros* (5 species). An overview of each soft tick species in China is presented below. Additionally, information on the deposition of type material of tick species first discovered in China is presented in this study. The administrative and biogeographical divisions of China are based on Chen et al. (52).

Argas assimilis Teng & Song, 1983

This species was first described in Jiangxi Province of China (45). The meaning of the specific name "*assimilis*" is "similar and closely resembling" (45).

Type depositories

Institute of Zoology, Chinese Academy of Sciences (IZAS) (holotype ♀, allotype ♂, paratypes 2♀♀ 2♂♂ and 1 nymph); Jiangxi Medical College of China (paratypes 4♀♀).

Local distribution

Oriental Region (Jiangxi, Guizhou) (45, 51–54).

Natural host

Passeriformes: swallow (*Hirundo daurica japonica*) (45, 51–54).

Habitats

Swallows' nest.

Molecular data

No record.

Tick-borne pathogens

No record from China.

Remarks

This species is closely related to *A. japonicus*, but can be distinguished by the following characters: Integumental ridges relatively narrower and markedly raised (integumental ridges thick, and not markedly raised in *A. japonicus*); peripheral integumental ridges narrower and more elongate, and regularly arranged (peripheral ridges thick and short, and irregularly arranged in *A. japonicus*); hypostome of female extending to mid-length of palpal article 3 (extending to mid-length of palpal article 2 in *A. japonicus*); article 3 shorter than article 4 (article 3 equal to article 4 in *A. japonicus*); each tarsus of nymph with a prominent dorsal subapical protuberance (no dorsal subapical protuberance in *A. japonicus*) (45).

Argas beijingensis Teng, 1983

This species was first described in Beijing, China (55). The specific name *beijingensis* is derived from "Beijing," China, the origin of the type species, plus the Latin adjectival suffix "-ensis," meaning "belonging to."

Type depositories

Institute of Zoology, Chinese Academy of Sciences (IZAS) (holotype ♀, allotype ♂, paratypes 3♀♀ 2♂♂ 4 nymphs and 4 larvae).

Local distribution

Palearctic Region (Beijing, Hebei, Shandong) (51–55).

Natural host

Columbiformes: pigeon (*Columba livia*), *Streptopelia chinensis*; Passeriformes: sparrow (*Passer montanus*), swallow (*Hirundo rustica*); Galliformes: chicken (*Gallus gallus domesticus*) (51–55).

Habitats

Avian nests and their surroundings.

Molecular data

No record.

Tick-borne pathogens

No record from China.

Remarks

According to Teng (55), this species is closely related to *A. reflexus*, but can be distinguished by the following characters: In adults, body slightly broader posteriorly (markedly broadened in posterior one-third in *A. reflexus*), the fixed digit of chelicera with two teeth (three teeth in *A. reflexus*), the setae on tarsi I–IV different in number between *A. beijingensis* and *A. reflexus*; in larva, body oval with an approximate oval plate of the dorsum (body subcircular with a relatively narrower and longer plate in *A. reflexus*), eight pairs of seta in posterolateral quadrants of the dorsum (nine pairs in *A. reflexus*). *Argas beijingensis* is also related to *A. vulgaris*, but can be distinguished by the following characters: In adults, the anus slightly posterior to the center of venter (much more separated from the middle of ventral body surface in *A. vulgaris*), peripheral integumental ridges short and sinuous (relatively narrower and longer in *A. vulgaris*); in larva, body oval, and its dorsolateral margin with 24–25 pairs of setae (body subcircular, and its dorsolateral margin with 19–21 pairs of setae in *A. vulgaris*) (55).

Sun et al. (53) reported specimens from Inner Mongolia, Beijing, Hebei, Shanxi, Shandong, Shaanxi, Jiangsu, Shanghai, Anhui, Fujian, Taiwan, and Sichuan in China as *A. beijingensis*, which were initially recorded as *A. reflexus*. However, according to the descriptions of Sun et al. (53), they only checked the specimens from Shandong; other specimens were not re-examined, thus the distribution of *A. beijingensis* should be further investigated.

Argas japonicus Yamaguti, Clifford & Tipton, 1968

Local distribution

Palearctic Region (Beijing, Hebei, Jilin, Liaoning, Inner Mongolia, Ningxia, Xinjiang); Oriental Region (Taiwan) (33, 51–58).

This species has also been reported in Japan and Korea (59), and has been studied more in depth in Japan.

Natural host

Columbiformes: *Streptopelia* spp.; Passeriformes: swallow (*Hirundo daurica japonica*, *Deliclion dasypus*), sparrow (*Passer* spp.); Galliformes: chicken (*Gallus gallus domesticus*) (33, 51, 53–58).

It has been reported that the overwhelming majority of specimens have been collected from swallows and swallow nests (54, 60–69). Researchers rarely collected *A. japonicus* ticks from hosts other than wild birds, although it has been found that this species successfully sucks blood from chickens and many mammals in the laboratory (62, 64). Zhao et al. (56) first reported *A. japonicus* collected from cattle in nature, and found this species actively infesting livestock from February to March in spring in Xinjiang. They also screened the pathogens of fed *A. japonicus* ticks from cattle and found spotted fever group *Rickettsia* spp. and "*Candidatus* Anaplasma boleense" in this species (56). In Japan, Uchikawa (62) used chicken skin as a feeding membrane to study feeding behavior of *A. japonicus*. The results indicated that most *A. japonicus* ticks fed on chicken, rabbit, sheep, and bovine blood could develop successfully but those fed on human, horse, and pig blood showed high mortality rates (68–77%) (62). However, the reason for this difference remains unknown. Several human infestations by this species have been reported in China and Japan (33, 57, 70). In China, the first reported case of human dermatitis caused by *A. japonicus* biting was recorded in Liaoning, China in 1986 (34). In April 2016, several human cases of *A. japonicus* ticks biting were reported in Inner Mongolia Autonomous Region of China, and the patients appeared to have fever, skin rash, swelling, itching and inoculation eschars (57). Subsequently, the microbiota of free-living *A. japonicus* in the affected community was explored (57). In Japan, a group of elderly patients with physical disabilities experienced infestation with *A. japonicus* coming from sparrow nests located under the eaves of a rehabilitation hospital. The tick bites were painful and accompanied by pruritus (70). Therefore, *A. japonicus* selects birds, especially swallows, as its primary and preferred hosts. Human and other mammals may act as accidental hosts.

Habitats

This species often inhabits the nests of birds, occasionally hencoops, poultry and livestock yards, and attacks people at night.

Molecular data

China: 16S rDNA (MH782636), 12S rDNA (MG668793–MG668795).

Other countries

Japan: 16S rDNA (AB819156, AB819157), mitochondrial genome (MT371799).

Tick-borne pathogens

Spotted fever group rickettsiae (56, 57), *Alcaligenes faecalis* (57), "*Candidatus* Anaplasma boleense" (56).

There are a few studies on the pathogens and diseases transmitted by *A. japonicus*. Further investigations of *A. japonicus* and its pathogens should be conducted in China.

Argas persicus (Oken, 1818)

Local distribution

Palearctic Region (Beijing, Xinjiang, Gansu, Qinghai, Hebei, Jilin, Liaoning, Heilongjiang, Inner Mongolia, Shandong, Shanxi, Shaanxi); Oriental (Shanghai, Hubei, Fujian); Paleozoic–Oriental ecotone (Anhui, Sichuan, Jiangsu) (17, 52–54, 71–76).

Natural host

Columbiformes: pigeon (*Columba* spp.); Passeriformes: sparrow (*Passer monatanus*), swallow (*Hirundo* spp.); Galliformes: chicken (*Gallus gallus domesticus*) (17, 52–54, 71–76).

This species appears to be mainly a parasite of domestic fowl and arboreal nesting birds (77). It commonly attacks humans, causing it to have an evil reputation especially in early Persia (77). Additionally, this species can sometimes be found in domestic animals, especially sheep and cattle, in China (51), which has not been reported in other countries or areas (77–83). This is mainly because domestic animals are often mixed and housed together with poultry in the rural areas of China.

Habitats

In the crevices of poultry houses and nearby human houses, or in the cracks or under the bark of trees frequented by their wild avian hosts.

Life cycle

Qi et al. (31) and Tian (30) thoroughly studied the life history of *A. persicus* in a laboratory. Di (39, 43) reported on its life habits and the seasonal and diurnal activities. In Shandong, *A. persicus* was found from early March to mid-October with an active period from May to September and peak prevalence in July (75). The overwintering period for this species was from November to February. This species endures for long periods, with larvae starving up to 8 months, nymphs 24 months and 3 years in adults. Any developed stage of *A. persicus* could overwinter, and the longevity was reported to be 10–20 years (43). The activity of *A. persicus* larvae was not limited by day and night. In contrast, the activity of *A. persicus* nymphs and adults was affected by light and mostly were active at night. Usually, the larva is attached to the featherless part or near the feather roots of poultry, where they can suck blood, whereas

the nymphs and adults are attached to the featherless toes of poultry. There were two to seven instars in the nymphal stage with similar morphological features, but they increased in size. Ticks began seeking for hosts at 7–8 pm, reaching a peak around midnight from late July to mid-September (39). The mating behavior between males and females was carried out after sucking a small amount of blood during the day or night (30). Ticks only climbed to the host when sucking blood and left the host immediately after completion of the blood meal. Most of the larvae usually fed for 2–7 days, while very few fed for 10 days. Each nymphal instar fed for several minutes to several hours, and the adults were generally replete in 15 min to 3 h. The molting period of larva was 6–18 days (30) or 4–17days (31), while the molting time of first instar nymph was 7–12 days (30) or 10–97 days (31), and that of second instar nymph was 9 days (30) or 12–63 days (31) under 26–28°C with 65–85% relative humidity. The various molting period of nymph may be related to individual differences and blood engorgement levels (30, 31).

According to the experimental observations, the preoviposition stage of females has been reported to range from 3 to 160 days (30, 31). Oviposition time seems to be related to the month in which females are fed. The preoviposition period of females sucking blood from June to August was the shortest, whereas that of females sucking blood in January was the longest. They generally oviposited in 4–21 days after engorgement, and the number of eggs was related to the amount of bloodsucking. Generally, 50–200 eggs were laid at a time, and more than 1,000 eggs could be laid in the lifetime of a female.

Molecular data

China: 16S rDNA (MN894073, MK555333, KR297208, KR297209, LC209197, LC209198, KX258880); COI (LC209195, LC209196, MN900726, MK571448); mitochondrial genome (OM368319, OM368320, MT012684, NC_053794).

Other countries

Australia: 16S rDNA (AY436769, AY436770, AY436772); Egypt: 16S rDNA (AF001402); COI (OM177661); Iran: Cathepsin L-like protein (MN175238, MN175239); COI (KX879770); Italy: 16S rDNA (GU451248); Kazakhstan: COI (MN900726); Kenya: 28S rDNA (KJ133607); 18S rDNA (KJ133633); ITS1 (KJ133633); ITS2 (KJ133607); mitochondrial genome (KJ133581); Pakistan: 16S rDNA (MZ496987, MT002847); Romania: COI (FN394341); NAD5 (FN394358); South Africa: 16S rDNA (GU355920); USA: 18S rDNA (L76353); 16S rDNA (L34321); 12S rDNA (GU355920); COI (U95864).

Tick-borne pathogens

Borrelia anserina, *Francisella tularensis*, *Coxiella burnetii*, *Rickettsia hoogstraalii*, *Coxiella*-like endosymbiont, *Pseudomonas geniculata*, *Sphingomonas koreensis*, *Acinetobacter haemolyticus*, *Streptococcus suis*, *Staphylococcus aureus* (84–87).

In China, *A. persicus* has been reported to carry many pathogens, as described above, and only *B. anserina* is well-known to cause fowl spirochetosis. There are many cases of illness in chickens, geese, and ducks bitten by *A. persicus* in China (24–27, 32, 72, 87). In 2006, there was an outbreak of goose spirochetosis in Inner Mongolia, which caused mortality in nearly half of the sick geese, and many adults of *A. persicus* were found in goose housings. Clinical symptoms, pathological anatomy, and microscopic examination indicated that the goose disease was caused by *B. anserina,* transmitted by the vector *A. persicus* (87). Additionally, it was also reported that most chickens lost their appetite, were emaciated, and even died within a week in Gansu China, because of the infestation by *A. persicus* larvae. The chickens were observed for depression, fluffy feathers, liquid stools, crowns, beards, feet visible mucous membranes of pale color, and unstable standing or paralysis. However, the cause of the disease or pathogens has not yet been reported (71).

In other countries or regions, this species has also been reported to transmit *Aegyptionella pullorum* (Aegyptianellosis), Slovakia virus, Kyasanur forest disease virus and *Francisella persica*, which all have not been detected in China (88–92).

Remarks

Argas persicus is considered native to Turanian–Central Asia but with human activities it become established throughout most continents except Antarctica (78). Many records report the presence of this species in Taiwan (51, 54, 72). However, Robbins (93) believed that published references to *A. persicus* in Taiwan were misidentifications (93–98). Indeed, *A. persicus* listed in Taiwan by Teng (72) may represent the morphologically similar *A. robertsi* (93). Thus, records of *A. persicus* from the Oriental region should be further determined.

Zhou and Meng (99) studied the karyotypes of 56 *A. persicus* ticks and found 51 ticks were diploids, i.e., $2n = 26$ (24 + XY) (♂); $2n = 26$ (24 + XX) (♀). Interestingly, it was also discovered that four ticks were tetraploid ($4n = 52$) and one tick was octoploid, i.e., $8n = 104$ (96 + XXXXYYYY). Zhou and Meng (99) speculated that the reason of this polyploidy could be related to the use of colchicine during the sample processing (99). Additionally, they also reported that the Y-chromosome of *A. persicus* from Xinjiang was 37.8% the length of the X-chromosome, and the average length of all autosomes was 14.7% the length of the X-chromosome (99). Goroschenko (100) reported those ratios of *A. persicus* from the former USSR as 54.4 and 26.5%, respectively. These differences might be related to tick strains from different geographical areas.

Argas pusillus Kohls, 1950

Local distribution

Oriental (Taiwan) (51–53, 93).

Argas pusillus is a typical southeastern Asian species that has been reported in Philippines, China, Thailand, Malaysia, and Singapore (5, 51, 93, 101–104).

Natural host

Chiroptera: bats (*Scotophilus temminckii*, *Pipistrellus imbircatus*).

This species mainly parasitizes bats, specifically *Scotophilus* spp.

Habitats

Near bat caves.

Molecular data

No record.

Tick-borne pathogens

No record from China.

Studies on *A. pusillus* and its pathogens all over the world are very limited, mainly including species examination, distribution, hosts and a few on pathogen detections (101–106). To date, Issyk–Kul fever virus and Keterah virus have been reported in this species (5, 105, 106).

Remarks

It is often confused with the bat tick, *A. vespertilionis*. Hoogstraal (letter No. 251, February 14, 1984 and letter No. 376, February 14, 1977) concluded that the samples of *A. vespertilionis* collected in Taiwan were *A. pusillus* (93). Robbins (93) stated that the published records of *A. vespertilionis* in Taiwan (72, 94, 95, 98, 107) may represent *A. pusillus* (93). In addition to Taiwan, this species probably also occurs in other areas of China; therefore, *A. vespertilionis* collected from southern China should be further re-examined.

Argas reflexus (Fabricius, 1794)

Local distribution

Palearctic (Gansu, Qinghai, Hebei, Henan, Inner Mongolia, Ningxia, Shandong, Shaanxi, Xinjiang, and Heilongjiang); Palaeozoic–Oriental ecotone (Anhui).

Argas reflexus can be found in the Palearctic region between parallels 31°N and 51°N (108, 109). This species is widely distributed in Europe and has been reported in some regions of Asia (Israel, Turkey, Iran, Pakistan, Afghanistan and Kazakhstan), as described in detail by Pfäffle and Petney (108). Additionally, Hoogstraal and Kohls (110) found a single unengorged larva of *A. reflexus* in Egypt.

Natural host

Columbiformes: pigeon (*Columba livia domestica*, *Columba rupestris*); Passeriformes: sparrow (*Passer* spp.), swallow (*Hirundo* spp.), chough (*Pyrrhocorax graculus*); Galliformes: chicken (*Gallus gallus domesticus*) (19, 22, 23, 42, 51, 53, 55, 73, 111, 112).

Argas reflexus predominantly parasitizes domestic pigeons (*Columba livia domestica*) and bites other birds, including rock pigeons (*Columba livia*), rock swallow (*Ptyonoprogne rupestris*), turtle doves (*Streptopelia turtur*), fan–tailed ravens (*Corvus rhipidurus*), jackdaw (*Corvus monedula*), swifts, swallows, owls, crows, several passerine birds, chickens and even humans (108–110, 113).

Habitats

Inhabit pigeon and other bird nests, and the vicinity of its hosts.

Molecular data

China: No record.

Other countries

Luxembourg: arg-r-1 (AJ697694); Poland: 16S rDNA (AF001401); Spain: 16S rDNA (MW289075, MW289076, MW289084); COI (MW288388); USA: 16S rDNA (L34322); 12S rDNA (U95865).

Tick-borne pathogens

No record from China.

It has been reported that *A. reflexus* is a vector of *Aegyptianella pullorum*, Crimean–Congo hemorrhagic fever virus, Uukuniemi virus, Grand Arbaud virus, Ponteves virus, Tunis virus, West Nile virus, Chenuda virus, Nyamanini virus, and Quaranfil virus (98, 114–118).

Remarks

Teng (55) concluded that *A. reflexus* published in "Economic Insect Fauna of China Fasc 15" was misidentified: specimens collected from Xinjiang should be *A. vulgaris* while those collected from Beijing should be *A. beijingensis*. However, he did not mention the specimens collected from other regions. Yu et al. (73) reported this species in Xinjiang. Based on the geographical location of China and the distribution area and

host characters of *A. reflexus*, it is possible for this tick species to appear in China. Therefore, this species has been kept in the valid tick list of China until conclusive evidence is obtained.

Argas robertsi Hoogstraal, Kaiser & Kohls, 1968

Local distribution

Oriental (Taiwan) (52–54, 93).

Argas robertsi is common in Australia (Queensland, Northern Territory, New South Wales) and the Indo–Malaya region, including Indonesia (Java), China (Taiwan), Thailand, India (West Bengal), and Sri Lanka (82, 119, 120).

Natural host

Galliformes: chicken (*Gallus gallus domesticus*), Pelecaniformes: cormorant (*Phalacrocorax* spp.), ibis (*Threskiornis* spp.); Ciconiiformes: heron (*Ardea* spp., *Ardeola* spp., *Bubulcus* spp., *Nycticorax* spp., *Egretta* spp., *Plegadis* spp.), stork (*Anastomus* spp.) (29).

Habitats

Often inhabits bird nests, occasionally occur in hencoops.

Life cycle

Hoogstraal et al. (119) studied the life cycle of *A. robertsi* collected from Taiwan, using domestic pigeons as experimental hosts at 28–30°C and 75% RH. The life cycle of *A. robertsi* was 2–10 months and included two to five nymphal instars in Taiwan, similar to other *A. robertsi* populations from different regions. The nymphs and adults fed within a few days of molting. Many males molted from the earlier nymphal instars. Most females needed to suck blood twice to lay eggs, while few needed to suck blood only once (119).

Molecular data

China: No record.

Other countries

Australia: 16S rDNA (AY436768).

Tick-borne pathogens

Kuo Shuun virus (29).

Other viruses, including CSIRO 1499 virus, Lake Clarendon virus, Nyamanini virus and Pathum Thani virus have also been detected in other countries or regions (82, 105).

Remarks

Barker and Walker (82) stated that *A. robertsi* and *A. persicus* lived in sympatry in Australia. Although *A. persicus* is very common in China, *A. robertsi* has only been reported in Taiwan. Further investigations on *A. robertsi* and *A. persicus* should be conducted in China.

Argas sinensis Jeu & Zhu, 1982

The specific name "*sinensis*" means "belonging to China."

Type depositories

Department of Parasitology, Chongqing Medical College, Chongqing, China (holotype one unfed larva, paratypes two unfed larvae, two partly engorged larvae, and four engorged larvae).

Local distribution

Oriental (Sichuan) (36, 38, 52).

Natural host

Chiroptera: bat (*Pipistrellus abramus*).

Jeu (36) stated that larvae could feed successfully on white rats and mice. Nymphs and adults could feed well on a wide range of vertebrate animals (including *Rattus tanezumi*, *Rattus norvegicus*, *Mus musculus*, guinea pig, rabbit, dog, cat and monkey) and poultry (including chicken, goose, duck, and pigeon) under laboratory conditions. Jeu even contributed his skin to verify that humans are also suitable hosts for ticks (36).

Habitats

Occurs in bat colonies, often can be found in bat infested buildings (38).

Life cycle

Jeu (36) carefully investigated the life history of *A. sinensis* collected from Chongqing, under laboratory conditions from 1973 to 1977 (36). There were two to four nymphal instars for this species. The molting nymph could be divided into the following three types: (1) composed of two instars that sucked blood twice; (2) composed of three instars that sucked blood 3 times; and (3) composed of four instars, the first instar nymph could molt into the second instar nymph directly without sucking blood, then sucked blood 3 times. Females laid eggs several times, with prolonged oviposition periods, but delaying the time between oviposition periods progressively. They were able to deposit four to eight batches of eggs, totaling 144–423 eggs (36).

Molecular data

No record.

Tick-borne pathogens

No record from China.

Remarks

The larva of this species is closely related to *A. vespertilionis* and *A. daviesi*, but differs from them in the following characters: (1) dorsal setae numbering 14 pairs; (2) body with 11 pairs of dorsoexternal setae and micro setae; and (3) relative distance between postpalpal and posthypostomal setae 2.3:1 (38).

Argas vespertilionis (Latreille, 1796)

Local distribution

Palearctic (Hebei, Shandong, Henan, Gansu, and Xinjiang); Oriental (Hubei, Hunan, Guangdong, Zhejiang, Guizhou, Fujian, Guangxi, and Yunnan); Paleozoic–Oriental ecotone (Sichuan, Jiangsu) (37, 51–53, 55, 121, 122).

Natural host

Chiroptera: bat (*Vespertilio* spp.) (37, 51–53, 55, 121, 122).

This species parasitizes bats, and occasionally attacks humans.

Habitats

Associated with bats and bat habitats.

Molecular data

China: 16S rDNA (MW132811, MF106219–MF106221, KY657240, OK047498, OK054512); COI (KY657239); mitochondrial genome (OM368317, OM368318).

Other countries

Belgium: COI (MK140084, MK140088); France: 12S rDNA (JX233821); Hungary: 16S rDNA (KX831484–KX831489); COI (KX431953–KX431955); Italy: 16S rDNA (KX831496–KX831498, HM751841); Japan: 16S rDNA (AB819158); mitochondrial genome (MT762370); Kenya: 16S rDNA (KX831491); COI (KX431956); Netherlands: COI (MK140082, MK140083, MK140085–MK140087); Pakistan: 16S rDNA (MK571555); COI (MK571553); Romania: 16S rDNA (KX831490); Spain: 28S rDNA(MT739330, MT739331); 18S rDNA(MT739410, MT739411); 5.8S rDNA(MT739330, MT739331); ITS1(MT739410, MT739411, MT739330, MT739331); ITS2(MT739330, MT739331); mitochondrial genome (MT680027, MT680028, NC_060373); United Kingdom: 16S rDNA (MF510175–MF510177); COI (MF510173, MF510174); Viet Nam: 16S rDNA (KX831492–KX831495); COI (KX431957–KX431960).

Tick-borne pathogens

Babesia vesperuginis, *Rickettsia raoultii, Rickettsia rickettsia* (121, 123, 124).

In other parts of the world, this tick species has been reported as a vector of Issyk–Kul, Keterah, and Sokuluk viruses, Q fever rickettsia, *Coxiella burnetii*, *Ehrlichia* sp. AvBat, *Rickettsia* sp. AvBat, *Borrelia burgdorferi sensu lato* and an unknown *Borrelia* species closely related to *B. recurrentis, B. crocidurae* and *B. duttonii* (101, 125).

Remarks

Argas vespertilionis is confused with morphologically similar species, therefore, the global distribution of this species is not clear. It appears that *A. vespertilionis* is widely distributed in Africa, Europe, the Palearctic parts of Asia, and a few parts of the oriental region, including some parts of India, Cambodia (126), Vietnam (123) and southern China (5, 51, 52, 93). Hoogstraal (5) stated that reports of *A. vespertilionis* from other parts of the oriental region (Bangladesh, Malaysia, and Philippines) were misidentifications with *A. pusillus*. Robbins (93) excluded *A. vespertilionis* from the checklist of tick species in Taiwan and corrected it to *A. pusillus*. Then, identifications of *A. pusillus* in China are all from Taiwan, and those oriental records of the *A. vespertilionis* are currently doubtful. Therefore, the occurrence of these two species in China should be reconsidered.

Argas vulgaris Filippova, 1961

Local distribution

Palearctic (Xinjiang, Jilin, Gansu, Ningxia, Liaoning, Beijing, Hebei, Inner Mongolia, Shanxi, Shandong, Shaanxi) (51–53, 55).

Filippova (8) indicated that this species was widely distributed in the Palearctic region and was common in the former Soviet Union.

Natural host

Columbiformes: pigeon (*Columba* spp.); Passeriformes: sparrow (*Passer* spp.) (51–53, 55).

Habitats

Often inhabits bird nests.

This species inhabits lowland and foothill meadow steppes, dry steppes, and deserts. Its vertical distribution ranges from sea

level (lower reaches of the Talghinka River in Dagestan) to 900 m above sea level (Karabil, Turkmenistan). Its favorite habitats are ground nests or burrows of birds in outcrops of loess, sandstone, and limestone, as well as the steep banks of rivers and lakes (8).

Molecular data

China: No record.

Other countries

Poland: 16S rDNA (AF001404).

Tick-borne pathogens

No record from China.

Few studies have been conducted on the pathogens of *A. vulgaris*. Hissar virus (*Bunyaviridae*) and Tyulek virus (*Orthomyxoviridae*) were isolated from this tick species in Tadjikistan and Kyrgyzstan, respectively (127, 128).

Remarks

Teng (55) stated that *A. reflexus* from Xinjiang published by Teng (72) should be *A. vulgaris*. Yu et al. (73) reported only *A. reflexus* in Xinjiang. In terms of geographic location and climate, both species have the potential to be distributed in Xinjiang. Therefore, the tick specimens of Xinjiang need to be re-examined.

Ornithodoros capensis (Neumann, 1901)

Local distribution

Oriental (Taiwan) (93).

This species is globally distributed along the coasts and islands of the Pacific, Atlantic and Indian Oceans; the Caribbean and Coral Seas and the lakes of the eastern African Rift Valley system (5, 129–131). Except for Taiwan, very few surveys have been conducted in other parts of China along the coastline, especially in the southern part where the species might also be distributed.

Natural host

No record from China.

Habitats

Inhabits in seabird nests.

Molecular data

China: No record.

Other countries

Algeria: 16S rDNA (KP776644); Australia: 16S rDNA (AH011497); COI (AH011497); NAD1 (AH011497); Brazil: 16S rDNA (KU757069); Cape Verde: 18S rDNA (JQ824327–JQ824368); 16S rDNA (JQ824295–JQ824326); Japan: 16S rDNA (AB819266, AB242431, AB242431, AB057537–AB057540, AB076080–AB076082); mitochondrial genome (AB075953, NC005291); USA: 16S rDNA (EF636462, EF636466).

Tick-borne pathogens

No record from China.

It has been reported that this species can transmit Soldado virus, West Nile virus, Johnston Atoll virus, Upolu virus, Nyaminini virus, Quaranfil virus, Saumarez Reef virus, Hughes virus, *Rickettsia* spp. and *Borrelia* spp. (129, 132).

Remarks

Although China has many islands scattered along the seashore, studies on seabird ticks are scarce, with the exception of *O. capensis*. It is known that both seabird ticks *O. sawaii* and *O. maritimus* are distributed in Palearctic region. *O. maritimus* is distributed in Great Britain, Ireland, France (Corsica), Tunisia, Portugal, Italy (off Sardinia), southwestern USSR, and Senegal (133). *Ornithodoros sawaii* is reported from Republic of Korea and Japan (133, 134). Therefore, these two species might also be distributed in the islands of China.

Ornithodoros huajianensis Sun, Xu, Liu & Wu, 2019

The specific epithet is in allusion to the habitat where this species was found (16).

Type depositories

Medical Entomology Gallery of Academy of Military Medical Sciences, Beijing, China (AMMSC) (holotype ♀, paratypes 2♀♀ 3♂♂ and 3 nymphs).

Local distribution

Palearctic (Gansu) (16).

Natural host

Rodentia: *Marmota bobak sibirica* (16).

Habitats

Prefer semiarid hilly steppes.

Molecular data

China: 16S rDNA (MK208992–MK208994).

Tick-borne pathogens

No record.

Remarks

This species belongs to the subgenus *Ornithodoros*. It was diagnosed by its broad rectangular tongue and triangular tongue–shaped posterior lip in the male genital apron, a shallow camerostome with definite folds, and smaller mammillae with a single seta mixed with larger ones in nymphs and adults (16).

Ornithodoros lahorensis (Neumann, 1908)

Local distribution

Palearctic (Xinjiang, Inner Mongolia, Shandong, Gansu, Liaoning, and Tibet) (18, 20, 41, 51–53, 56, 72, 135, 136).

This species is widely distributed in the Palearctic region, including Armenia, Dagestan, Kazakhstan, Uzbekistan, Turkmenistan, Kyrgyzstan, Tajikistan, Russia, Kosovo, Republic of Macedonia, Syria, Turkey, Iran, Iraq, Saudi Arabia, Afghanistan, Lebanon, Syria, Pakistan, Bulgaria, Greece, Israel, China, and India (5, 8, 132, 137–141).

Natural host

Carnivora: dog (*Canis* spp.); Artiodactyla: cattle (*Bos* spp.), sheep (*Ovis* spp.), goat (*Capra* spp.), camel (*Camelus* spp.); Perissodactyla: horse (*Equus* spp.) (18, 20, 41, 51–53, 56, 72, 135, 136).

This species was originally as a parasite of the Asiatic mouflon, *Ovis orientalis arkal*, and other wandering ungulates resting beside cliffs. However, nowadays, it is a notorious parasite of sheep, camels, and cattle, especially in primitive stables and dwellings in steppes and mountain deserts (5). This species has also been reported to infest human in Turkey and the former Soviet Union (138–140).

Habitats

Living mainly in sheep pens or other livestock sheds (also found in chicken coops). It is rarely reported from natural habitats.

Molecular data

China: 18S rDNA (KX530878, KX530879); 16S rDNA (MG651950–MG651959, KX530872–KX530877, ON159478–ON159502, MN564903–MN564909, OM673115–OM673125, OL444952–OL444957); 12S rDNA (MG651960–MG651967); COI (KX530866–KX530871).

Other countries

Afghanistan: 18S rDNA (L76354); Iran: COI (MK318148, MG582607).

Tick-borne pathogens

"*Candidatus* Anaplasma boleense" and *Anaplasma ovis* (35, 56).

Other pathogenic associations include Crimean–Congo haemorrhagic fever (CCHF) virus, *Rickettsia sibirica*, *R. conorii*, *Brucella abortus*, *F. tularensis*, and *C. burnetii*, which have not been detected in China (139).

Life cycle

Ornithodoros lahorensis is one of the most studied species of soft tick in China. Shao (28) studied the biology of *O. lahorensis* feeding on rabbit under laboratory conditions in Xinjiang. After hatching, it took more than one month for larvae at room temperature before they were able to attach to a host, and then took a total of 24–42 days for blood–sucking larvae to become engorged third instar nymphs (28). Engorged third instar nymphs molted into males and females for 113–149 days and 110–147 days, respectively. Newly molted adults needed 1–1.5 months before attaching to hosts. Engorged females laid eggs between June to August, peaking in July. In Xinjiang, adults and third-instar nymphs could overwinter in the wall crevices of a sheep fold. The larvae infested sheep in late September and October. Zhao et al. (56) reported that *O. lahorensis* ticks infested livestock from late February to early April in southern Xinjiang.

Ornithodoros papillipes (Birula, 1895)

Local distribution

Palearctic (Shanxi, Xinjiang, Inner Mongolia, and Shaanxi) (40, 44, 48, 51, 53, 72).

The species is widely distributed in the Mediterranean and Central Asian subregions of the Palearctic, including Kazakhstan, Uzbekistan, Turkmenistan, Kyrgyzstan, Tajikistan, eastern Libya, western Egypt, Turkey, Cyprus, Syria, Lebanon, Israel, Early Jordan, Iraq, Saudi Arabia, Iran, Afghanistan, Pakistan (Kashmir and western Punjab), and China (8). However, owing to confusion in systematics, some of these data require clarification (8).

Natural host

Carnivora: dog (*Canis* spp.), fox (*Vulpes* spp.); Artiodactyla: sheep (*Ovis* spp.); Lagomorpha: hare (*Lepus* spp.); Erinaceomorpha: hedgehog (*Erinaceus* spp.); Soricomorpha: scilly shrew (*Crocidura suaveolens*); Anura: toad (*Bufo viridis*) (40, 44, 48, 51, 53, 72).

Habitats

It usually selects caves, grottoes, and burrows inhabited by small and medium-sized animals in desert and semi-desert areas along its distribution. In some regions, it often occurs in livestock stables and human houses.

Life cycle

In China, many studies on the biology of *O. papillipes* have been carried out by early researchers (48), which will be very important for distinguishing *O. papillipes* from *O. tholozani*. Engorged females oviposit eggs in summer and autumn (48). Feng et al. (48) reported that there were three to six nymphal instars for this species using mice (*Mus musculus*) and guinea pigs (*Cavia porcellus*) as hosts. A few engorged third instar nymphs molted to adults with the number of males > females; most engorged fourth instar nymphs molted to adults with the number of females > males; a few engorged fifth instar nymphs molted to adults and very few fifth instar nymphs still molted to sixth instar nymphs. The whole process from egg to adult took 5 months to 1 year, which was determined by external temperature and other conditions (48). Additionally, guinea pig (*Cavia porcellus*), chicken (*Gallus gallus domesticus*) and grassland tortoise (*Testudo horsfieldii*) were used as hosts. The results showed that tick development was different under the same laboratory conditions. According to the average weight and volume of engorged ticks, guinea pig is the best host, followed by chicken and then turtle (48).

Molecular data

China: No record.

Czech: Defensin (FJ222575–FJ222577).

Tick-borne pathogens

Borrelia persica (40).

In the 1950–1980s, many cases of tick-borne relapsing fever were reported in Xinjiang. In southern part of this province, the pathogen was *Borrelia persica* transmitted by *O. papillipes* (40, 48, 142). Feng et al. (48) stated that the natural infection rate of spirochetes was very high in *O. papillipes* with spirochetes isolated from 12 of 13 tick groups collected from wall crevices of human houses and burrows of *Bufo viridis*. Additionally, the authors collected many *Bufo viridis* from the same habitats as *O. papillipes*. They then dissected the internal organs (liver, spleen, etc.) of *Bufo viridis*, prepared a suspension emulsion with normal saline, and injected intraperitoneally into guinea pigs. Spirochetes were found in the blood of guinea pigs, which proved that *Bufo viridis* was the natural carrier of tick-borne relapsing fever pathogen (48). Another clinical experiment indicated that 13 guinea pigs suffered from relapsing fever after being bitten by naturally infected *O. papillipes* ticks (80–150 ticks per guinea pig). The incubation period was 4 to 6 days, and the course of the disease lasted 15–20 days. Spirochetes appeared in large numbers in the peripheral blood of these animals. On average, more than 20 spirochetes were observed per field in thick blood smears and in some cases, they were so abundant that could not be reliably counted. During the course of the disease, two guinea pigs died when a large number of spirochetes appeared (48). Shao (40) stated that *O. papillipes* is in close contact with human beings in Xinjiang. They surveyed 50 households in a village and found 49 households were infested by this species. Therefore, in the 1980s, the harm caused by tick-borne relapsing fever in Xinjiang was notable.

Filippova (8) stated that *O. papillipes* was the main vector of tick-borne relapsing fever in the republics of Central Asia and Kazakhstan as well as in neighboring foreign countries. By testing spontaneous carriage, experimental infection and the precipitation reaction a wide range of wild, domestic, and farm animals, carriers of spirochetes in natural and village foci have been established. However, some domestic animals, such as sheep and goats, were characterized by low spirochetemia, resulting in these animals serving only as secondary sources of spirochetes (8). Ticks are capable of taking up spirochetes at any phase and stage, and transmitting them both transstadial and transovarial. The bite of a single infected tick is sufficient to infect humans with spirochetosis (8).

Under experimental conditions, *O. papillipes* can acquire *C. burnetii*, store it for a long time period, transmit the pathogen transstadially, and infect healthy animals during subsequent feeding (8).

Remarks

This species is considered a synonym of *O. tholozani* (Laboulbène and Mégnin, 1882) by Neumann (143, 144), which was subsequently accepted by many Western scientists (3, 5). Currently, *O. tholozani* is reported from India, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Afghanistan, Iran, Iraq, Syria, Jordan, Turkey, Greece, Israel, Egypt, Cyprus, Libya, and Lebanon (51, 145–147). Nuttall et al. (77) considered *O. papillipes* a dubious species, but noted that Birula's figures were difficult to reconcile with the description of *O. tholozani*, especially with regard to the sides of the camerostome and the tarsi, thus they inserted the original description of *O. papillipes* in their book. Filippova (8) indicated that from the diagnosis

and drawings of Laboulbene and Mégnin (1882), it follows that when establishing this species, they had an admixture of species among the type specimens. Indeed, the absenece of cheeks, the structure of the peritremes, hypostome, chelicerae, and legs, as well as larval morphology, suggests that the second species could have been an *Alveonasus* sp. (8). Moreover, Filippova (8) did recognize differences between *O. tholozani* and *O. papillipes*, and thought that Neumann's synonymy relied on the examination of more than one species, likely *O. tholozani* and *O. lahorensis*. She also pointed out that in the literature the species *O. tholozani* should be morphologically similar species *O. papillipes*, *O. verrucosus*, *O. lahorensis*, and other West Asian species (8). In Russian literature, the most common name is *papillipes*. Therefore, Eastern European workers strongly defend the validity of the name *O. papillipes* with scientifically sound arguments. Guglielmone et al. (3) pointed out that the uncertain status of these taxa led them to treat *O. tholozani* and *O. papillipes* both as provisionally valid.

Ornithodoros tartakovskyi Olenev, 1931

Local distribution

Palearctic (Xinjiang, Inner Mongolia, and Shaanxi) (40, 48, 51–53, 72).

This species is distributed in the Palearctic region including Kazakhstan, Uzbekistan, Turkmenistan, Kyrgyzstan, Tajikistan, Iran and China (8, 148).

Natural host

Rodentia: *Rhombomys opimus*; Testudines: tortoise (*Testudo horsfieldii*) (40, 48, 51–53, 72).

Habitats

Mainly inhabit desert and semi-desert areas.

Molecular data

China: No record.

Other countries

Czech: Defensin (FJ222581, FJ222582).

Tick-borne pathogens

Borrelia latyschewii (40).

The pathogen *Borrelia latyschewii* is spread by *O. tartakovskyi* in northern Xinjiang of China (40, 48, 142). *Ornithodoros tartakovskyi* plays a much smaller role in the spread of spirochetosis among humans than *O. papillipes* and *O. verrucosus*, due to its confinement almost exclusively to natural habitats, particularly to burrows of small diameter (8). This species also transmits *Coxiella burnetii* and *Acanthocheilonema viteae* (8, 149).

Conclusions

With the increasing number of new emerging and reemerging tick-borne diseases over the past 20 years, an increasing number of people are paying attention to ticks and tick-borne pathogens. Geographically, China is located in the southeastern part of the vast Eurasian continent, including the Palearctic and Oriental realms and has a variety of ecological types. However, soft ticks and their associated pathogens remain largely unstudied in China. Toward the end of 2021, the argasid tick fauna of China comprised 15 valid species (6.88% of the world's argasid species). Four species are endemic from China: *A. (Argas) assimilis*, *A. (Argas) beijingensis*, *A. (Carios) sinensis* and *O. (Ornithodoros) huajianensis*. Although there are currently no reports of these Chinese endemic argasid species in other countries and regions, it is still possible for those species to be distributed in adjacent regions. Except for *O. capensis*, all other *Ornithodoros* species in China are found in the Palearctic region. Except for *A. vulgaris,* which is limited to the Palearctic Region, the greatest number of *Argas* species is present in the Oriental Region or the Oriental + Palearctic Region. *A. persicus* and *O. lahorensis* most often inhabit nearby human houses and commonly attacks people that makes them the two most thoroughly studied argasid ticks in China.

In total, 47 vertebrate species have been recorded as hosts for Argasidae in China. The most commonly reported hosts of soft ticks in China are birds, followed by mammals. Anurans are rare hosts for *O. papillipes*; however, they can harbor infectious relapsing fever *Borrelia* spp. transmitted by this soft tick (48, 51). The fact that amphibians are implicated as reservoirs of relapsing fever spirochetes is interesting, unprecedented in the eco–epidemiology of these agents, and highlights the need to re–study the disease in China. Additionally, *A. japonicus* and *A. persicus* are always reported to infest birds and domestic fowl abroad, while these two species are often found in livestock in China, which might be because domestic animals are often mixed and housed with poultry in Chinese rural areas. Nine species (60%) were recorded parasitizing humans in China (*A. beijingensis*, *A. japonicus*, *A. persicus*, *A. sinensis*, *A. vespertilionis*, *A. vulgaris*, *O. lahorensis*, *O. tartakovskyi*, and *O. papillipes*). Therefore, soft ticks are no less harmful to humans than hard ticks are.

It is worth noting that some clinical cases have been reported in China. These cases were caused by ticks or tick-borne pathogens such as *A. japonicus*, *A. persicus*, *O. lahorensis*, *O. tartakovskyi*, and *O. papillipes*. However, the pathogens in each case have seldom been investigated.

Additionally, molecular research and investigation of soft ticks and their pathogens, especially on species parasitizing birds and bats remains scarce in China. Except for studies on their morphological characters, research in other areas has not been done for *A. assimilis*, *A. beijingensis*, *A. pusillus*, *A. vulgaris*, *O. capensis*, *O. tartakovskyi*, and *O. huajianensis* in China. Therefore, it is necessary to carry out comprehensive research on soft ticks and associated pathogens in the future.

Author contributions

ZC and JL conceived, designed, and drafted the manuscript. Both authors read and approved the submitted manuscript.

Funding

This review was supported by the Natural Science Foundation of Hebei Province (C20220516), Science Foundation of Hebei Normal University (L2020B17), and Science and Technology Project of Hebei Education Department (QN2020162).

Acknowledgments

We greatly appreciate editor Sebastián Muñoz–Leal and the two reviewers for their positive and constructive comments and suggestions. We are also grateful to Wei Pei from Guangdong Testing Institute of Product Quality Supervision for providing literature of ticks in Japan.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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