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Wildlife parasitology: Emerging diseases and neglected parasites

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Wildlife parasitology: Emerging diseases and neglected parasites

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Editorial: Wildlife parasitology: emerging diseases and neglected parasites

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Editorial on the Research Topic
Wildlife parasitology: emerging diseases and neglected parasites

Introduction

The scientific literature involving wildlife and parasites has been mainly focused on the most common carnivore species, with the majority of publications originating in Europe. The present Research Topic was edited by parasitologists from different geographical areas (Romania, Canada, Costa Rica, and Iran), who managed to gather an impressive number of papers focused on wildlife parasitology from various countries, contributing to the extension of the knowledge in this field as well as highlighting the importance of research in this research area.

Overall, 15 original research papers and four case reports were included in this Research Topic, published by 147 different authors based in 25 countries. Among these, 3 (15.8%) papers were focused on carnivore species, 1 (5.3%) investigated parasites in primates, 3 (15.8%) papers reported parasites of wild birds, 1 (5.3%) on armadillos, 2 (10.5%) on wild boars, 1 (5.3%) on bats, 1 (5.3%) on invasive frogs, 1 (5.3%) on marsupials, and 4 (21%) investigated parasites in ruminants. In addition, one paper (5.3%) was focused on cats and one (5.3%) on fleas. Of note in this Research Topic two novel parasite species namely *Sarcocystis funereus* (Apicomplexa, Sarcocystidae), and *Delicata tatouay* (Molineidae, Anoplostrongylinae) were described, and the existence of the ancient deer-specific *Cooperia ventricose* was confirmed.

Bellow we summarize 19 articles sorted by their subject to three classical categories i.e., helminths, protozoa, and arthropoda.

Articles on helminth parasites

Wild canids are known as important reservoirs of zoonotic parasitic infections. Uribe et al. investigated the presence of zoonotic helminths in wild canids from the Amazonian and Andean regions in Colombia and identified three species with a zoonotic

potential (*Dipylidium caninum*, *Spirometra mansoni*, and *Lagochilascaris cf. minor*). A better knowledge of epidemiology and transmission routes of these neglected helminths is advocated.

Among wild carnivores, mustelids represent a less studied group, although very abundant and widely distributed. Deak et al. conducted a study on the species diversity and distribution of *Crenosoma* species infecting mustelids in Romania and showed that badgers were infected by *C. melesi* and *C. petrowi*, while beech martens were infected with *C. petrowi* and *C. vulpis*. The authors reported new host-parasite associations and sequenced *C. melesi* and *C. petrowi* for the first time.

Zoonotic parasitic infections are very diverse and can also be caused by metastrongyloid nematodes. One example of such a parasite infecting an atypical host is presented by Solorzano-Scott et al. in an opossum from Costa Rica. The authors presented the first case of cerebral infection by *Angiostrongylus costaricensis*, underlying the difficulties in diagnosing neuroangiostrongylosis and the importance of molecular methods to confirm the identity of parasites.

Felids are important hosts for many parasites, some of which have a severe or even lethal effect. Such parasites, like the neglected angio-neurotropic *Gurltia paralysans* nematode, can cause severe disease in domestic cats and can pose a risk for endangered wild felids. Feline gurltiosis cases from South America were presented by Gómez et al. in an original research paper with a focus on a specific case of infection in a domestic cat.

Non-human primates are commonly observed in captivity. Cuccato et al. reported a lethal case *of Cysticercus longicollis* infection in a captive ring-tailed lemur (*Lemur catta*) from Italy. The exact source of infection was not identified, but it was assumed to be correlated with carnivores from the biopark, underling the importance of the control of parasitic diseases as well as the implementation of biosecurity measures.

Magdálek et al. studied the seasonality and anthelmintics susceptibility of *Ashworthius sidemi*, an alien nematode which has emerged in captive fallow deer in Central and Eastern Europe over the last decade. Negligible seasonal patterns of parasite egg shedding indicated adaptation of this non-native parasite to the current climatic conditions of the Czech Republic.

Albrechtová et al. revised the trichostrongylid nematode *Cooperia* from red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) and confirmed the existence of the deer-specific *Cooperia* species *C. ventricose* which was described only in 1809 and is similar in morphology to *C. pectinata* parasitizing bovids.

Pikula et al. reported the filarial nematode *Litomosa* sp. in the abdominal cavity of a parti-colored bat (*Vespertilio murinus*) and its microfilariae in bat semen suggesting semen-borne transmission of this worm in addition to the known life cycle pattern that involves blood-sucking ectoparasites.

de Oliveira Simões et al. described a new roundworm *Delicata tatouay* in the small intestine of the greater naked-tailed armadillo (*Cabassous tatouay*) that inhabits Uruguay, Northeastern Argentina, Eastern Paraguay, and South, Central, and Northeastern Brazil. This novel species is the 14th member of the genus *Delicata* that infects armadillos.

Finally, Lykins et al. reported encysted larvae *Pterygodermatites whartoni* in invasive Cuban treefrogs (*Osteopilus septentrionalis*) in Central Florida, United States. Authors demonstrated that Cuban treefrogs can serve as potential paratenic hosts of *P. whartoni*, that the spirurid nematode is not restricted to Southeastern Asia, and that this invasive frog can play a role in the distribution and transmission of the invasive parasite.

Articles on protozoan parasites

Wild boars have a wide geographical distribution and are susceptible to many parasitic infections, representing an important reservoir for pathogen transmission to animals and humans. In Europe, the population of wild boars has increased, and as a consequence, the risk of emerging vector-borne diseases is higher. Sgroi et al. screened over 200 wild boars from Italy for *Babesia*/*Theileria* infections and identified for the first time *Babesia vulpes* and *Babesia capreoli* in 13 and 2 tested wild boars, respectively. In the same animal species, but this time in Asia (Korea), Lee and Kwak explored the public risk of infection with *Giardia duodenalis* by examining 612 wild boar fecal samples using the PCR technique. Overall, they identified 20.4% prevalence in wild boars underlying the seasonal factor as an important risk factor.

The pudus (*Pudu puda*) are the world's smallest deers, and distributed only in the Southern Andes of Chile and Argentina. Hidalgo-Hermoso et al. reported DNA of *Bartonella* spp., hemotropic *Mycoplasma ovis*-like, and *Coxiella burnetii* from this near threatened Cervidae all of which are potentially zoonotic. They presented the first report of *B. henselae*, the causative agent of zoonotic cat scratch diseases, in a wild ungulate.

Máca et al. identified the Tengmalm's owl (*Aegolius funereus*) as the definitive host of a novel *Sarcocystis* species named as "*Sarcocystis funereus* sp. nov." Authors isolated oocysts and sporocysts of the parasite from the intestinal mucosa of the bird host, and experimentally fed them to a mouse model (as intermediate host) to observe the sarcocysts in the skeletal muscle.

Articles on arthropod parasites

Bahiraei et al. reported 31 chewing lice species collected from 612 examined wild birds representing 16 orders, 33 families, 60 genera, and 78 species in different regions of Iran. They also presented an updated checklist of louse species reported from the country according to their avian hosts.

Liu et al. worked on the mitochondrial genomes of two flea species *Frontopsylla spadix* and *Neopsylla specialis* which are the main flea vectors for the transmission of wild rodent plague into rats. Data generated using long-range PCR and next-generation sequencing technologies made the basis for future molecular evolution, taxonomy, and systematics of the flea species.

Sarcoptic mange in the Spanish wild goat Iberian ibex (*Capra pyrenaica*) was the subject of a study by Valldeperes et al.. They found that the local skin immune response is a determinant factor in the clinical responses to *Sarcoptes scabiei* infestation in this species.

Wechtaisong et al. studied the diversity of *Anaplasma* and *Bartonella* species in *Lipoptena fortisetosa* keds collected from captive Eld's deer (*Rucervus eldii thamin*) in Thailand. Authors discovered five *Bartonella* lineages including a new independent lineage of novel *Bartonella* species, *Anaplasma bovis* and other ruminant-related *Anaplasma*. They suggested the implementation of preventative measures in areas surrounding wild animals in order to prevent pathogen transmission among animals and humans.

Ali et al. reported for the first time the soft tick *Alectorobius coniceps* collected from nests of highly aerial birds "swifts" in Pakistan with morphological and genetic data expanding its reported geographical distribution.

Conclusion

In conclusion, this Research Topic successfully addressed a variety of parasitic groups in a myriad of hosts from different regions of the world. This richness demonstrates the fertile ground wildlife parasitologists are working on and the importance of One Health approaches to keep this important research.

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Diversity of *Crenosoma* species in mustelids with the first molecular characterization of *C. melesi* and *C. petrowi*

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Species of genus *Crenosoma* have a wide distribution and are reported in Europe, the Americas, and Asia. Currently, the genus includes 14 nominal species, out of which 9 are parasitic in mustelids. Two species are mostly reported in mustelids from Europe, namely *C. melesi* and *C. petrowi*. Up to now, no genetic sequences are deposited in GenBank for any of the two. The aims of this study were to investigate the distribution, prevalence, and diversity of *Crenosoma* spp. infecting mustelids in Romania and to genetically characterize the species. Mustelids ($n = 247$) were collected over a period of 7 years from different locations in Romania and the respiratory tract was removed and examined for nematodes. Detected nematodes were morphologically identified and fragments of two genes were sequenced. Sampled mustelids included Eurasian badger, *Meles meles* ($n = 102$), Eurasian otter, *Lutra lutra* ($n = 20$), beech marten, *Martes foina* ($n = 36$), European pine marten, *Martes martes* ($n = 5$), steppe polecat, *Mustela eversmanii* ($n = 1$), European mink, *Mustela lutreola* ($n = 1$), least weasel, *Mustela nivalis* ($n = 2$), European polecat, *Mustela putorius* ($n = 78$), and marbled polecat, *Vormela peregusna* ($n = 1$). Nematodes from Eurasian badgers were morphologically identified as *C. melesi* ($n = 13$, 12.74%) and *C. petrowi* ($n = 3$, 2.94%). Nematodes from the beech martens were identified as *C. petrowi* ($n = 6$, 16.66%), *C. vulpis* ($n = 1$, 2.78%) and *Crenosoma* spp. ($n = 3$, 8.33%). Co-infections with two *Crenosoma* species were detected in one beech marten (*C. petrowi* + *C. vulpis*, $n = 1$, 2.77%) and in one European pine marten [*C. petrowi* + *C. vulpis* ($n = 1$, 20%)]. Two genes of *Crenosoma melesi* and *C. petrowi* were partly sequenced for the first time. We report new host-parasite associations for *M. martes* and *C. vulpis*. However, further studies are needed in order to determine the host-parasite associations and to improve the understanding of the epidemiology of *Crenosoma* nematodes.

KEYWORDS

animal-host association, *Crenosoma* spp., *Meles meles*, mustelids, Romania

Introduction

Nematodes of the family Crenosomatidae are found in the respiratory tract and sinuses of various mammals (1). The family includes five genera: *Paracrenosoma* Yun and Kontrimavichus, 1936 (in the respiratory system of insectivores), *Troglostrongylus* Vevers, 1923 (in the respiratory system of felids),

Prestwoodia Anderson, 1978 (in the sinuses of opossums of genus *Didelphis*), *Otostrongylus* de Bruyn, 1933 (in the respiratory tract of seals), and *Crenosoma* Molin, 1861 (in the trachea, bronchi, and bronchioles of carnivores and insectivores) (2). *Molinofilaria* Vuylsteke 1956 (in the bronchi and veins of pinnipeds) is considered similar to *Otostrongylus*, but the classification of this genus remains unclear (Anderson, 1978).

The genus *Crenosoma* is morphologically distinguishable by the presence of a striated and folded cuticle (3). Species belonging to the genus are distributed in Europe, the Americas, and Asia (2, 4–8, 10) (Table 1). The latest review of the genus *Crenosoma* lists 14 nominal species (43), out of which 9 are found in mustelids. Out of these, three species are found in the New World (*C. brasiliense* Vieira et al. 2012, *C. goblei* Dougherty, 1945, *C. hermani* Anderson, 1962) and six in the Old World [*C. melesi* Jančev and Genov, 1988, *C. petrowi* Morozov, 1939, *C. schachmatovae* Kontrimavichus, 1969, *C. schulzi* Gagarin, 1958, *C. taiga* Skrjabin and Petrov, 1928, *C. vulpis* (Dujardin, 1844)].

There is a relatively large body of literature, often confusing, with reports of various *Crenosoma* species in mustelids. For instance, Stunženas and Binkiene (43) list *C. petrowi* as a species distributed in Eurasia but among the hosts, they list two American mustelids. Such a wide distribution over two biogeographical regions is often related to a poor species definition and the absence of genetic data, as most reports are based on morphological identifications. The two most common species of *Crenosoma* reported in mustelids in Europe are *C. melesi* and *C. petrowi*. Surprisingly, prior to the present study, no gene sequences were known for any of the two.

In Romania, *Crenosoma vulpis* infection in carnivores was documented only in foxes (51, 52), and *Crenosoma* spp. in bears (53, 54) with limited knowledge regarding the species diversity and distribution range among other carnivores. Wild carnivores are important reservoirs for parasites that can infect domestic animals and humans (55–58). At the same time, badgers are the least studied group in this direction, even though they are reservoirs for *Mycobacterium bovis* (Infantes-Lorenzo et al., 2019). Romania has a remarkable diversity and abundance of mustelids, with nine extant species recorded in the country (59).

Considering the very limited knowledge and the existence of a wide variety of *Crenosoma* spp. parasites in mustelids, the present paper aimed to investigate the distribution, prevalence, and diversity of these species among mustelids in Romania. Additionally, identified species were also characterized by partial sequencing of two genes, and the risk factors related to sex, age, and geographical localization were analyzed simultaneously.

Materials and methods

Samples

Between March 2014 and March 2021, 247 carcasses of mustelids were collected by hunters or found as roadkills in different regions of Romania (Supplementary material 1). Details regarding the sex, age, date and locality of collection were recorded and the carcasses were sent to the Department of Parasitology and Parasitic Diseases of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca where they were kept individually in labeled sealed plastic bags at −20°C until examination. The entire respiratory tract of each animal was removed, and the trachea, the large bronchi, and the bronchioles were thoroughly checked for the presence of nematodes under a stereomicroscope. The lungs were immersed in tap water for a few hours and manually compressed to extract the remaining nematodes. The water was then filtered through sieves and scrutinized for parasites. All nematodes were collected using fine entomological tweezers and washed in physiological saline solution. Nematodes were placed in 4% formalin (for further morphological identification) and 70% ethanol (for molecular analysis). When only one specimen was detected, it was placed in 70% ethanol. A coproscopic and larvoscopic examination was not done due to the freezing and decomposition of the carcasses.

Morphological identification

Each nematode was temporary mounted on a glass slide in mineral oil and identified based on the morphological descriptions (8). Photographs and measurements of the specimens were taken using an optical microscope (Olympus BX61) connected to a digital camera (DP72 with Cell^F imaging software Olympus Corporation, Tokyo, Japan). The following morphometric features were evaluated in males: body length, body width, number of anterior rings, length and maximum width of the esophagus, lengths of spicules, and length and width of gubernaculum. In females, the following morphological features were evaluated: body length, body width, number of anterior rings, length and maximum width of the esophagus, tail length, and egg size. All sizes are given in micrometers (μm).

Sequencing and phylogenetic analysis

Genomic DNA was isolated from one or more specimens preserved in ethanol, using a commercial kit (Isolate II Genomic DNA Kit, meridian Bioscience, London, UK), according to the manufacturer's instructions. The samples were processed by means of PCR amplification and bidirectional sequencing of three genetic markers, as previously described (Table 2). Only the samples that yielded high quality sequences of all three markers were further analyzed using MEGA X software (63). The pairwise distances were evaluated, and the evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (64).

Statistical analysis and maps

The statistical analysis was performed using EpiInfo 7 software (CDC, USA). The prevalence of infection and its 95% Confidence

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TABLE 1 Species of *Crenosoma* identified in mustelids: hosts and geographical distribution.

Species	Host	Country or region	References
C. brasiliense Vieira et al. 2012	***Galictis cuja****	Brazil	(2)
C. goblei Dougherty, 1945	*Procyon lotor psora** *Procyon l. lotor*	USA	(5)
	Martes americana	USA	(11)
	Lutra canadensis	USA	(12)
		USA	(13)
	Procyon l. lotor	Canada	(6)
	Procyon l. lotor	USA	(14)
C. taiga Skrjabin and Petrow, 1928	***Mustela sibirica****	Former USSR	(4)
	M. putorius	Russia	(15)
	Martes martes	Former USSR	(16)
	Martes foina		
	Meles meles		
	Mustela nivalis		
	Mustela erminea		
	Mustela altaica		
	Mustela zibelina		
	Gulo gulo		
C. schachmatovae Kontrimavichus, 1969	***Mustela erminea****	Former USSR	(17)
	Martes foina	Lithuania	(18, 19)
	Neovison vison		
	Mustela putorius		
	Neovison vison	Lithuania	(19)
	Mustela putorius		
C. hermani Anderson, 1962	***Neovison vison****	Canada	(20)
C. petrowi Morozov, 1939	***Martes zibellina****	Russia	(21)
	Martes martes ruthega	Russia	(5)
	Neovison vison	Former USSR	Zueva and Belyrov, 1965
	Gulo-gulo	Karelian Republic	(22)
	Martes martes	Former USSR	(23)
	Martes foina		
	Mustela vison		
	Mustela erminea	Kazakhstan	(24)
	Pekania pennanti	USA	(6)
	Taxidea taxus	Canada	
	Martes martes	Former USSR	(25)
	Martes foina		
	Martes zibellina	Former USSR	
	Martes foina	Former USSR USA Canada	(16)

(Continued)

TABLE 1 (Continued)

Species	Host	Country or region	References
	Ursus americanus	Canada	(17)
	Martes americana	Canada	(26)
	Martes foina	Italy	(27)
	Martes martes	Spain	(28)
	Vulpes vulpes	Russia	(29)
	Nyctereutes procyonoides		
	Meles meles		
	Canis lupus familiaris		
	Martes zibellina	Russia	(30)
	Neovison vison	Russia	(31)
	Martes foina	Bulgaria	(25)
	Ursus americanus	Canada	(32)
	Martes foina	Bulgaria	(33)
	Martes foina	Romania	Current study
	Martes martes		
	Meles meles		
C. melesi Jancev and Genov, 1988	***Meles meles****	Bulgaria	(8)
	Mustela nivalis	Spain	(9)
	Meles meles	Italy	(34)
	Meles meles	Spain	(35)
	Meles meles	Norway	(36)
	Mustela putorius	France	(10)
	Mustela putorius	Germany	(37)
	Neovison vison	Spain	(38)
	Meles meles	Ireland	(39)
	Meles meles	Romania	Current study
C. schulzi Gagarin, 1958	***Meles meles****	USSR	(40)
	Meles meles	The Republic of Moldova	(41)
	Meles meles	Kirghizstan Uzbekistan Karelian Republic	(16)
C. vulpis (Dujardin, 1844)	*Ursus arctos*	Former Yugoslavia	(42)
	Ursus americanus	Canada	(7)
	Taxidea taxus	Former USSR	Reviewed by (43)
	Canis lupus familiaris	Chile	(44)
	Many canid hosts	Eurasia North America	(43)
	Martes zibellina	Europe	
	Lutra lutra	Former USSR	(16)
	Gulo gulo	North America	
	Meles meles	Germany	(45)

(Continued)

TABLE 1 (Continued)

Species	Host	Country or region	References
	Martes foina	Germany	(46)
	Meles meles	Poland	(47)
	Meles meles	Italy	(48)
	Martes foina	Portugal	(49)
	Neovison vison	Denmark	(50)
	Mustela putorius		
	Martes martes	Romania	Current study
	Martes foina	Romania	Current study

Species marked with "*" represent the type hosts. Species in bold are members of Mustelidae.

TABLE 2 Primers used for amplification and sequencing of *Crenosoma* specimens.

Gene	Product (bp)	Primer sequence	References
Cytochrome oxidase subunit 1 (*cox*1)	~700	LCO1490: GGTCAACAAATCATAAAGATATTGG	(60)
		HCO2198: TAAACTTCAGGGTGACCAAAAAATCA	
Large subunit (LSU) rRNA gene	850-950	391 F: AGCGGAGGAAAAGAAACTAA	(61)
		501 R: TCGGAAGGAACCAGCTACTA	
	850-900	537 F: GATCCGTAACTTCGGGAAAAGGAT	(62)
		531 R: CTTCGCAATGATAGGAAGAGCC	

Interval (95% CI) were established and the differences among various categories (age, sex, bioregions) were evaluated by chi-square test, and considered significant at $p \leq 0.05$.

The distribution map was generated using ArcMap 10.6.1 software.

Results

The examined mustelids were morphologically identified as Eurasian badger, *Meles meles* (n = 102), Eurasian otter, *Lutra lutra* (n = 20), beech marten, *Martes foina* (n = 36), European pine marten, *Martes martes* (n = 5), steppe polecat, *Mustela eversmanii* (n = 1), European mink, *Mustela lutreola* (n = 1), least weasel, *Mustela nivalis* (n = 2), European polecat, *Mustela putorius* (n = 78), and marbled polecat, *Vormela peregusna* (n = 1). *Crenosoma* spp. nematodes were detected in the trachea, bronchi and bronchioles (Figure 1) of 26 mustelids (10.6%; 95% CI 7.0–15.1), namely: 16 Eurasian badgers (15.7%; 95% CI 9.2–24.2), 9 beech martens (25%; 95% CI 12.1–42.2), and one European pine marten (20%; 95% CI 0.5–71.6). The nematodes collected from the Eurasian badgers were morphologically identified as *C. melesi* (n = 13, 12.7%) and *C. petrowi* (n = 3, 2.9%). In beech martens *C. petrowi* (n = 6, 16.7%), *C. vulpis* (n = 1, 2.8%) and *Crenosoma* spp. (n = 3, 8.3%) were identified, the latter being in a very bad condition, which rendered them unidentifiable to species level. None of the Eurasian badgers, was co-infected with *C. melesi* and *C. petrowi*. Co-infections with two *Crenosoma* species were detected in one beech marten (CJ007577) [*C. petrowi* + *C. vulpis* (2.8%)] and the only positive European pine marten (CJ007578) [*C. petrowi* + *C. vulpis* (20%)].

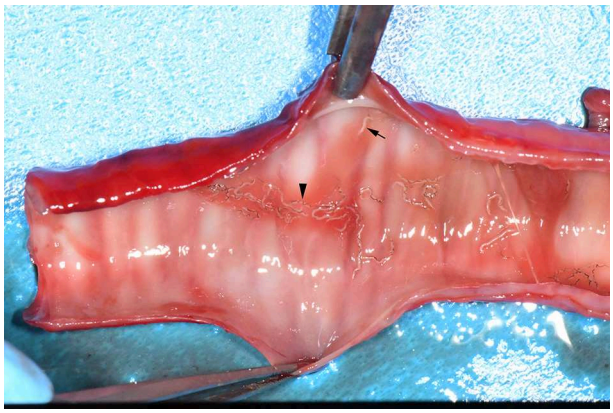

FIGURE 1
Adult nematodes of *C. petrowi* in the trachea of a badger collected in Borod locality. Arrow head: Female nematodes with a thin, black tube inside; Black arrow: Male nematodes, smaller and white.

Adult *C. melesi* presented specific circular folds visible along the length of the body (Figure 2a), starting from the middle of the esophagus until after the middle of the body length, with a region in which they were less obvious, followed by their reach back in the region of the anal opening (Figure 2b). The females' body length was between 4.6 and 14.9 mm and their width from 249.9 to 971 μm. From a lateral view, the vulva protruded significantly above the cuticle. Inside the two uteri, eggs in different development stages were observed. The tail has a conic shape with two evident subterminal papillae. The adult males were 4.1

FIGURE 2
Morphological characteristics of *C. melesi*. **(a)** The specific circular folds. **(b)** Caudal extremity of a female with the cuticular folds visible and the anal opening marked with an arrow head. **(c, d)** Morphological characteristics of a male *C. melesi*. **(c)** The trilobated copulatory bursa with the presence of terminal globular papilla in each ray (asterix); **(d)** note the spicules splited in two branches.

to 5.9 mm in length and 149 to 434.3 µm in width. The cuticular folds were visible until almost half of the body length, after which they disappeared. The copulatory bursa had three lobes, with the lateral ones more developed than the median one. The bursa was sustained by rays which ended in large globular papillae (Figure 2c). The two spicules were almost equal in length, and each was split in two branches in their middle and distal part (Figure 2d).

Crenosoma petrowi adults had a transparent cuticle that formed evident folds that protrude in their posterior part (Figure 3e). The rings formed by the cuticle were visible until half of the body length for female worms and only in the anterior third in males, where the folds stretch and become unapparent. The females detected in Eurasian badger hosts were 6.7–9.2 mm in length and in 402.2–406 µm in width. The ones detected in the two *Martes* species were 3.6 to 5.8 mm in length and 283.3–384.1 µm in width. The vulva was lacking the appendage above the cuticle, and it was localized in the anterior third of the worm. Eggs and larvae were visible inside the uteri. The anal opening was very close to the posterior extremity of the nematode (110–210 µm) (Figure 3d). Male nematodes collected from badgers were 3.4–4 mm in length and 220.3–262.3 µm in width. The male specimens collected from both species of martens were slightly smaller, 2.4–3.2 mm in length and 154.8–224.8 µm in width. The copulatory bursa had three well distinctive parts, and it is sustained by rays. The spicules were almost equal in length and slightly curved at their caudal end (Figures 3a, b). On the dorsal side of the spicules there was a thin protrusion visible in the second third of the length (Figures 3a, c). The gubernaculum had the shape of a barque from a lateral view (Figure 3b).

Crenosoma vulpis adults have a cuticular sheath that formed evident folds visible in the anterior part and were stretched in the posterior extremity. The females detected in *Martes foina* were 9.4–10.3 mm in length and 348.6–427 µm in width. The ones collected from *Martes martes* were 4.6 to 6.7 mm in length and 357.3–455.4 µm in width. The vulva was positioned almost in the middle of the body, closer to the posterior extremity and the anus was at 117.3–189.3 µm from the caudal extremity (Figure 4a). Two papillae (phasmids) are visible on the lateral parts of the tail. The eggs containing a larva were visible and measured only in one specimen collected from *Martes foina* and had a size of 75.2–76 × 39.7–42.7 µm (Figure 4b). No male specimens of *C. vulpis* were found.

The measurements of all the nematodes that were morphologically characterized are available in the Supplementary material 2.

High-quality sequences were obtained for all of the three markers for a total of 21 *Crenosoma* specimens, belonging to 14 hosts: ten Eurasian badgers, three beech martens, and one pine marten. The LSU sequences were highly conserved and insufficient for a clear differentiation among species (Supplementary material 3). However, the differences and distances between the *cox* 1 isolates were in agreement with the morphological identification of the species. The phylogenetic analysis revealed that *C. melesi* formed a separate clade, while *C. petrowi* clustered with *C. vulpis* and is more closely related to *C. goblei* (Figure 5).

According to the bioregion, overall, the differences in the prevalence of infection were significant ($X^2 = 13.18$; d.f. $= 4$; $p = 0.01$).

In the Eurasian badgers, juveniles were significantly more frequently infected as compared to adults ($X^2 = 8.95$; d.f. $= 1$; $p = 0.002$), while the differences between sex and bioregion were

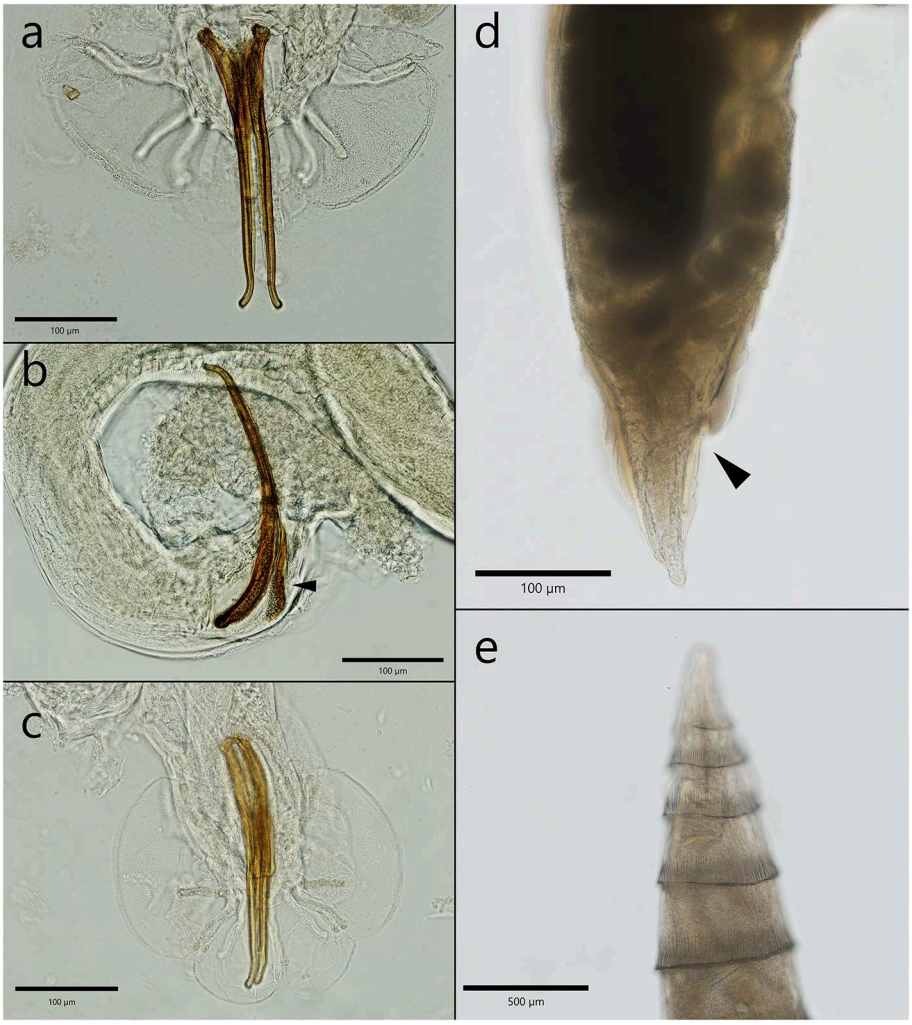

FIGURE 3
The morphological characteristics of male and female *C. petrowi*. **(a)** Posterior extremity of a male with the two spicules—note the thin protrusion; **(b)** lateral view of a male caudal bursa—the characteristic shape of the gubernaculum (arrow head); **(c)** the posterior extremity of a male with the typical caudal bursa; **(d)** posterior extremity of a female nematode; **(e)** specific folds in the anterior extremity.

not significant. For the beech martens, no significant differences were identified.

Thirteen (12.75%) of the Eurasian badgers were infected with *C. melesi*, while the other three (2.94%) harbored *C. petrowi*. Among beech martens, *C. petrowi* was identified in six (16.67%) individuals, of which one (2.78%) was co-infected with *C. vulpis*. The remaining three (8.33%) were positive for *Crenosoma* spp. The statistical data is available in Supplementary material 4. The distribution map of the *Crenosoma* species and the positive hosts is shown in Figure 6.

Discussion

The genus *Crenosoma* is widely distributed and its species infect a wide variety of mammal hosts. *Crenosoma vulpis*, infecting mainly canid hosts, is the most studied species of the genus, followed by *C. striatum*, parasitic in hedgehogs (Table 1). Although there are numerous studies which report the infection with *Crenosoma*, the identification was based only on the morphological characteristics, correlated with the assumed host specificity. The morphological identification of species is mainly based on the number and aspect of the anterior cuticular folds, the aspect of the female tales and the dimensions and aspects of the copulatory bursa and rays in male nematodes. Nowadays, when genetic tools are largely available, the lack of molecular data could be considered a limitation of the published studies, which could have affected the knowledge on parasite-host associations. Interestingly, up to now, there is very little information about the genetic sequences of *Crenosoma* species. Partial *cox*1 gene sequences are available for only three species in the GenBank (*C. striatum*, *C. vulpis,* and *C. goblei*), while SSU and/or LSU sequences are known for four species (*C. striatum*, *C. vulpis*, *C. goblei,* and *C. mephitidis*).

The present study brings important details regarding the diversity of *Crenosoma* species in mustelids and host-parasite association. Moreover, two species, namely *C. melesi* and *C. petrowi* were reported for the first time in Romania. *Crenosoma melesi* is a respiratory strongyle typically infecting Eurasian badgers, which was initially described in Bulgaria, followed by few reports in other

European countries. *Crenosoma melesi* was identified also in other mustelid species (Table 1). Although in our study we found a higher prevalence of infection in juvenile Eurasian badgers, as no data is available from other studies across the range of this host, we cannot conclude that there is an age risk. Generally, higher prevalence in young animals has been documented for other nematode species and it is believed that this could be due to their curious behavior, increased immunity gained with the age, or differences in their food habits (52, 65).

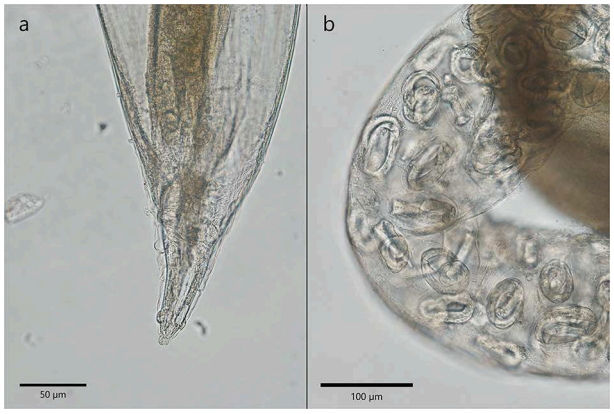

FIGURE 4
The morphological characteristics of *C. vulpis*. **(a)** The posterior extremity of a female with stretched cuticular folds. Anal opening is visible; **(b)** Note the presence of eggs containing larva in the uterus of a female.

Crenosoma petrowi was identified in three mustelid species: *Meles meles*, *Martes foina*, and *Martes martes*. Additionally, this species is much more similar to *C. vulpis* in regard to its wide range of parasitized animals, including canids and *Ursus americanus,* and also to its wide distribution range (Table 1). Moreover, the present results are in accordance with this statement as based on the phylogenetic analysis, *C. petrowi* is closely related to *C. vulpis* (Supplementary material 3), and genetically distant to *C. melesi*. However, the reports of *C. petrowi* in canid hosts are questionable, as artificial infections of red foxes were unsuccessful (17).

Previously, only *C. vulpis* was morphologically identified in red foxes from Romania (52), and is now morphologically and molecularly confirmed in two mustelid species, *M. martes* and *M. foina*. In red foxes, the abundance of *C. vulpis* has a strong positive relationship with the presence of wetlands, and environmental factors mostly act on the intermediate hosts, regulating the distribution pattern (52). However, animals examined in the present study were grouped based on bioregions, and both animals

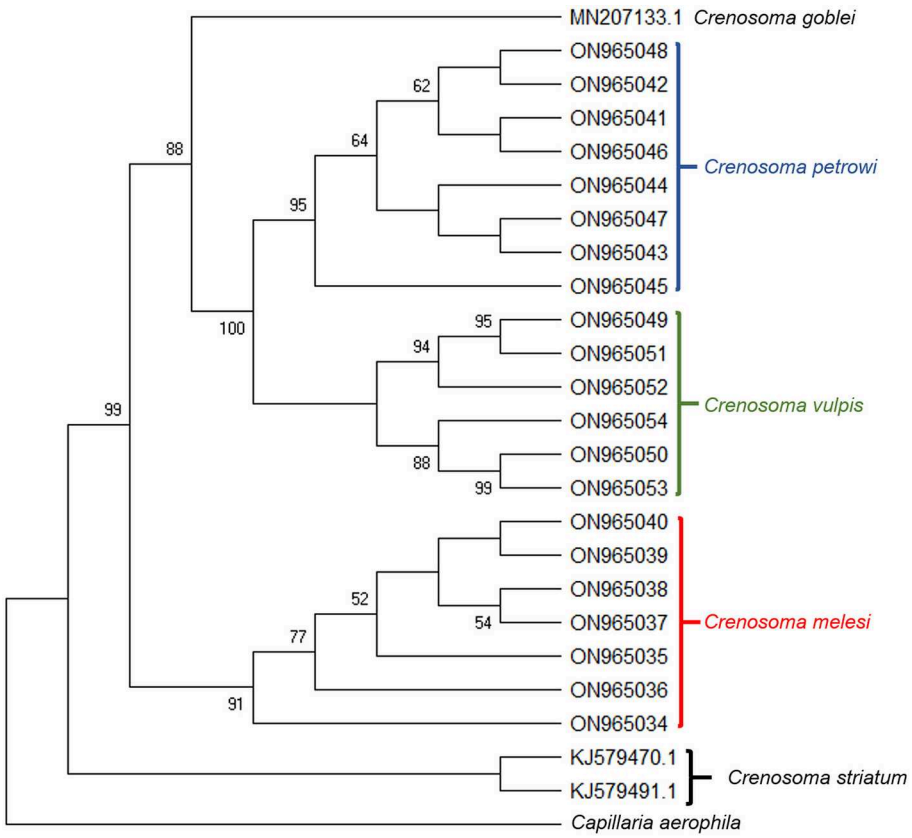

FIGURE 5
Bootstrap consensus tree inferred from 1000 replicates, taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The analysis involved 24 *Crenosoma cox* 1 nucleotide sequences obtained during the present study (21) or retrieved from the GenBank database (3), and one sequence of *Capillaria aerophila*, used as outgroup.

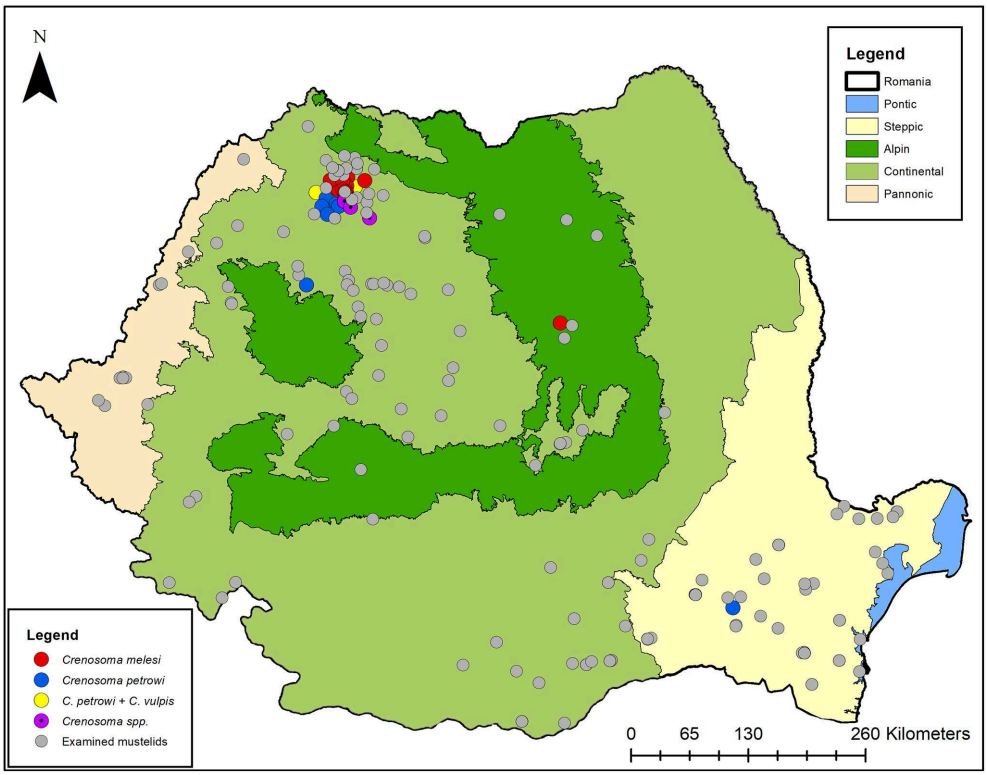

FIGURE 6
The distribution map of *Crenosoma* spp. in Romanian mustelids.

infected with *C. vulpis* originated in a continental bioregion, located in the same county (Maramureș). This infection could be associated with an endemic area of *C. vulpis* rather than a specific parasite-host association, with mustelids being only accidental hosts.

The absence of *C. vulpis* in Eurasian badgers could be related to a previous misidentification of this species in badgers' hosts, or to a negative parasite-host association. Until more information is available on this topic, we can hypothesize that badgers are unsuitable hosts that may accidentally get infected in endemic areas. The only positive *M. martes* was found to be co-infected with *C. vulpis* and *C. petrowi*, which to the best of our knowledge is the first report of a co-infection with two species of the *Crenosoma* genus in the same host. Similarly, in one *M. foina* we detected the same species association. These findings underline the importance of complementary identification methods, such as molecular typing of specimens, especially for genera that are known to have more than one species parasitic in a particular host. Ideally, morphological identification should be followed by molecular confirmation.

In addition, *C. vulpis* was detected for the first time in a European pine marten. The morphological descriptions of *Crenosoma* specimens from the present study are in accordance with data previously reported (8, 16), with slight differences in morphometrics.

Besides the two species of martens and the badgers, all the other mustelids were negative for infection with *Crenosoma* spp. For *M. nivalis*, *M. lutreola*, *M. eversmanii* and *V. peregusna* the lack of infection could be attributed to the low number of examined animals. However, 31 *L. lutra* and 78 *M. putorius* were examined with no *Crenosoma* nematodes detected. Both mustelids are suitable hosts and were previously found to be infected with *Crenosoma* spp. (Petrov, 1940; Kontrimaviciius et al., 1976; Nugaraitė et al., 2014; Kretschmar, 2016). There is only one report of infection with *C. vulpis* in one otter, in the Asian part of the former USSR (16). Most likely, due to its habitat and food preferences consisting mainly in crustaceans and fish (66), this host is not ecologically exposed to infective *Crenosoma* larvae. Infection with *C. taiga* was identified only once in one European polecat from a zoo in Moscow in 1940 (15) and since then, no other reports are available. More recently, infection with *C. schachmatovae* was reported in polecats from Lithuania (18, 19). In the present study, all the examined polecats were negative, and we could speculate that *C. schachmatovae* is absent in Romania (Table 1).

The present paper presents the first complex study on *Crenosoma* parasitic in mustelids and points up the need of respecting specific identification protocols when dealing with multiple-species parasites. Moreover, the study highlights the need for further studies in order to elucidate the host-parasite associations, pathological implications on mustelid hosts, as well as the exploration of other gene sequences and their suitability for phylogenetic taxonomy.

Conclusions

There are three species of the genus *Crenosoma* infecting Romanian mustelids. Co-infections with two species of the genus

in the same animal host are possible. We report a new host-parasite association for *M. martes* and *C. vulpis*. Sequences of *C. melesi* and *C. petrowi* were described for the first time. The *cox1* difference between the identified species is in accordance with the morphological identification. Further studies are needed in order to determine the host-parasite associations and to improve the understanding of the epidemiology of *Crenosoma* nematodes.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the ethical decision nr. 232 23.11.2020, approved by Bioethics Committee of USAMV Cluj-Napoca.

Author contributions

GD helped with the collection of the carcasses, performed the necropsies, morphologically identified the nematodes, analyzed the data, and wrote the manuscript. AI performed necropsies, the molecular work, and the statistical analysis. CG performed the necropsies and revised the manuscript. AM coordinated the study, financially supported the molecular work, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1094554/full#supplementary-material

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Molecular detection and assemblage analysis of the intestinal protozoan *Giardia duodenalis* in wild boars in Korea

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Giardia duodenalis (syn. *G. intestinalis, G. lamblia*) is the only *Giardia* species that infects humans and most other mammals. Wild boars are a reservoir of many viruses, bacteria, and parasites that can be transmitted to livestock and humans. This study examined the infection rate of *G. duodenalis* in wild boars and confirmed its specificity by comparing assemblages through PCR amplification of the 18S rRNA, *gdh*, and β-giardin genes. Fecal samples were collected from roadkilled or trapped wild boars from April 2016 to December 2021 in Korea. DNA was extracted directly from 612 wild boar fecal specimens using a commercial kit. PCR was performed targeting the 18S rRNA region, β-giardin, and glutamate dehydrogenase genes of *G. duodenalis*. Some PCR-positive samples were selected for sequencing analysis. The obtained sequences were subsequently used for phylogenetic tree construction. Of the 612 samples tested, 125 (20.4%) were positive for *G. duodenalis*. The highest infection rate was detected in the central region (12.0%) and in autumn (12.7%). Among the risk factors, the seasonal factor was statistically significant (p=0.012). Phylogenetic analysis revealed three genetic assemblages: A, B, and E. Assemblages A and B exhibited 100% identity with *Giardia* sequences isolated from human and farmed pigs in Korea and Japan. This result cannot be ignored because it indicates the possibility of zoonotic transmission. Therefore, continuous management and monitoring of this pathogen are necessary to prevent transmission and protect animal and human health.

KEYWORDS

genotype, infection rate, phylogenetic analysis, wild boar, *Giardia duodenalis*

1. Introduction

Giardia is a flagellated protozoan parasite that infects various vertebrates (1, 2). Currently, seven nonhuman-infecting species (*Giardia agilis*, *G. ardaea*, *G. psittaci*, *G. muris*, *G. microti*, *G. peramelis,* and *G. cricetidarum*) and one species infecting humans and other mammals, *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*), have been identified (2, 3).

The infection caused by *G. duodenalis*—known as giardiasis—is important from veterinary and public health perspectives. This parasite has a wide range of hosts, including wild animals, and giardiasis is a common disease in livestock and companion animals (1, 4, 5). Additionally, *Giardia* infections are prevalent in areas with poor hygiene, where the ingestion of cysts is high. However, cases are emerging worldwide because infection occurs when cysts are ingested through contaminated water or direct person-to-person contact (6–10).

Molecular studies have classified *G. duodenalis* into eight distinct genetic groups, known as assemblages A–H (3, 5, 7–9). These assemblages are morphologically similar but exhibit genetic

heterogeneity (7). Assemblages A and B are predominant in humans; however, they have been reported to have zoonotic potential as they have been detected in several other mammals, and their host range is comprehensive (3, 4, 7, 11, 12). In contrast, assemblages C–H have been identified in nonhuman hosts (4, 6, 11, 12), with a few exceptions (13–15). Assemblages C and D have been identified in canines, E in hoofed livestock, F in cats, G in rodents, and H in pinnipeds (12, 15, 16).

Wild boars are widely distributed worldwide and are edible wild animals. However, they are susceptible to several parasites (e.g., helminths/protozoa, viruses, or bacteria), making them potential reservoirs for disease transmission (17, 18). Some studies have investigated the relationship between wild boar contact and disease transmission and the role played by wild boars in foodborne zoonoses (19, 20). Studies of *Giardia* infections in wild boars and domestic pigs have been conducted worldwide (17, 21–24). However, research on *Giardia* in Korea has mainly focused on environmental samples—including drinking water, cattle-like livestock animals, and companion animals, such as dogs—and studies on wild animals are lacking (25–29). No studies have been conducted on *Giardia* infections in wild boars, and only one study is available on domestic pigs (30).

Therefore, this study aimed to confirm the rate of *Giardia* infection, genetic diversity, and potential for zoonotic transmission in wild boars and to compare them with *Giardia* assemblages in domestic pigs.

FIGURE 1
Map of the sample collection regions. The country was divided into three regions based on the boundaries of administrative districts as follows: northern (provinces of Gangwon and Gyeonggi), central (provinces of Chungbuk, Chungnam, Gyeongbuk, and Jeonbuk), and southern (provinces of Gyeongnam, Jeju, and Jeonnam).

2. Materials and methods

2.1. Study area and collection of fecal samples

From April 2016 to December 2021, fecal samples were collected from deceased wild boars found trapped in forests or on roads after being hit by vehicles across the country. Wild boar feces were collected from the intestines after a veterinary performed the carcass necropsy. This procedure was supervised by the National Institute of Environmental Research in Korea. Because the collection of feces from the carcass was unrelated to research ethics and did not cause hazards to any animals, approval from Kyungpook National University's Institutional Animal Care and Use Committee was not required for the present study. Samples were individually placed in tubes and delivered to the laboratory, where DNA was extracted. The primary data for fecal samples, including region, season, and sex, were recorded for each individual, and any unclear or suspicious data were logged as "unknown." The samples were collected in spring, summer, and autumn. No samples were collected in winter. The sampled areas were divided into three regions—northern, central, and southern—based on the boundaries of the administrative districts (Figure 1).

2.2. DNA extraction and PCR assay

DNA was extracted using a commercially available kit (QIAamp® Fast DNA Stool Mini Kit; QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was placed in a sterile tube and stored at −20°C.

Nested PCR was performed to amplify the three target genes related to *Giardia* detection. Initially, primers amplifying the 18S rRNA region were used to screen positive samples. The primers used were RH11 (5′-CAT CCG GTC GAT CCT GCC-3′) and RH4LM (5′-GTC GAA CCC TGA TTC TCC G-3′) in the first round and GiarF (5′-GAC GCT CTC CCC AAG GAC-3′) and GiarR (5′-CTG CGT CAC GCT GCT CG-3′) in the second round (31). These primer sets were used to amplify the 18S rRNA gene for a length of approximately 170 bp. A commercial premix kit (AccuPower® HotStart PCR PreMix; Bioneer, Daejon, Korea) was used for regular PCR. The reaction was conducted in a volume of 20 µL containing 1 µL of each primer, 3 µL of sample DNA or first-round PCR products, and 15 µL of sterile distilled water. PCR amplifications were performed using Mastercycler nexus (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation, annealing, and extension at 95°C for 30 s, 59°C for 30 s, and 72°C for 20 s, respectively; and final extension at 72°C for 5 min.

Subsequently, additional PCR was performed to obtain positive sequences by amplifying the β-giardin (*bg*), glutamate dehydrogenase (*gdh*), and triosephosphate isomerase (*tpi*) genes of the *Giardia*-positive DNA samples screened via 18S rRNA PCR. Nested PCR was performed to amplify three genes. The expected fragment sizes were approximately 510 bp for *bg* (32, 33) and approximately 530 bp for *gdh* and *tpi* (34). For the PCR amplification of *gdh*, gdh1/2 (TTC CGT RTY CAG TAC AAC TC/ACC TCG TTC TGR GTG GCG CA) and gdh3/4 (ATG ACY GAG CTY CAG AGG CAC GT/GTG GCG CAR GGC ATG ATG CA) primer sets were used. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles

of denaturation, annealing, and extension at 95°C for 30 s, 55°C for 30 s (nested PCR at 59°C for 30 s), and 72°C for 30 s, respectively, and a final extension at 72°C for 5 min. For the PCR amplification of *bg*, G7/G759 (AAG CCC GAC GAC CTC ACC CGC AGT GC/GAG GCC GCC CTG GAT CTT CGA GAC GAC) and G7n/G759n (GAA CGA GAT CGA GGT CCG/CTC GAC GAG CTT CGT GTT) primer sets were used. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation, annealing, and extension at 95°C for 30 s, 65°C for 30 s (nested PCR at 55°C for 30 s), and 72°C for 30 s, respectively, and a final extension at 72°C for 5 min. For the PCR amplification of *tpi*, gtp1/2 (AAA TIA TGC CTG CTC GTC G/CAA ACC TTI TCC GCA AAC C) and gtp3/4 (CCC TTC ATC GGI GGT AAC TT/GTG GCC ACC ACI CCC GTG CC) primer sets were used. The reaction conditions included an initial denaturation at 95°C for 5 min; 35 cycles of denaturation, annealing, and extension at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, respectively, and a final extension at 72°C for 5 min. All PCR products were loaded in the electrophoresis unit with 1.5% agarose gel stained with ethidium bromide. The gel was run for 30 min at 135 V. Images were acquired using an ultraviolet transilluminator. PCR-positive samples were sent to Macrogen (Daejeon, Korea) for direct DNA sequencing.

2.3. Statistical and phylogenetic analysis

The data were subjected to χ^2 test using SPSS version 26.0 (IBM Corporation, Armonk, NY, United States), and p values of <0.05 were considered statistically significant. Unknown data were excluded from the calculations as missing values.

For the phylogenetic analysis, MEGA7 software was used to construct each phylogenetic tree using the *Giardia* 18S rRNA, *bg*, and *gdh* sequences obtained in this study and the GenBank-accessed sequence. Phylogenetic inference was conducted using the maximum likelihood method with 1,000 bootstrap replications.

3. Results

3.1. *Giardia* infection rates in wild boars based on 18S rRNA amplification

The PCR results were evaluated based on region, season, and sex. An overall *Giardia* infection rate of 20.4% (125/612) was observed (Table 1).

The infection rates in the northern, central, and southern regions were 9.0% (19/210), 12.0% (22/183), and 9.6% (9/94), respectively, while the rate was 60.0% (72/125) for the local unknown sample. The infection rate in the central region was higher than that in other regions. However, the values did not differ significantly among groups, calculated by excluding unknown regional samples ($p = 0.635$). The infection rate was 9.4% (19/202) in summer and 12.7% (32/252) in autumn. No positivity was detected in spring. Among the 125 positive samples, 19 were identified in summer (15.2%) and 32 in autumn (25.6%). The differences in infection rates among the groups were statistically significant ($p = 0.012$). Infections were detected in 9.3% of males, 9.0% of females, and 40.6% of wild boars with unknown sex, with no significant differences among groups ($p = 0.544$).

TABLE 1 *Giardia duodenalis* infection in wild boars based on 18S rRNA amplification.

Group			No. tested	No. positive (%)	value of *p*
Sex	Male		227	21 (9.3)	
	Female		166	15 (9.0)	0.544
	Unknown		219	89 (40.6)	
Region[a]	Northern	GW	109	9 (8.3)	
		GG	101	10 (9.9)	
		Subtotal	210	19 (9.0)	
	Central	CB	49	5 (10.2)	
		CN	32	3 (9.4)	
		GB	69	8 (11.6)	
		JB	33	6 (18.2)	0.635
		Subtotal	183	22 (12.0)	
	Southern	GN	58	6 (10.3)	
		JN	32	3 (9.4)	
		JJ	4	0 (0.0)	
		Subtotal	94	9 (9.6)	
	Unknown		125	75 (60.0)	
Season	Spring		44	0 (0.0)	
	Summer		202	19 (9.4)	
	Autumn		252	32 (12.7)	0.012
	Unknown		111	73 (65.8)	
Total			612	125 (20.4)	

[a]CB, Chungbuk; CN, Chungnam; GB, Gyeongbuk; GG, Gyeonggi; GN, Gyeongnam; GW, Gangwon; JB, Jeonbuk; JJ, Jeju; and JN, Jeonnam.

3.2. Sequencing and phylogenetic analysis

Of the 125 18S rRNA-positive samples, we obtained 19 successfully aligned nucleotide sequences through 18S rRNA sequencing. Phylogenetic analysis based on the *Giardia* 18S rRNA sequence revealed three assemblages: A, B, and E. Four sequences (OM943184–OM943187) from positive samples and a reference sequence obtained from GenBank were used for phylogenetic analysis. The obtained sequences are shown in bold in Figure 2. Three *bg* locus-positive (2.4%, 3/125) samples (OM937920–OM937922) and four *gdh* locus-positive (3.2%, 4/125) samples (OM937923–OM937926) were successfully sequenced, and assemblages A and E were confirmed following phylogenetic analyses based on the sequences of *bg* and *gdh* (Table 2; Figures 3, 4). However, *tpi* was not detected in any of the samples.

4. Discussion

Giardia infection rates have been studied in many countries, both in humans and animals (5, 7, 11, 15, 35). Notably, the rates of *Giardia* infection in wild boars have been reported to be 40.7% in Poland, 22.5% in southern Spain, 3.1% in China, 1.7% in Croatia, and 1.3% in northwestern Spain (17, 23, 24, 36, 37). However, no studies have been

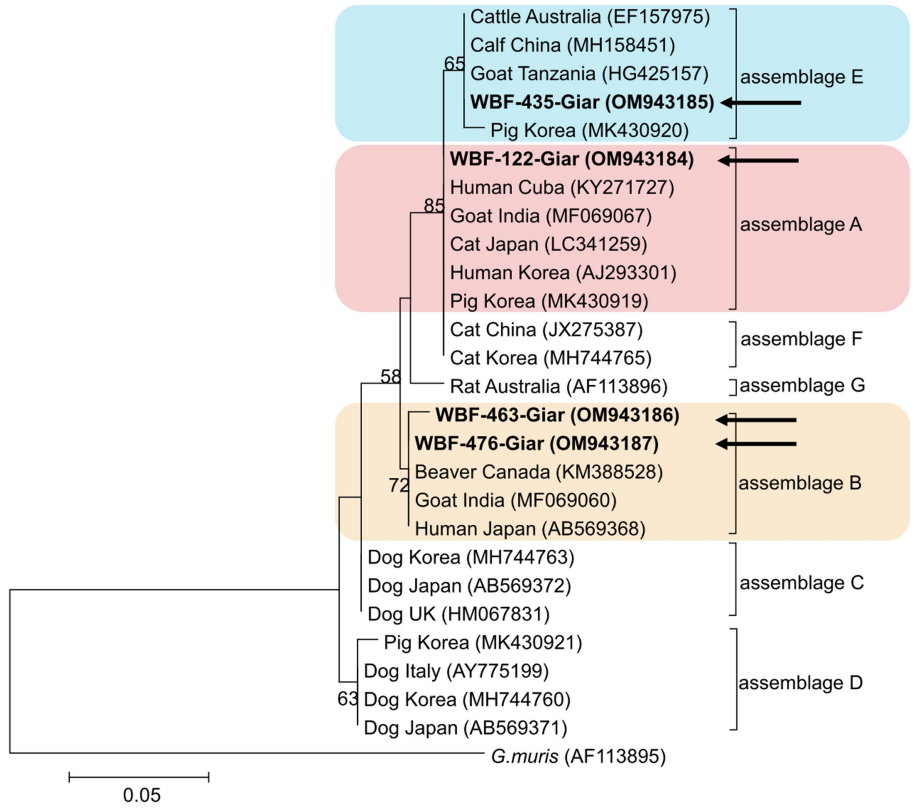

FIGURE 2
Phylogenetic tree of *Giardia* (18S rRNA) isolated from Korean wild boars. The sequences obtained from the phylogenetic analysis based on the *Giardia* 18S ribosomal RNA are indicated in bold.

TABLE 2 Genotyping of *Giardia duodenalis* based on 18S rRNA, *β-giardin*, and *gdh* amplification.

Genotypes	*ssu* rRNA (*n*=19)	*β-giardin* (*n*=3)	*gdh* (*n*=4)
Assemblage A	16	2	4
Assemblage B	2	0	0
Assemblage E	1	1	0

conducted on *Giardia* infection in Korean wild boars. Even when extending the scope of investigation to livestock pigs, only one infection study has been conducted, with a reported infection rate of 14.8% (110/745) (30). However, this result was obtained from a survey targeting only the central and southern regions of Korea. The infection rate in wild boars confirmed in the present study was 20.4% (125/612), which is higher than that in other animals (e.g., livestock and companion animals) in Korea. For instance, infection rates in calves were reported to be 13.1% (77/590) in 2016 (29), 12.7% (40/315) in 2018 (38), and 5.6% (44/792) in 2021 (39). The value was 3.8% in cats (6/158) (40) and 15.5% (99/640) in dogs (26). Only one study of Korean wild animals was conducted with small sample sizes, and the following infection rates were reported: 14.3% (1/7) in Eurasian otter, 31.8% (7/22) in leopard cat, and 9.1% (1/11) in raccoon dog (27). Various factors, such as the host, number of samples, country or region, surrounding environment, and diagnostic methods, result in differences in the obtained values. For example, a large difference in the positivity rate between direct fluorescence assay and PCR methods has been reported in a study conducted in Poland (14.8% vs. 40.7%, respectively) (37).

In the present study, DNA sequences were extracted from 612 fecal samples and analyzed based on the sampling region, season, and sex using PCR. The infection rates were the highest in the central region (12.0%) and the lowest in the northern region (9.0%). However, this difference was not statistically significant, which is in contrast with the results of previous studies conducted on dogs and pigs (26, 30). In pigs, the infection rate in the southern region (16.3%) was reported to be twice that in the central region (8.1%), which was statistically significant. In dogs, the infection rate in the southern region (40.7%) was significantly higher than that in other regions (northern 11.5%, central 7.9%). Therefore, it is assumed that other external factors within the entire area, such as the living environment of the host, may cause differences in the infection rate by region, and additional research is needed to explore this hypothesis further.

With regard to season, the results revealed a positive rate of 0.0% in spring, 9.4% in summer, and 12.7% in autumn. However, the lack of winter samples and the absence of infections in spring require further validation through additional samples. *Giardia* infection rates have been reported to be the highest during the rainy season in several previous studies (41, 42), confirming that the illness is waterborne. Furthermore, Lee et al. (30) reported higher infection rates in autumn

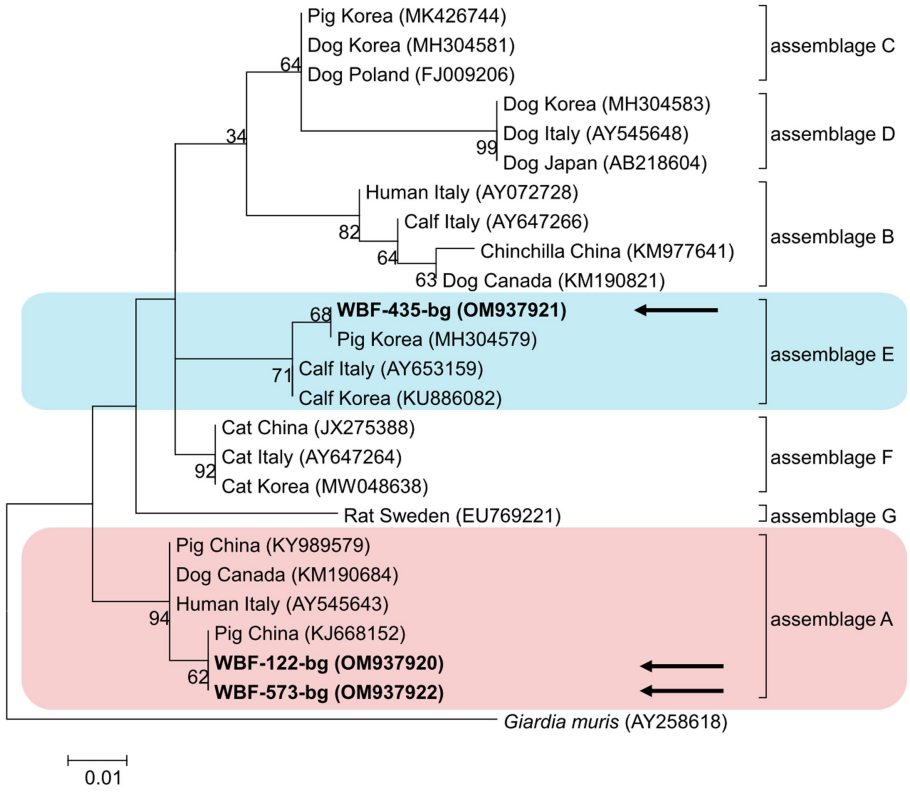

FIGURE 3
Phylogenetic tree of *Giardia* (β-giardin) isolated from Korean wild boars. The sequences obtained from the phylogenetic analysis based on the *Giardia* β-giardin gene are indicated in bold.

(21.2%) than in summer (12.0%) for pigs, which is consistent with the results of wild boar infections reported in our study.

In terms of sex, the infection rates did not differ significantly between male (9.3%) and female wild boars (9.0%), which is consistent with the results reported for Nigerian pigs (21), where the infection rate was 25.0% for males and 25.7% for females. The infection rates did not differ significantly in studies conducted on other animals in Korea. The values were 17.6 and 13.8% for male and female dogs, respectively, and 4.8 and 3.1% for male and female cats, respectively (26, 40). However, the relationship between *Giardia* infection and sex remains unclear, as most infections were detected in wild boars of unknown sex (40.6%). Therefore, further studies are needed to clarify this aspect.

In this study, *bg* or *gdh* showed a lower positivity rate than 18S rRNA; even *tpi* was not detected. This is a characteristic of the protein-coding gene. Because the protein-coding gene is a single-copy gene (43), previous studies of *Giardia* infection in Korean dogs and pigs showed low sensitivity (26, 30). Nevertheless, it was used to compare the genotype results of 18S rRNA and *bg* or *gdh* with each other.

In the present study, the genotypes obtained based on 18S rRNA analysis were identified as assemblages A, B, and E. Assemblages A and B were distinguished as potential zoonotic groups, owing to their wide and diverse host ranges (12, 44). Assemblage A in wild boars was first identified in Croatia (23), whereas assemblage B was first identified in Poland (37). As few studies are available on wild boars, the results of studies on pigs were used for comparisons. Assemblage E was predominantly identified, assemblage A was partly identified, and assemblage B was rarely identified (5, 45–48). The results of a study on Korean pigs are similar (30). Assemblages A and E as well as C and D were confirmed in Korean pigs. Assemblage E was identified as the predominant one, while assemblage B was not detected (30). However, assemblage B, which had not been previously identified in pigs, was detected, and assemblage A was more common than the other assemblages. As the human infection of assemblage E has been confirmed in a recent Brazilian study (49), the possibility of zoonotic transmission in all types identified in this study should be considered. Notably, the same assemblage A was confirmed in the *bg* sequence (OM937920) in the case of sample WBF-122, and the WBF-122 18S rRNA sequence (OM943184), the Korean human sequence (AJ293301), and the Korean pig sequence (MK430919) showed 100% sequence similarity. Additionally, the WBF-122 *bg* (OM937920) and the Chinese pig (KJ668152) sequences showed 100 and 99.3% similarity to the Italian human sequence (AY545643), respectively. Assemblage A was also confirmed in the *gdh* sequence. The WBF-556 *gdh* sequence (OM937925) showed 99.8% similarity to the Australian sequence (L40510). Assemblage E was also identified in the sequences of 18S rRNA (OM943185) and *bg* (OM937921) of the WBF-435 sample. The WBF-435 *bg* sequence (OM937921) showed 100% identity with the sequence identified in Korean pigs (MH304579). However, the *gdh* sequences of other assemblages were not identified, possibly because the *bg* and *gdh* protein-coding genes have lower sensitivity than 18S rRNA (43).

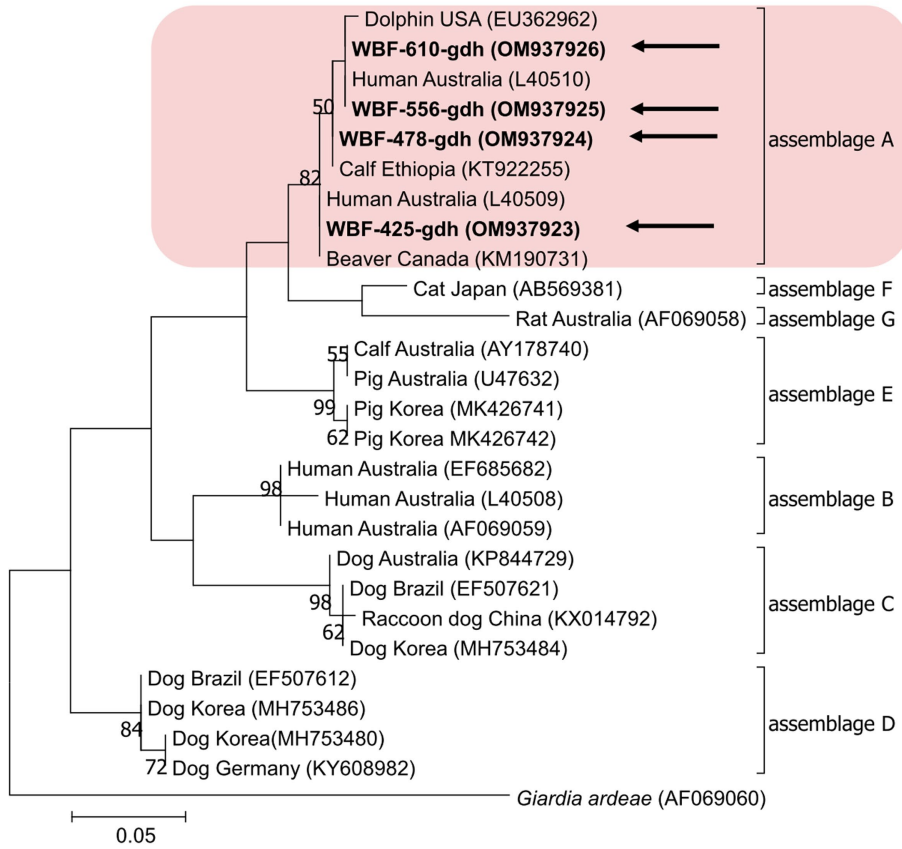

FIGURE 4
Phylogenetic tree of *Giardia* (gdh) isolated from Korean wild boars. The sequences obtained from the phylogenetic analysis based on the *Giardia* gdh gene are indicated in bold.

Therefore, this is a limitation of the study and a subject for future research.

5. Conclusion

The overall *Giardia* infection rate in Korean wild boars was 20.4%. This study analyzed differences in infection rates based on region, season, and sex to determine the risk factors for infection. Only season was identified as a statistically significant factor. However, assemblages A, B, and E were identified in the fecal samples, and assemblage A was confirmed to be 100% identical to the genotype found in human and farmed pigs in Korea. This indicates the possibility of *Giardia* transmission from a range of animals to other animals, or from animals to humans. Assemblages A and B were confirmed to be zoonotic, and assemblage E was confirmed to be zoonotic as well; however, human infections are rare. Therefore, their zoonotic potential should be studied.

To the best of our knowledge, this was the first nationwide study of *Giardia* infections affecting wild boars that provided basic data on genetic diversity. However, infection rates should be further confirmed by analyzing more samples. Additionally, comparative analysis with protein-coding genes should be conducted to identify genetic characteristics in future studies. The results obtained in this study, which indicate the possibility of zoonotic transmission, cannot be ignored. Furthermore, continuous management and monitoring of this pathogen are necessary to prevent transmission and protect the health of animals and humans.

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/, OM943185; https://www.ncbi.nlm.nih.gov/genbank/, OM943184; https://www.ncbi.nlm.nih.gov/genbank/, OM943186; https://www.ncbi.nlm.nih.gov/genbank/, OM943187; https://www.ncbi.nlm.nih.gov/genbank/, OM937921; https://www.ncbi.nlm.nih.gov/genbank/, OM937920; https://www.ncbi.nlm.nih.gov/genbank/, OM937922; https://www.ncbi.nlm.nih.gov/genbank/, OM937926; https://www.ncbi.nlm.nih.gov/genbank/, OM937925; https://www.ncbi.nlm.nih.gov/genbank/, OM937924; and https://www.ncbi.nlm.nih.gov/genbank/, OM937923.

Ethics statement

Since the collection of feces from carcass was not related to research ethics and did not cause hazard to any animals, the approval from Kyungpook National University's Institutional Animal Care and Use Committee was not required for the present study.

Author contributions

DK designed the study. HL conducted the experiment, wrote the manuscript, performed statistics, and analyzed the data. HL and DK edited the article. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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The local skin cellular immune response determines the clinical outcome of sarcoptic mange in Iberian ibex (*Capra pyrenaica*)

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Introduction: Sarcoptic mange, caused by *Sarcoptes scabiei*, is a disease with implications for wildlife conservation and management. Its severity depends on the host's local skin immune response, which is largely unknown in Iberian ibex (*Capra pyrenaica*), a mountain ungulate dramatically affected by mange. In this species, the clinical outcome of sarcoptic mange varies among individuals, and the local immune response could be key to controlling the infestation. This study aims to characterize the local cellular immune response and its relationship with the clinical outcome.

Methods: Fourteen Iberian ibexes were experimentally infested with S. scabiei and six more served as controls. Clinical signs were monitored, and skin biopsies were collected from the withers at 26, 46, and 103 days post-infection (dpi). The presence and distribution of macrophages (including M1 and M2 phenotypes), T lymphocytes, B lymphocytes, plasma cells, and interleukine 10 were quantitatively evaluated using immunohistochemical techniques.

Results: An inflammatory infiltrate that decreased significantly from 26 to 103 dpi was observed in all the infested ibexes. The predominant inflammatory cell population in the skin of the mangy ibexes was formed by macrophages (mainly the M2 phenotype) followed by T lymphocytes, with lower numbers of B lymphocytes and plasma cells. Three clinical courses were identified: total recovery, partial recovery, and terminal stage. The inflammatory infiltrates were less pronounced in the fully recovered ibexes than in those that progressed to the terminal stage throughout the study.

Discussion: The results suggest an exacerbated but effective Th1-type cellular immune response controlling mange in Iberian ibex. Furthermore, the local immune response appears to determine the variability of the clinical responses to *S. scabiei* infestation in this species. This first report on the progression of local skin immune cells is relevant not only for individuals but also for population management and conservation.

KEYWORDS

***Capra pyrenaica*, clinical outcome, experimental infestation, Iberian ibex, immunohistochemistry, inflammatory cell, sarcoptic mange (*Sarcoptes scabiei*), skin immune response**

1. Introduction

Sarcoptic mange is an emerging parasitic transmissible disease caused by the mite *Sarcoptes scabiei* and affects humans and animals worldwide (1–4). It can cause significant declines in wildlife populations; therefore, it is relevant for wildlife conservation and management (2). In Spain, sarcoptic mange has been reported in wild carnivores (5, 6), lagomorphs (7, 8), and ungulates (9–11), but the most dramatic effects of the disease have been observed in mountain ungulates, including Cantabrian chamois (*Rupicapra pyrenaica parva*) and Iberian ibex (*Capra pyrenaica*), in which outbreaks have led to demographic declines of over 95% (12–16).

Despite sarcoptic mange having been known for a long time and the number of studies on this disease conducted in humans, domestic animals, and wildlife, its clinical course and progression remain not yet fully understood. *Sarcoptes scabiei* burrows galleries in the epidermis, feeding on host cells and lymphatic fluid (17) and inducing antigenic reactions with imbalances in Th1/Th2/Th17 immune responses (18, 19). These responses drive the pathogenesis and clinical signs of sarcoptic mange, which range from mild erythema to more severe lesions, such as dermatitis, hyperkeratosis, alopecia, and systemic signs, which sometimes eventually cause death (6, 20–23). However, two research questions regarding the immune response to *S. scabiei* in different wildlife species remain not yet fully clarified: (1) what is the effect of immunological responses on mange severity, and (2) what are the primary drivers in host–parasite interactions for both positive and negative clinical outcomes of mange? (24, 25).

The pathogenesis of sarcoptic mange has been characterized in humans, in which the local skin immune response defines the severity of the parasitosis (18, 26, 27). A Th1-mediated skin immune response with higher T CD4+ lymphocyte infiltrates leads to milder ordinary scabies, while a response skewed toward Th2 with predominantly T CD8+ lymphocyte infiltrates leads to a more severe crusted scabies (18, 26, 27). Therefore, the detailed study of the inflammatory response to *S. scabiei* in skin biopsies can reveal interspecific and/or interindividual variability in the severity of clinical signs, as well as in the development of resistance and the existence of asymptomatic carriers. This has led to the study of immunological and inflammatory local skin response to sarcoptic mange both by conventional histological techniques and by immunohistochemical methods in domestic animals such as goats (*Capra hircus*), sheep (*Ovis aries*), dogs (*Canis lupus familiaris*), and pigs (*Sus scrofa domesticus*), as well as in wildlife, including chamois (*Rupicapra* spp.), red deer (*Cervus elaphus*), wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), roe deer (*Capreolus capreolus*), Iberian lynxes (*Lynx pardinus*), wild boars (*Sus scrofa*), and wombats (*Vombatus ursinus*) (6, 19, 28–35). These studies have demonstrated not only interspecific but also intraspecific differences in the local skin immune response to *S. scabiei* infestation (1), which can also vary between naturally and experimentally infested animals (28). However, skin lesions and inflammatory infiltrate also depend on the stage of infestation; therefore, to precisely histologically characterize the local inflammatory response, a representative number of histological sections taken throughout the disease course must be studied. Thus, identifying inflammatory cell populations in experimentally induced scabietic lesions during the sensitization and challenge infection phases, and characterizing the timing and intensity of the immunological response during the challenge phase, could help elucidate their role and the mechanism for the immune response in wildlife (25, 29).

The features of the humoral and cellular immune responses, both local in the skin and systemic in the bloodstream, are relevant for the course of the disease. Consequently, they also influence sarcoptic mange management at both an individual and population level, as strategies and their associated costs must be weighed against the risks, hazards, and impact of the disease on the population (36). The management of sarcoptic mange in wild Caprinae, and in Iberian ibex in particular, is challenging and controversial, with different and even contradictory measures being applied and a lack of consensus not only on the management options but even on the criteria for deciding which measure to implement (16). Understanding individual immune responses that affect population dynamics is not only relevant for individual health and welfare, but also has population, ecological, and management implications.

Although Iberian ibex has repeatedly been reported to be capable of recovering and surviving sarcoptic mange (37–39), previous studies suggest that the systemic humoral immune response is not effective at preventing the development of advanced clinical stages of sarcoptic mange and eventual death. Rather, it is a non-specific indicator of the inflammatory process associated with the disease (40–42). Conversely, also in Iberian ibex, the ability to cope with sarcoptic and survive may depend on the skin local cellular immune response rather that on the both skin local and systemic humoral immune response (1, 3, 21, 43).

Therefore, the aim of this study is to immunohistochemically characterize the cellular immune skin response of Iberian ibex to experimental infestation with *S. scabiei*, describe the progression of the inflammatory infiltrate throughout the course of the disease, and investigate the potential link between this response and the clinical outcome.

2. Materials and methods

2.1. Animals

Twenty healthy free-ranging Iberian ibexes (10 females and 10 males, aged between 1 and 11 years) were captured in the Sierra Nevada Natural Space (36° 55′- 37°10′N, 2° 56′- 3° 38′W) and the Sierras de Cazorla, Segura y Las Villas Natural Park (37° 53′-37° 88′N, 2° 53′-2°88′W), in southern Spain. The ibexes were captured and immobilized with a combination of xylazine (3.0 mg/kg) and ketamine (3.0 mg/kg) (44), using a Teleinject G.U.T 50® anesthesia

Abbreviations: AIC, Akaike's information criterion; AICc, corrected Akaike's information criterion; AICWt, Akaike's information criterion weight; CD4, Cluster of differentiation 4; CD8, Cluster of differentiation 8; CD3, Cluster of differentiation 3; CD20, Cluster of differentiation 20; CD204, Cluster of differentiation 204; Dpi, Days post-infection/infestation; ELISA, Enzyme linked immunosorbent assay; GLMM, General linear mixed model; H&E, Hematoxylin and Eosin; HRP, Horseradish peroxidase; Iba-1, ionized calcium-binding adapter molecule 1; IL, interleukin; iNOS, inducible nitric oxide synthase; K, degrees of freedom; KOH, potassium hydroxide; LMM, linear mixed model; PBS, phosphate buffered saline; Th1, type 1T helper; Th2, type 2T helper; Th17, type 17T helper.

gas-applicator[1]. After firing a single dart from a distance of 10–20 m, the anesthesia was maintained for approximately 15 min, at which point a first clinical inspection was conducted. The ibexes were inspected for clinical signs compatible with sarcoptic mange, and the presence of antibodies against *S. scabiei* was assessed using a validated enzyme-linked immunosorbent assay (ELISA) (42). Only ibexes negative for sarcoptic mange by both physical examination and serological diagnosis were retained for the study and transported to specific experimental facilities located in the Sierra de Huétor Natural Park (37° 18′-37° 30′N, 3° 28′-3° 47′W). The ibexes were divided into groups of four to six individuals of mixed sexes and ages. Each group was kept in a separate pen measuring 30 m^2 with access to food and water *ad libitum*. These ibex groups remained constant throughout the entire experimental period.

This study adhered to all legal requirements and guidelines related to animal welfare and experimentation in Andalusia, Spain and Europe. The handling procedures and sampling frequency were designed to minimize stress and its impact on the health of the subjects, in accordance with European (2010/63/UE) and Spanish (R.D 53/2013) standards. The study was approved by the Ethics on Animal Welfare Committee of the University of Jaén and authorized by the Dirección General de Producción Agrícola y Ganadera of the Consejería de Agricultura, Pesca y Medio Ambiente of the Junta de Andalucía (Ref: SA/SIS/MD/ps/ October 25, 2012). The Sierra Nevada Natural Park staff also approved this study.

2.2. Experimental infestation

After an 8-week adaption period in the facilities, 14 of the 20 ibexes were experimentally infested with *S. scabiei*. Two cm^2 skin fragments were attached to the withers from a naturally infested free-ranging Iberian ibex, as described previously (21). Mite density was calculated in skin pieces adjacent to those used for the infestation using a stereomicroscope after overnight digestion in 5% potassium hydroxide (KOH) solution at 40°C (45). A thermal gradient was then induced by shining a light on Petri dishes with black bottoms and transparent central areas (46). The resulting estimated dose received by each ibex was 750 ± 440 mites (mean ± standard deviation). The remaining six ibexes served as non-infested controls.

2.3. Clinical assessment

The clinical signs and the extension of mange-compatible skin lesions were monitored for 103 days post-infection (dpi) and classified as 0 (no visible skin lesions), 1 (focal skin lesions affecting less than 50% of the body surface), or 2 (extended skin lesions affection more than 50% of the body surface), as described previously (14). None of the control ibexes developed any lesions or clinical signs compatible with mange. Conversely, both lesions and clinical signs were observed in all the infected ibexes, in which three different clinical courses were identified: four ibexes had mild skin lesions affecting less than 50% of the body surface that healed completely before the end of the 103-day experimental period ("totally recovered"); three ibexes had progressive skin lesions spreading over 70% of the body surface, but with signs of recovery, such as hair growth, reduction of skin thickening, and skin smoothing ("partially recovered"); and seven ibexes had advanced skin lesions, including alopecia, skin desquamation, thickening and crusting, and pruritus, spreading over 70% of the body surface without signs of recovery ("terminal") (21).

2.4. Skin sampling

Skin biopsies were collected from the withers of the infested ibexes at 26, 46, and 103 dpi using an 8-mm diameter biopsy punch (KRUUSE® Biopsy Punch, Langeskov, Denmark), after the interscapular region was shaved. The control ibexes were sampled from the same body region using the same procedure only at 103 dpi. Each ibex was individually restrained in a handling crush, blindfolded, and locally anesthetized with a combination of 10 mg of lidocaine hydrochloride and 0.01 mg of adrenalin (ANESVET®, Ovejero Lab, León, Spain). After the skin samples were collected, the resulting skin wounds were topically treated with Bactrovet® antiseptic sprays, composed of a mixture of micronized aluminum and silver and rosehip oil.

A total of 48 skin samples were obtained (Table 1). Each biopsy was placed into 10% neutral buffered formalin for 48–72 h, then transferred to 60% ethanol and stored at 4°C until histological analysis. The biopsies were embedded in paraffin and 4-µm thick sections were either stained with hematoxylin and eosin (H&E) for skin lesion assessment (21) or used for immunohistochemical studies.

2.5. Immunohistochemistry

Different primary antibodies raised against antigens expressed by macrophages (Iba-1), including M1 (iNOS) and M2 (CD204) subpopulations, T lymphocytes (CD3), B lymphocytes (CD20), and plasma cells (Kappa-Lambda), as well as the cytokine interleukin 10 (IL-10), were used to quantitatively assess different cell populations that play a relevant role in the local skin immune response (Table 2).

Heat-mediated antigen retrieval was performed on 4-µm-thick sections placed onto poly-L-lysine-coated slides, using the PT Link® system (PT-Link, Agilent®, Santa Clara, CA, USA) or proteinase K, depending on the specific antibody used (Table 2). After deparaffinization, rehydration, and drying, endogenous peroxidase

TABLE 1 Number of skin biopsies from the Iberian ibexes experimentally infested with *Sarcoptes scabiei* and controls used for immunohistochemical analyses.

Group	Day post-infestation (dpi)			Total
	26	46	103	
Terminal	7	7	7	21
Partially recovered	3	3	3	9
Totally recovered	4	4	4	12
Control			6	6
Total	14	14	20	48

1 https://www.telinject.de/en/products/vario-product-line/g-u-t-50/

TABLE 2 Primary antibodies and protocols used to characterize the different cellular types analyzed in the skin of the Iberian ibexes experimentally infested with *S. scabiei* and controls.

Target	Specificity (clone)	Source	Epitope unmasking	Dilution
Iba-1	Macrophage (rabbit polyclonal)	Wako®	96° 20′ buffer Dako pH6	1:2,000
CD-204	MØ Scavenger receptor A (mouse monoclonal) (clone SRA-ES) M2 marker	TransGenic Inc.® (KAL-KT022)	96° 20′ buffer Dako pH6	1:400
iNOS	Inducible nitric oxide synthase (rabbit polyclonal) M1 marker	Novus®	96° 20′ buffer Dako pH6	1:150
IL-10	Interleukin-10 (mouse monoclonal)	Biorbyt® (Orb10892)	96° 20′ buffer Dako pH9	1:100
CD3	T lymphocyte (rabbit polyclonal) (clone A0452)	Dako®	96° 20′ buffer Dako pH6	1:300
CD20	B lymphocyte (rabbit polyclonal)	Thermo® (RB9013P)	No unmasking	1:150
Kappa-Lambda	Plasma cells (rabbit polyclonal)	Dako® (0191–0193)	Proteinase K 0.05%	Kappa: 1:7,000 Lambda: 1:15,000

was blocked by immersing the sections in 3% oxygen peroxide in methanol solution for 30 min at room temperature in the dark. The sections were then incubated with the specific primary antibodies diluted in a commercial reagent (Antibody Diluent, Agilent®, Santa Clara, CA, USA) (Table 2) overnight at 4°C in a humidified chamber. After washing, immunolabeling was performed using a ready-to-use kit EnVision System® (Agilent®, Santa Clara, CA, USA) for the appropriate monoclonal or polyclonal antibodies, and slides were incubated for 40 min at room temperature. After two washes in phosphate-buffered saline (PBS), antibody localization was determined using 3,3-diaminobenzidine (Agilent®, Santa Clara, CA, USA) as a chromogenic substrate for horseradish peroxidase (HRP) of the secondary antibody. The reaction was stopped with tap water after 3–4 min or, for plasma cells, using the commercial ImmPact® Vector® kit (Red Substrate Kit) (Vector Lab, Newark, CA, USA). Finally, the slides were counterstained with Harris's hematoxylin. Appropriate species- and isotype-matched immunoglobulins were used as controls, including sections with an isotype control for the primary antibody and those for which the primary antibody was omitted. The same examined sections were used to determine the optimal dilution and incubation pH.

2.6. Evaluation of the immunostained slides

A total of 356 skin sections immunolabeled for the seven cellular markers analyzed in the 48 skin samples were evaluated, corresponding to the 20 ibexes included in the study (Table 1). Ten randomly chosen fields were selected and photographed at a magnification of 400× (Nikon® Eclipse Ci microscope, coupled with a MicrosCopiaDigital MDE3-6-3 digital camera) on each slide. The immunolabeled cells were counted on digital images using the Cell Counting add-on in ImageJ (National Institutes of Health, Bethesda, MD, USA). The average value for the 10 fields counted in each slide was calculated for each immunolabeled cell subtype. Additionally, the distribution of the immunostained cells in the different skin areas was assessed.

The immunostained cell count and distribution was independently evaluated by two of the authors (J. Espinosa and V. Pérez, the latter a European College of Veterinary Pathology Diplomate), and any discordant results were reviewed using a multiheaded microscope to reach a consensus.

2.7. Statistical analysis

Data normality was initially assessed for each cellular type and total cellular counts using graphical methods, skewness checks, and Kolmogorov -Smirnov tests. As all the variables did not conform to normality, non-parametric statistical methods were used.

Generalized linear mixed models (GLMMs) and linear mixed models (LMMs) were fitted using the packages "nlme,"" lme4," "mgcv," and "MuMIn," depending on the distribution of the variable and using the individual identity as a random term (due to non-independency of the measures) for total inflammatory cell, total macrophage, M2 macrophage, and T lymphocyte counts. The models were selected based on the Akaike information criterion (AIC) (47). The explicative variables included in the models were dpi, the experimental group (control or infested), and clinical outcome within the infested group (recovered, partially recovered, and terminal). The effects of individual variables, such as age and sex, did not affect cell number and were considered part of the individual identity in the random term. When the interaction between the dpi and outcome was significant, different GLMMs were applied to detect specific differences between groups.

B lymphocyte and plasma cell counts were too low to perform any statistical models. Therefore, Mann–Whitney *U* tests were carried out to compare these two variables between the experimental groups (control vs. infested). Differences in B lymphocyte and plasma cell counts among dpi (26, 46, and 103 dpi) within the infested clinical outcome groups (healthy, totally recovered, partially recovered, and terminal) were assessed using non-parametric Friedman's tests for repeated measures, whereas the differences among the three different clinical outcome groups for each dpi were assessed through Kruskall–Wallis tests and pairwise comparisons using a Wilcoxon rank sum test with Bonferroni correction (48). Finally, Spearman's rank correlation test was applied to establish possible correlations among the different cell types analyzed.

All the statistical procedures were performed with R 4.1.2 software using the functions "ggdensity," "stats_overlay_normal_density," and "skewness," and the packages "nortest," "plotrix," "coin," "lme4," and

"AICcmodavg" (R Development Core Team 2022). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Differences between control and infested ibexes

Throughout the experimental infestation, five cell types (macrophages; M2 macrophages; T lymphocytes; B lymphocytes; and plasma cells) were detected in the skin of the infested (26, 46, and 103 dpi) and control ibexes (103 dpi) (Figure 1; Table 3). The identification of M1 macrophages through iNOS marker and IL-10-immunomarked cells was anecdotal. The positively immunolabeled cells were identified by their morphology and deep brown (macrophages and lymphocytes) or red (plasma cells) granular stain. When analyzing the cell counts of all the dpi altogether, all the cell types were significantly ($p < 0.001$) more abundant in the skin of the infested ibex than in the control ones. When analyzing only the samples from 103 dpi, the difference between the control and the infested ibexes was significant only for total macrophage and M2 macrophage counts (Table 3).

In the infested ibexes, total macrophages (Iba-1) and M2 (CD204) macrophages were observed in the superficial and intermediate dermis as well as in the epidermis, but not in the control ibexes. T lymphocytes (CD3+ immunolabeled) were observed in all the layers of the dermis, forming multifocal to confluent aggregates interspersed with macrophages. Additionally, intraepidermal foci of lymphocytic exocytosis were observed, mainly in samples from 26 dpi. Finally, antibody-producer cells (B lymphocytes and plasma cells) were less abundant in the immune cellular skin infiltrates identified in all the groups and from all the time points (Figure 1; Table 3). Both cell types were distributed mainly at the intermediate and deep dermis, but while B lymphocytes were diffusely distributed, plasma cells were seen mostly as perivascular infiltrates, mainly in the deep dermis (Figure 1).

Macrophages, both overall and especially M2, were the predominant cell type in the inflammatory infiltrate in the skin of the infested ibexes (Figure 2; Table 3). Consequently, total (Iba-1) and M2 (CD204) macrophage counts significantly correlated ($R^2 = 0.827$, $p < 0.001$). T lymphocytes were the second most abundant cell population. Finally, B lymphocytes and plasma cell numbers were less abundant and significantly correlated ($R^2 = 0.707$; $p < 0.001$).

3.2. Comparison among clinical outcomes in the infested ibexes

3.2.1. Total cell counts

According to the most parsimonious model, total inflammatory cell count trend was explained by the interaction between dpi and outcome (corrected AICc [AICc] = 5072.0; degrees of freedom $K = 10$; AIC weight [AICcWt] = 1.0) (Supplementary Table S1).

The variables significantly influencing total inflammatory cell count were all the time points; the terminal outcome; the interaction of all the time points with the terminal outcome; and the interaction of 103 dpi with the partially recovered outcome. The interaction of 46 dpi with the partially recovered outcome approached significance (Table 4).

Total cell counts consistently decreased throughout the study in all the infested groups (Figure 1; Supplementary Table S2). Despite this decrease, the total cell counts of the infested ibexes at 103 dpi were still higher than those of the control ibexes at 103 dpi ($p = 1.4e\text{-}08$) (Figure 2; Table 3). However, this difference from the controls was only significant for the partially recovered and terminal groups, but not for the totally recovered ibexes (Supplementary Table S3).

At 26 dpi, the total cell counts were higher in the terminal ibexes than in the partially recovered ones. At 46 dpi, the total cell counts of the terminal group were higher than those of the totally and partially recovered groups. At 103 dpi, the totally recovered ibexes had significantly lower total cell counts than the partially recovered and terminal groups (Figure 3; Table 3; Supplementary Table S4).

3.2.2. Total macrophage counts

Total macrophage counts were also explained by the interaction between dpi and outcome, according to the most parsimonious model (AICc = 4390.2, $K = 10$, AICcWt = 1.0) (Supplementary Table S5).

The effects of all the sampling time points, all the outcomes, and the interaction of 103 dpi with all the terminal outcomes were significant on the total macrophage counts (Table 5).

In all the infected groups, the total macrophage counts consistently decreased for each time point (Supplementary Table S6; Figures 1, 4). Despite this decrease, the total macrophage counts of the infested ibexes at 103 dpi were still higher than those of the control ibexes ($p = 5.0e\text{-}09$) (Figure 2; Table 3). However, this difference from the controls was only significant for the partially recovered and terminal groups, but not for the totally recovered ibexes (Supplementary Table S7).

At 26 dpi, the total macrophage counts of the terminal ibexes were higher than both those of the totally and partially recovered groups. At 46 and 103 dpi, the total macrophage counts of the terminal ibexes were only significantly higher than those of the totally recovered group (Supplementary Table S8; Figure 4).

3.2.3. M2 macrophage counts

As for total cell counts and total macrophage counts, the M2 macrophage counts were again explained by the interaction between dpi and clinical outcome, according to the most parsimonious model (AICc = 3931.3, K = 10, AICcWt = 1.0) (Supplementary Table S9).

The effects of 103 dpi, the interaction of 46 dpi with the terminal outcome, and the interactions of 103 dpi with the partially recovered and the terminal group outcomes were significant in terms of the M2 macrophage counts, while the effect of the terminal outcome approached significance (Table 6).

The M2 macrophage counts decreased consistently in all the study periods in the terminal group, while such a decrease was only significant for 103 dpi in the totally and partially recovered ibexes (Supplementary Table S10; Figures 1, 5). Despite these decreases, the M2 macrophage counts of the infested ibexes altogether and for each clinical outcome group were still higher than those of the control ibexes at 103 dpi ($p = 2e\text{-}14$) (Figure 2; Table 3; Supplementary Table S11).

Statistically significant differences in the M2 macrophage counts among the clinical outcomes were only found at 26 dpi, when the values of the terminal ibexes were significantly higher than those of

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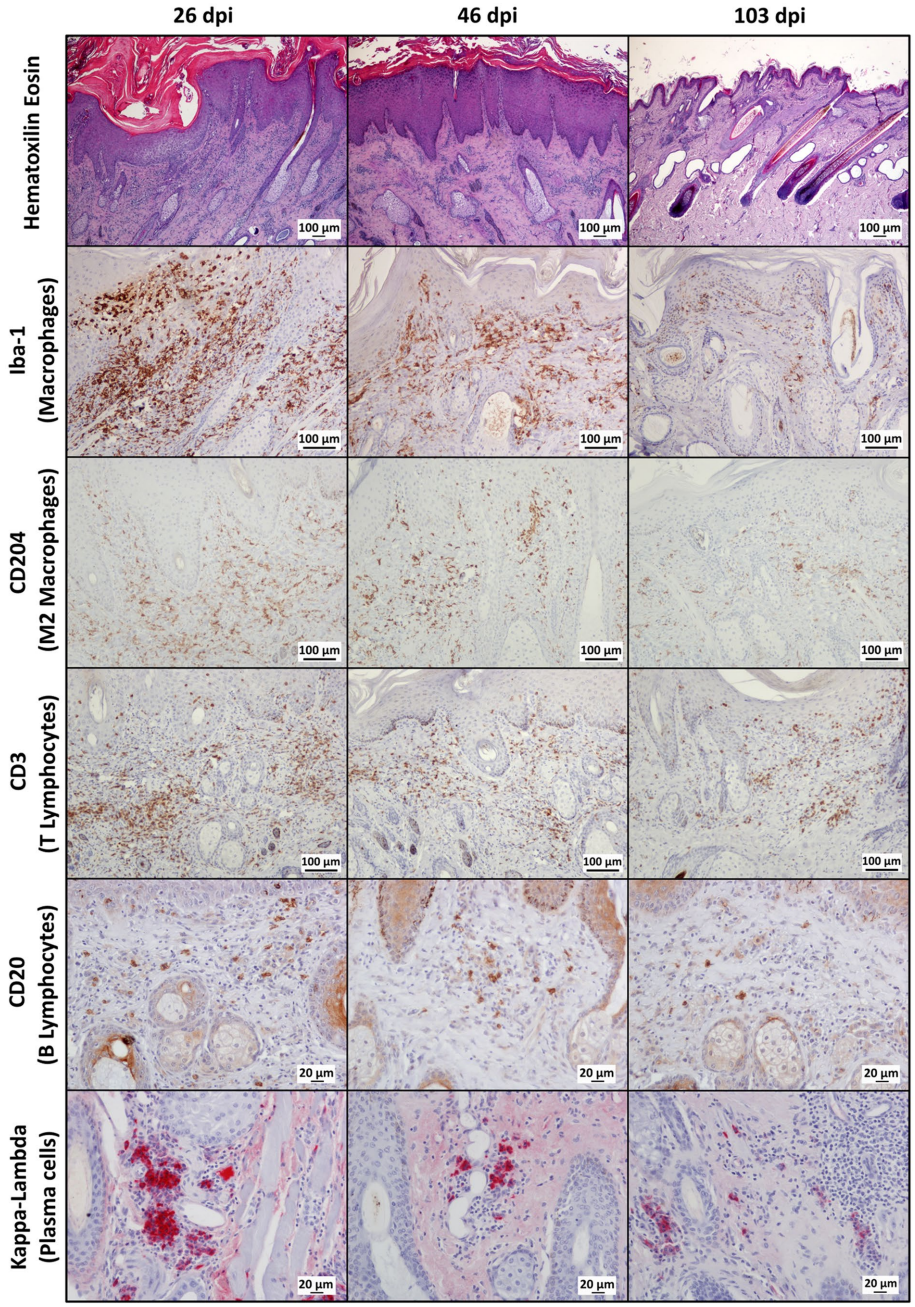

FIGURE 1
Photomicrographs of sections of mangy skin of Iberian ibexes experimentally infested with *Sarcoptes scabiei* at 26, 46, and 103days post-infestation (dpi), showing immunolabeling of total macrophages (Iba-1 marker), the M2 macrophage subtype (CD204), T lymphocyte (CD3), B lymphocytes (CD20), and plasma cells (Kappa-Lambda). The positively immunostained macrophages (total and the M2 phenotype) and T and B lymphocytes appear brown, whereas plasma cells are red. Magnification: 200×.

the partially recovered group, approaching significance with the totally recovered ibexes (Supplementary Table S12; Figure 5).

3.2.4. T lymphocyte counts

The T-lymphocyte count trend was also explained by the interaction between clinical outcome and dpi according to the most parsimonious model (AICc = 4587.3, K = 10, AICcWt = 1.0) (Supplementary Table S13).

The effects of all the dpi and the partially recovered outcome were significant on T lymphocyte counts, as were the interactions between the clinical outcomes and the sampling dpi (Table 7).

The T lymphocyte counts decreased consistently in each sampling interval in all the experimentally infested groups, except for the partially recovered ibexes between 46 dpi and 103 dpi (Supplementary Table S14; Figures 1, 6). The T lymphocyte counts of the infested ibexes altogether were not significantly different at any

TABLE 3 Total cell counts for the control Iberian ibexes at 103days post-infestation (dpi) and the infested Iberian ibexes altogether at 26, 46, and 103 dpi.

	Number of cells per field at 400× (mean±standard error)			
	Control (103 dpi)	Infested (26 dpi)	Infested (46 dpi)	Infested (103 dpi)
Macrophages (Iba-1)	7.29 ± 1.79*	45.97 ± 3.73^{a}	26.09 ± 2.02^{b}	13.98 ± 2.08^{c*}
M2 macrophages (CD204)	1.83 ± 0.42*	31.76 ± 2.73^{a}	25.57 ± 2.17^{b}	11.50 ± 2.24^{c*}
T lymphocyte (CD3)	11.03 ± 0.70	33.01 ± 3.04^{a}	19.11 ± 2.19^{b}	15.36 ± 1.48^{b}
B lymphocyte (CD20)	0.11 ± 0.06	2.66 ± 0.69^{a}	1.24 ± 0.21^{ab}	0.33 ± 0.09^{b}
Plasma cells (Kappa-Lambda)	0.19 ± 0.10	2.84 ± 0.56^{a}	2.95 ± 1.55^{a}	0.26 ± 0.22^{b}
Total cells	20.44 ± 2.63*	116.24 ± 8.08^{a}	74.96 ± 3.64^{b}	41.44 ± 4.67^{c*}

a, b, and c means with different superscripts are significantly different from each other among time points within the infested Iberian ibexes; *statistically significant differences between the control and the infested Iberian ibexes at 103 dpi.

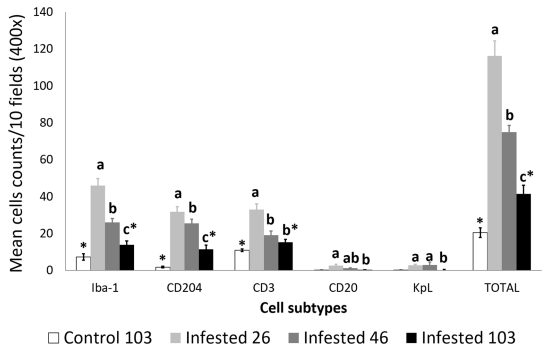

FIGURE 2
Cell counts (mean±standard error) of positively inmunolabeled total macrophages (Iba-1 marker), M2 macrophages (CD204), T lymphocytes (CD3), B lymphocytes (CD20), and plasma cells (Kappa-Lambda) in the skin of the non-infested ibexes and the mangy skin of the infested ibexes altogether at 26, 46, and 103days post-infestation (dpi). The total cell counts are shown as "TOTAL." a, b, and c means with different superscripts are significantly different from each other among time points within the infested ibexes; *statistically significant differences between the control and the infested ibexes at 103 dpi.

time point from those of the control ibexes at 103 dpi (Figure 2); however, the terminal group had higher T lymphocyte counts than the control ibexes at 103 dpi (Supplementary Table S15; Figure 6).

At 26 dpi, the partially recovered ibexes had lower T lymphocyte counts than the totally recovered and terminal groups. At 46 dpi, no statistically significant differences in T lymphocyte counts were detected among the three clinical outcomes. At 103 dpi, the recovered ibexes had significantly lower values than the terminal group, approaching significance versus the partially recovered ibexes (Supplementary Table S16; Figure 6).

3.2.5. B lymphocyte and plasma cell counts

The differences in B lymphocyte counts among clinical outcomes were only significant at 26 dpi, when they were higher in the terminal than in the partially recovered ibexes ($p = 0.023$). The decrease in B lymphocyte counts was consistent in the totally recovered (26 dpi to 46 dpi $p = 0.0499$; 46 dpi to 103 dpi $p = 0.0056$) and terminal (26 dpi to 46 dpi $p = 0.00272$; 46 dpi to 103 dpi $p = 6.2e\text{-}06$) groups, while they decreased more progressively in the partially recovered ibexes (statistically significant differences were only found between 26 dpi and 103 dpi, $p = 0.016$) (Figure 7).

Plasma cell counts were higher in the terminal group than in both the totally ($p = 0.0450$) and partially ($p = 0.0350$) recovered groups at 26 dpi. While plasma cell counts remained stable in the totally recovered group ($p = 0.174$), they decreased consistently throughout the study period in the other two groups (partially recovered: 26 dpi to 46 dpi, $p = 0.0098$; 46 dpi to 103 dpi, $p = 0.0346$; terminal: 26 dpi to 46 dpi, $p = 0.00056$; 46 dpi to 103 dpi, $p = 0.00036$). Nevertheless, the plasma cell counts of the terminal group were still higher than those of the partially recovered group at 46 dpi ($p = 0.043$) and the totally recovered group at 103 dpi ($p = 0.026$) (Figure 8).

4. Discussion

The capacity of Iberian ibexes to recover from and survive sarcoptic mange has been repeatedly reported (37–39). The underlying pathophysiological mechanisms of this recovery seem to rely on the local skin cellular immune response, which is driven by differences in genomic expression, rather than on any humoral immune response, either local or systemic (1, 3, 41–43, 49, 50). This longitudinal study describes for the first time the progression of the local skin immune cell response in Iberian ibexes experimentally infested with *S. scabiei*. Moreover, this is also the first report of such an analysis in any wild ungulate. More importantly, this study also provides the first evidence linking the clinical outcome of sarcoptic mange with the local skin immune cell response, specifically identifying the immune cell types involved and their relationship with the clinical outcome and eventual mortality or survival of the Iberian ibexes affected by this disease.

Compared with the control ibexes, sarcoptic mange caused a severe inflammatory response in the Iberian ibex epidermis and dermis, which can be attributed to the mechanical, excoriating, and allergenic action of the mites (51). This inflammatory infiltrate corresponded to the lesions previously reported in these same individuals, including severe hyperkeratosis, crusts, irregular epidermal hyperplasia with rete ridges, spongiotic areas with apoptotic keratinocytes, and lymphocyte exocytosis foci in the epidermis. Additionally, these lesions were related to the abundance of mites, their detritus, and galleries in the histological sections (21, 38). Such parallelism between skin lesions and inflammatory skin infiltrate has also been reported in other wildlife species, such as chamois and wombats (25, 28).

The absence of mites and the decrease of immune cells observed at 103 dpi is consistent with the previously reported tissue restoration (21) and corresponds with the decline in mite numbers (38). This reduction in mite numbers could be attributed to the exacerbated local inflammatory response observed at 26 dpi. Although this response causes severe tissue damage, it could control and eliminate the mite or promote mite displacement toward other dermal areas where the

TABLE 4 Summary of the most parsimonious model explaining the evolution of total inflammatory cells in the mangy skin of Iberian ibexes by days post-infection (dpi) and outcome (recovered, partially recovered, and terminal).

Fixed effects	Estimate	SE	*z*-value	*p*-value
dpi46	−0.58	0.030	−19.45	<2.2e-16
dpi103	−1.44	0.041	−35.13	<2.2e-16
Partially recovered	−0.16	0.115	−1.36	0.175
Terminal	0.22	0.094	2.36	0.018
dpi46* partially recovered	0.09	0.047	1.85	0.064
dpi103* partially recovered	0.63	0.058	10.85	<2.2e-16
dpi46* terminal	−0.08	0.036	2.18	0.030
dpi103* terminal	0.48	0.047	10.25	<2.2e-16

SE, standard error.

TABLE 5 Summary of the most parsimonious model explaining the evolution of total macrophage counts by days post-infection (dpi) and Iberian ibex outcome (recovered, partially recovered, and terminal).

Fixed effects	Estimate	SE	*z*-value	*p*-value
dpi46	−0.53	0.046	−11.68	<2.2e-16
dpi103	−1.42	0.063	−22.45	<2.2e-16
Partially recovered	0.24	0.112	2.17	0.030
Terminal	0.58	0.092	6.34	2.3e-10
dpi46* partially recovered	−0.34	0.067	−0.58	0.565
dpi103* partially recovered	0.28	0.086	3.28	0.001
dpi46* terminal	−0.05	0.053	−0.95	0.343
dpi103* terminal	0.25	0.071	3.64	2.7e-04

SE, standard error.

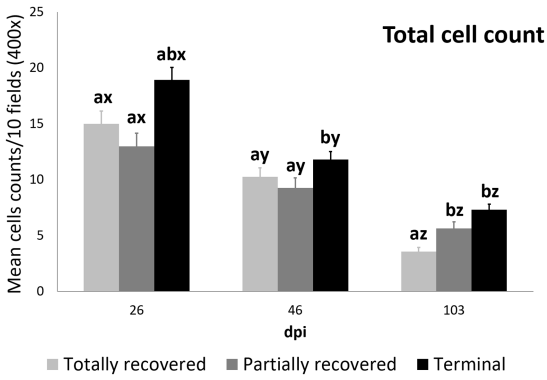

FIGURE 3
Cell counts (mean±standard error) of the all the inmunostained cell types grouped for the clinical outcome groups at 26, 46, and 103 dpi. a and b means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

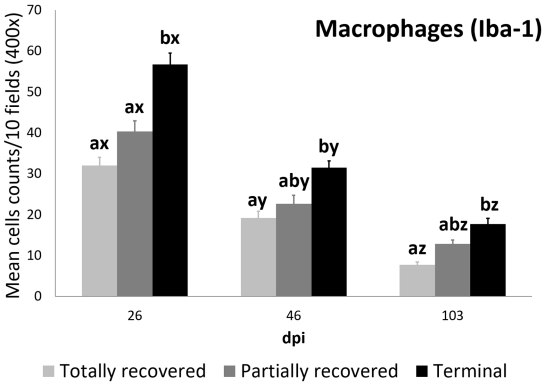

FIGURE 4
Cell counts (mean±standard error) of positively immunolabeled Iba-1 cells (macrophages) for the clinical outcome groups at 26, 46, and 103days post-infestation (dpi). a and b means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

inflammatory response is still reduced (18, 19). Alternatively, the reduction of the inflammatory infiltrate throughout the dpi may not have caused the decrease in mite numbers, but rather be the consequence of mites displacing toward unaffected skin areas richer in nutritional factors (38).

This skin inflammatory response observed in the infested Iberian ibex was characterized by a predominance of macrophages (mainly the M2 subtype), followed by T lymphocytes, with a lower involvement of antibody-producing cells (B lymphocytes and plasma cells) (Figures 2–8). This type of inflammatory response is similar to that reported in other mountain ungulate and wildlife species (25, 32, 33, 52). The higher abundance of intra-epithelial macrophages in the affected ibexes compared with the control group could be due to exocytosis foci of macrophages migrating from the dermis to phagocytise the mite or increased numbers of Langerhans cells, as described in chamois and related to antigen presentation to T lymphocytes (25, 32, 33, 53).

In humans, the more severe crusted scabies is associated with fewer macrophages infiltrating the skin and an unbalanced Th1/Th17 immune response, whereas in ordinary scabies, M2 macrophages are more abundant in the inflammatory infiltrate, indicating a balanced Th1/Th2 immune response (26). Both a suppressive effect mediated by a macrophage migration inhibitory factor expressed by *S. scabiei* and/or an induction of mixed M1/M2 macrophage polarization against the

TABLE 6 Summary of the most parsimonious model explaining the evolution of macrophage phenotype M2 counts by days post-infection (dpi) and Iberian ibex outcome (recovered, partially recovered, and terminal).

Fixed effects	Estimate	SE	*z*-value	*p*-value
dpi46	−0.02	0.042	−0.41	0.686
dpi103	−1.42	0.068	−20.92	<2.2e-16
Partially recovered	−0.14	0.171	−0.80	0.423
Terminal	0.26	0.140	1.87	0.062
dpi46* partially recovered	0.02	0.067	0.30	0.767
dpi103* partially recovered	0.56	0.096	5.88	4.2e-09
dpi46* terminal	−0.38	0.057	−7.21	5.6e-13
dpi103* terminal	0.53	0.078	6.87	6.6e-12

SE, standard error.

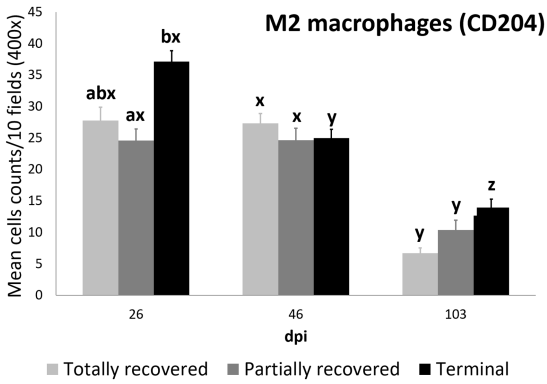

FIGURE 5
Cell counts (mean±standard error) of positively immunolabeled CD204 cells (M2 phenotype macrophages) for the clinical outcome groups at 26, 46, and 103days post-infestation (dpi). a and b means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

scabies mite have been suggested to explain the lower macrophage counts and shift to the M2 phenotype in scabietic humans (54–56).

Although differential staining of macrophage populations (iNOS targeting M1 and CD204 targeting M2) has been attempted previously for other parasitic diseases (57–61), to the authors' knowledge this is the first study analyzing macrophage differential expression in a parasitosis caused by an arthropod. This allowed the macrophages infiltrating the skin of the experimentally infested ibexes to be identified primarily as M2, which suppress local inflammatory response (54). Similarly, in wild carnivores, macrophages have been associated, along with neutrophils, with the milder alopecic form, interpreted as a delayed type IV hypersensitivity reaction (5, 25, 33, 62). This matches the dermis histopathology previously reported in these same Iberian ibexes, consistent with type I and type IV hypersensitivity responses (21). Overall, the consistent decrease of total and M2 macrophage counts in all the clinical outcome groups in the infested Iberian ibexes, combined with the consistently higher values in the terminal group (Figures 4, 5), indicate a successfully balanced Th1/Th2 immune response that resolves the inflammatory condition after the infestation and eliminates or displaces the mite to other areas of the body (18, 19). Such an immune response was more intense and, therefore, required a longer and more sustained response in the terminal ibexes than in either the fully or partially recovered ones (Figures 4, 5). The same explanation of a higher inflammatory response in the severely affected ibexes resolving later and more slowly than the milder increase in the totally recovered ibexes could account for the similar trend observed in T and B lymphocytes and plasma cells (Figures 6–8). To fully distinguish the local skin immune responses to sarcoptic mange determining the clinical outcome in the host, macrophage subpopulations and cytokines should be assessed and further characterized, not only in Iberian ibex but also in other species, to detect potential common inflammatory recovery patterns.

Higher counts of T lymphocytes have been described in response to sarcoptic mange in chamois, wild boars, goats, sheep, and pigs (19, 30, 32, 34, 35), whereas in foxes, wolves, red deer, and roe deer, T lymphocytes were less abundant in the inflammatory infiltrate (5, 33, 62, 63). Finally, the scarce infiltrate of B lymphocytes and plasma cells matches previous reports in mangy chamois and domestic goats and sheep (30, 34, 62). Although antibodies against *S. scabiei* have been broadly used for surveys of sarcoptic mange in wildlife, their role in providing protection against the disease is unclear. In Iberian ibex they increase with clinical severity and, generally, are an indicator of the contact and intensity of the infestation rather than protection (1, 3, 43, 63, 64), corresponding to the low involvement of antibody-producing cells in the skin found in this study.

Despite the common decreasing trend in all the cellular types observed in all three clinical outcome groups (totally recovered, partially recovered, and terminal), the differences among these three groups indicate a correlation between the local skin cellular immune response and the clinical outcome. The skin immune response to sarcoptic mange varies among species and individuals, although it usually corresponds to a type I or type IV hypersensitivity response. In the type IV hypersensitivity response, the memory of T lymphocytes seems to play a key role in limiting infection at the initial phase (18, 24, 25). Moreover, the type of T cells involved (CD4+ or CD8+) and the proportion of macrophages in the local skin immune cellular response is a differential trait between ordinary and crusted scabies in humans (26, 27). Similarly, in domestic goat and sheep, the T lymphocyte subpopulations shift to CD4+ over CD8+ lymphocytes in the local skin immune response to sarcoptic mange (30, 34). Thus, the higher initial (26 dpi) T lymphocyte count of the totally recovered ibexes (Figure 6), leading to lower total cell, macrophage (both total and the M2 phenotype), T lymphocyte, and plasma cell counts at the end of the study period (103 dpi) than in the terminal ibexes (Figures 3–6, 8), seems to confirm that an initial effective local skin T-cell immune response is crucial for controlling the spread of sarcoptic mange in Iberian ibex. Further characterization of this T-cell immune response, including the cell types, cytokines, and gene expression involved, should help us to fully understand the

TABLE 7 Summary of the most parsimonious model explaining the evolution of T lymphocyte counts by days post infection (dpi) and Iberian ibex outcome (recovered, partially recovered, and terminal).

Fixed effects	Estimate	SE	*z*-value	*p*-value
dpi46	−0.87	0.046	−19.15	<2.2e-16
dpi103	−1.37	0.055	−24.92	<2.2e-16
Partially recovered	−0.66	0.171	−3.89	1.0e-04
Terminal	−0.19	0.138	−1.44	0.151
dpi46* partially recovered	0.63	0.076	8.36	<2.2e-16
dpi103* partially recovered	1.10	0.082	13.43	<2.2e-16
dpi46* terminal	0.50	0.056	8.90	<2.2e-16
dpi103* terminal	0.77	0.065	11.86	<2.2e-16

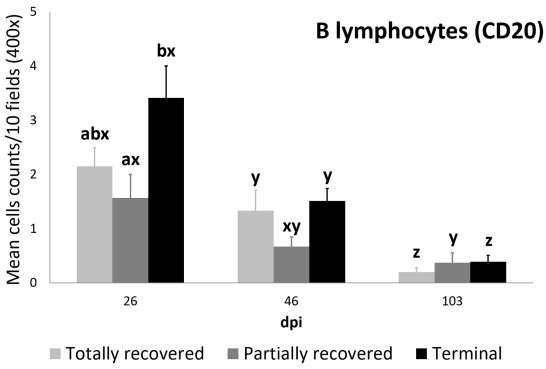

FIGURE 7
Cell counts (mean±standard error) of positively immunolabeled CD20 cells (B lymphocytes) for the clinical outcome groups at 26, 46, and 103days post-infestation (dpi). a and b means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

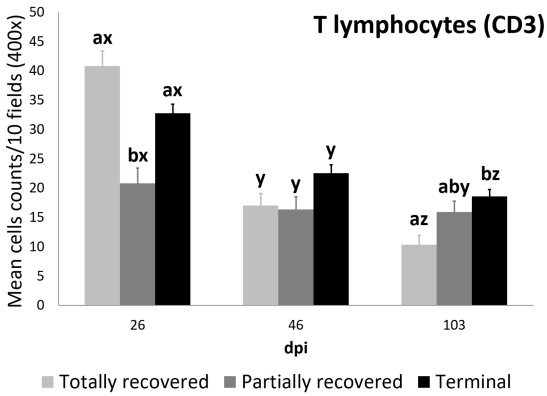

FIGURE 6
Cell counts (mean±standard error) of positively immunolabeled CD3 cells (T lymphocytes) for the clinical outcome groups at 26, 46, and 103days post-infestation (dpi). a and b, means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z, means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

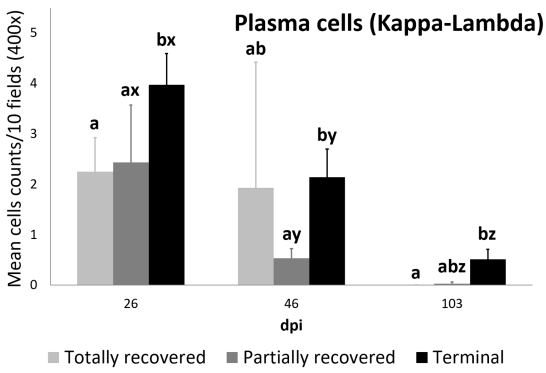

FIGURE 8
Cell counts (mean±standard error) of positively immunolabeled Kappa-Lambda cells (plasma cells) for the clinical outcome groups at 26, 46, and 103days post-infestation (dpi). a and b means with different superscripts are significantly different from each other among the clinical outcome groups at the same time point; x, y, and z means with different superscripts are significantly different from each other among time points within the same clinical outcome group.

pathophysiological and immune mechanisms responsible for the control or extension of sarcoptic mange in Iberian ibex (21, 24–26, 49, 50, 54).

To summarize, this study reports for the first time the progression of the local skin immune cell response over time, not only to *S. scabiei* infestation in Iberian ibex but to any ectoparasite in any wild ungulate species. The skin immune cell infiltrate elicited by mange in Iberian ibex, predominantly formed by macrophages and T lymphocytes, indicates a locally intense cellular inflammatory response. The evolution over time of mite presence and the inflammatory response must be considered when assessing mange lesions through skin biopsies, as false negative results could occur due to mite absence and the complete restoration of tissue integrity as soon as 3 months after the establishment of the mite (21, 49).

The M2 macrophage phenotype predominantly induced by *S. scabiei* has immunoregulatory effects and a reparative action, which could contain the mite, limit the inflammatory infiltrate, and aid in restoring tissue integrity. However, the decrease in inflammatory infiltrate could instead be a consequence of mite displacement to unaffected skin areas, rather than a direct effect of the M2 macrophages. The higher initial T lymphocyte counts in the ibexes that had a milder clinical outcome and completely healed suggest that this early T lymphocyte immune response plays a key role in controlling the spread and severity of sarcoptic mange in this species, limiting the inflammatory infiltrate and leading to a faster resolution of the disease. Moreover, even if this first antigen-presenting response fails, differences in the local inflammatory response and consequently in the clinical outcome occur, with some individuals recovering and

healing and others progressing to severe stages of the disease that ultimately lead to death.

Therefore, the development of severe sarcoptic mange and eventual death requires a sequence of different conditions. Firstly, the ibex must come into contact with an effective infective dose of *S. scabiei* mites to become infested. Secondly, the local immune skin response, which involves antigen presentation by T lymphocytes and is described in the totally recovered ibexes in this study, must fail to control the spread of the disease. Thirdly, the inflammatory response must be intense and not resolve or heal spontaneously, unlike the findings in the partially recovered ibexes in this study. Finally, the uncontrolled infestation must spread and become severe enough to exhaust the pathophysiological resources of the host.

Further investigation is necessary to gain a comprehensive understanding of the mechanisms underlying resistance to sarcoptic mange in Iberian ibex, as reported in this study. Immunological, immunohistochemical, genetic, and genomic research would be particularly informative in this regard. The present findings contribute to the growing body of evidence refuting the previously held belief that *S. scabiei* infestation in this species inevitably leads to mortality. Therefore, these findings have significant implications not only for individual disease outcomes but also for the management of the Iberian ibex populations affected by sarcoptic mange.

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.

Ethics statement

The animal study was reviewed and approved by the Ethics on Animal Welfare Committee of the University of Jaén and authorized by the Dirección General de Producción Agrícola y Ganadera of the Consejería de Agricultura, Pesca y Medio Ambiente of the Junta de Andalucía (Ref: SA/SIS/MD/ps/ October 25, 2012).

Author contributions

MV analyzed the data, drafted the original manuscript, and elaborated the final version of the manuscript. JG designed the experiment, obtained funding, performed the experimental infestation, obtained and curated the samples, and critically revised the manuscript. VP performed the immunohistopathological analyses, and critically revised the manuscript. JL-O designed the experiment, obtained funding, performed the experimental infestation, obtained and curated the samples, drafted the original manuscript, and elaborated the final version of the manuscript. AR-B performed the experimental infestation, obtained and curated the samples, and critically revised the manuscript. PF, JP, and GM designed the experiment, obtained funding, and critically revised the manuscript. ST participated in data analysis, and critically revised the manuscript. RS designed the experiment, obtained funding, and critically revised the manuscript. JE performed the experimental infestation, obtained and curated the samples, performed the immunohistopathological analyses, drafted the original manuscript, and elaborated the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1183304/full#supplementary-material

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First molecular detection of *Babesia vulpes* and *Babesia capreoli* in wild boars from southern Italy

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Introduction: Following the increase of wild boar (*Sus scrofa*) populations in Europe, a potential risk of emerging infections by vector-borne pathogens may occur. Despite this, the circulation of piroplasmid species in these ungulates is still a neglected topic, particularly in the Mediterranean basin. Therefore, this study aimed to investigate the presence of *Babesia*/*Theileria* spp. in wild boars from southern Italy to assess the epidemiological role of these ungulates in the circulation of piroplasmids.

Methods: By using a *citizen science* approach among hunters and veterinarians, wild boar spleen samples were collected in the Campania region (southern Italy) between 2016 and 2022. A combined semi-nested PCR/sequencing analysis targeting the V4 hyper-variable region of *18S* rRNA was run to detect *Babesia*/*Theileria* spp. DNA.

Results: Out of 243 boars, 15 (i.e., 6.2, 95% CI: 3.4–9.9) tested positive to *Babesia*/*Theileria* spp., *Babesia vulpes* ($n = 13$, 5.3, 95% CI: 3.1–8.9) the most prevalent, followed by *Babesia capreoli* ($n = 2$, 0.8, 95% CI: 0.2–2.9). Three different *B. vulpes* sequence types were identified (i.e., ST1, ST2, ST3), with the most representative as ST1 (60%), and a single *B. capreoli* sequence type. No statistically significant difference ($p > 0.05$) were found between the presence of the pathogens and boar age, sex, province and sample collection year.

Discussion: Data demonstrate for the first time the occurrence of *B. vulpes* and *B. capreoli* in wild boars, which may play a role in the biological cycle of piroplasmids. We emphasize the importance of monitoring these ungulates to prevent potential foci of *infection*. The engagement of hunters in epidemiological scientifically based surveys can constitute a technically sound control strategy of piroplasmids in a One Health perspective.

KEYWORDS

***Babesia capreoli*, *Babesia vulpes*, Italy, public health, wild boar**

1. Introduction

Piroplasmids of the genus *Babesia* and *Theileria* (Aconoidasida, Piroplasmida) are global emerging tick-borne apicomplexan protozoa infecting multiple wild species, as well as domestic animals and humans (1, 2). Among more than 100 different species identified so far, some of these intracellular parasites display a high host specificity in wild mammals (3). For instance, the role of some wildlife species has been ascertained in the maintenance of certain *Babesia* spp., such as red foxes (*Vulpes vulpes*) for *Babesia vulpes*, red deer (*Cervus elaphus*) for the zoonotic *Babesia divergens* and roe deer (*Capreolus capreolus*) for *Babesia capreoli* and the zoonotic *Babesia venatorum* (3–6). Despite this, piroplasmid surveillance in wild boar (*Sus scrofa*) populations is a neglected topic due to their apparent absence in this ungulate in Europe (7). The only two *Babesia* spp. infecting boars, also common in pigs, *Babesia trautmanni* and *Babesia perroncitoi*, have been detected mostly in the 1990s via morphology without any molecular confirmation (7). The unique cases of molecular detection of piroplasmids in boars are to date reported as unspecified *Theileria* spp. in Italy ($n = 3$ out of 117) (8) and Portugal ($n = 3$ out of 65) (9), *Babesia bigemina* in Italy ($n = 2$ out of 257) (10) and a single finding of *B. divergens* out of 550 in the Czech Republic (7). This negligible occurrence of piroplasmids in boars is likely due to a low prevalence and parasitaemia and low number of tested animals (i.e., <100 in several epidemiological surveys) (11–13), despite the use of highly sensitive qPCR/conventional PCR protocols (7, 14). However, the role of boars in the epidemiology of piroplasmids in Europe cannot be ruled out considering that their high density (15), territorial expansion (16) and spatial overlap with other wildlife populations may increase the chance of tick infestation and piroplasmid transmission (8). Indeed, a considerable risk for new foci of emerging *Babesia* and *Theileria* infections is now evident in Europe, especially in the south and in the Mediterranean basin where great diversity of piroplasmid species (17) and high biodiversity of ixodid ticks occur (18). Some areas in these regions are also associated with a great vocation for outdoor recreational activities exposing to the risk of piroplasmid infection, as demonstrated by the high seroprevalence in hunting dogs from rural areas of southern Italy (19), where wildlife, ticks and related pathogens overlap (20). Therefore, this study aimed to investigate the occurrence of *Babesia/Theileria* spp. in wild boars from southern Italy and to assess the epidemiological role of these ungulates in the circulation of piroplasmids.

2. Materials and methods

2.1. Study area and sampling

The study was run in the Campania region, southern Italy, characterized by a typical Mediterranean temperate climate and progressively continental features of mainland and mountainous landscapes. Under the frame of a surveillance plan of wildlife by the Italian Ministry of Health (authorization no. IZSME RC 05/16), spleens of wild boars were collected from October 2016 to December 2022. Field activities were carried out in collaboration with "trained persons" (i.e., regular boar hunters educated specifically on hunting hygiene, health and food safety through specific theorical and practical courses, according to Reg. EU 853/2004) (21). Hunters culled boars and collected spleens and information (age, sex, geographic origin) under supervision of veterinarians affiliated with the University of Naples Federico II and regional health systems. In order to minimize the risk of cross-contamination, whole spleens were collected and stored at ±4°C in separate plastic biohazard bags and delivered to the necropsy room of the Department of Animal Health, Experimental Zooprophylactic Institute of southern Italy (Portici, Italy). Each spleen was flamed on the surface before sampling an aliquot from the inner portion for DNA extraction. Classes related to boar age (i.e., piglet <1 years old, juvenile 1–2 years old, adult >2 years old) were estimated by the examination of the teeth (i.e., primary and permanent teeth eruption times and root hole diameter of incisors), according to Massei and Toso (22).

2.2. Sample size calculation

A minimum sample size of 243 wild boars was estimated using the opensource software OpenEpi (23), inserting the following data: a population size of 84,000 boars (data supplied by the regional emergency plan of wild boars in Campania region); expected prevalence of *Babesia/Theileria* spp. infection in the population of 5% ±3 (i.e., 2%–8%), according to Zanet et al. (10); confidence limits of 5% and desired absolute precision of 3%.

2.3. DNA extraction, PCR protocol, and sequencing

One gram of spleen was individually homogenized by tissue lysis (Qiagen) in sterile PBS buffer with two 4.8 mm glass beads (Diatech Lab Line, Salerno, Italy). Each DNA extraction session included a negative extraction control (represented by an equal volume of RNase/DNase free water instead of DNA extraction elute). From 200 µL of homogenized sample, extraction of nucleic acid was obtained using a commercial kit (QIAampDNA Blood & Tissue; Qiagen, Hilden, Germany), according to the manufacturer's instructions. A semi-nested PCR protocol targeting the V4 hypervariable region of the *18S* ribosomal RNA gene was used for the direct detection of *Babesia/Theileria* spp. DNA (10). In the first round, primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3′) and RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3′) were used in a final reaction volume of 25 µL, using Promega PCR Master Mix (Promega Corporation, WI, United States), 20pM of each primer, and ≈100 ng of DNA template measured with the Biofhotometer plus (Eppendorf, Hamburg, Germany), according to the manufacturer's instructions. The thermocycling conditions included initial denaturation for 5 min at 95°C, followed by 25 cycles of denaturation for 30s at 95°C, 45 s annealing at 50°C and 90s extension at 72°C and a final extension of 10 min at 72°C. Amplicons (1 µL) of the first PCR round were used as template in the second round with the same primer RLB-R2 plus RLB-FINT (5'-GACAAGAAATAACAATACRGGGC-3′). The reaction mix and cycling conditions were identical in first and second rounds, except for the total number of cycles (i.e., 40) and annealing temperature (55°C) in the second round. In all PCR runs, positive (i.e., *Babesia*

canis DNA of fox spleen from Italy) and negative (reaction mix plus sterile water) controls were used. All PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, United States). Amplicons were purified by the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced in both directions using the same primers of the second round by the BigDye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, United States). Consensus sequences were obtained by the Geneious software version 9.0 (Biomatters Ltd., Auckland, New Zealand) (24) and compared with those available in the GenBank database by the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Statistical analysis

An exact binomial 95% confidence interval (95% CI) was established for the proportions of infection found herein. The Chi-squared or Fisher's exact test were used, depending on the population size, to assess any statistical differences of infection by animal age, sex, province of origin and sample collection year, while odds ratio was used for the infection risk by sex. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed by using the online software Epitools—Epidemiological Calculators (25). The distribution of *Babesia*-positive wild boars according to provincial borders of the study area was determined using aerial imagery from Bing aerial maps software (Microsoft, Redmond, Washington, United States).

3. Results

A total number of 243 wild boar spleen samples from southern Italy between 2016 and 2022 were analyzed. Fifteen animals (i.e., 6.2, 95% CI: 3.4–9.9) tested positive to *Babesia* spp. DNA, 13 (i.e., 5.3, 95% CI: 3.1–8.9) and two (i.e., 0.8, 95% CI: 0.2–2.9) with *B. vulpes* and *B. capreoli*, respectively, using the combined semi-nested PCR/sequencing approach. The geographic distribution of *Babesia*-positive wild boars according to provincial borders of the study area is illustrated in Figure 1. Detailed data on prevalence, confidence intervals and statistical analyses are listed in Table 1. No statistically significant differences (i.e., $p > 0.05$) were found according to the boar's age, sex, province and collection year. Three different *18S* rRNA partial sequences of *B. vulpes* were identified (sequence types ST1, ST2, ST3), with the most representative type being ST1 (60%) and a single sequence of *B. capreoli*. Compared to ST1, there were single nucleotide polymorphisms in ST2 (a T instead of C in position 220) and ST3 (a C instead of G in position 71). All sequences had 99–100% nucleotide identity with those available in GenBank. Sequences obtained in this study were deposited in GenBank under the following accession numbers: OQ520218 for *B. vulpes* ST1, OQ520219 for ST2, OQ520220 for ST3 and OQ520222 for *B. capreoli*.

4. Discussion

This study helps to fill the gap on *Babesia* spp. presence in wild boars, as well as suggesting cooperation of health stakeholders and trained persons (*citizen science* approach) as an effective tool for monitoring wildlife and related pathogens (26, 27).

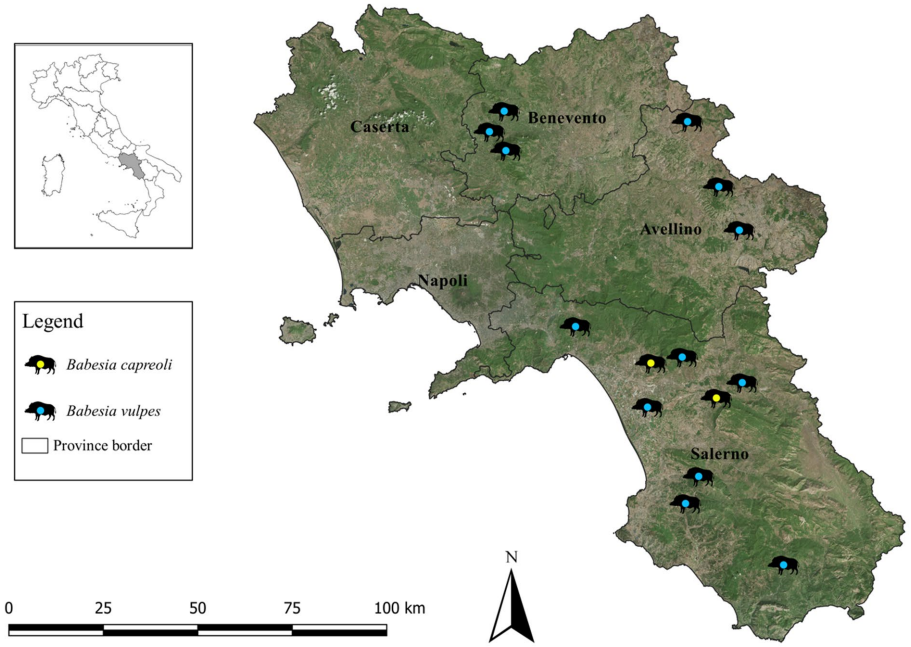

FIGURE 1
Distribution of wild boars ($n = 15$) positive to *Babesia vulpes* and *Babesia capreoli* by provinces (Avellino, Benevento, Caserta, Naples, Salerno) of the Campania region, southern Italy, 2016–2022.

TABLE 1 Wild boar spleen samples (n = 243) tested for *Babesia* spp. DNA in southern Italy, 2016–2022.

Variables	Pos/Tot[a]	%	95% CI[b]	Chi-squared; value of p	Odds ratio
Age (years old)					
Piglet (<1)	1/44	2.3	0.04–11.8		
Juvenile (1–2)	4/56	7.1	2.8–17.0		
Adult (>2)	10/143	7.0	3.8–12.4		
				$\chi^2 = 1.4$; $p = 0.230$	Not applicable
Sex					
Male	7/121	5.8	2.8–11.5		
Female	8/122	7.1	3.4–12.4		
				$\chi^2 = 0.1$; $p = 0.800$	0.88
Province					
Avellino	2/31	6.5	1.8–20.7		
Benevento	3/49	6.1	2.1–16.5		
Caserta	1/14	7.1	1.3–31.5		
Salerno	9/149	6.0	3.2–11.1		
				$\chi^2 = 0.1$; $p = 0.998$	Not applicable
Year					
2016	0/17	-	-		
2017	1/25	4.0	0.7–19.5		
2018	2/35	5.7	1.6–18.6		
2019	2/38	5.3	1.5–17.3		
2020	2/41	4.9	1.3–16.1		
2021	4/42	9.5	3.8–22.1		
2022	4/45	8.9	3.5–20.7		
				$\chi^2 = 2.9$; $p = 0.820$	Not applicable
Total	15/243	6.2	3.4–9.9		

[a]Pos/Tot: number of positive samples out of the total analyzed.
[b]95% CI: 95% confidence interval.

To date, the only piroplasmid DNA in boars of Europe have been reported in a publication of *B. bigemina* in Italy (10), unspecified *Theileria* spp. in Italy and Portugal (8, 9) and *B. divergens* in the Czech Republic (7).

However, the moderate infection prevalence of *Babesia* spp. herein found in southern Italy (6.2%), and northern regions of the country (from 2.6% to 4.7%) (8, 10), suggests an involvement of boars in the sylvatic life cycle of the parasite. The absence of statistically significant difference in prevalence by boar's age and sex in this study confirms that these variables do not influence the infection frequency, similar to findings of Zanet et al. (10). Again, the absence of significant differences in *Babesia* prevalence by province and collection year of samples suggests a stable circulation of infection in the study area.

Regarding *B. vulpes*, although the fox is the main reservoir in Europe (4, 28), the similar infection prevalence of this piroplasmid species in boars within this survey (13/243, 5.3%) and in foxes from the same study area (8/187, 4.3%) (29) indicates a potential involvement of this ungulate in pathogen maintenance. Despite roe deer being the most common host observed previously to be infected with *B. capreoli* (30, 31), its low prevalence (0.8%) in boars from this study should not exclude a role of these latter hosts in maintaining the pathogen considering the scant presence of other ungulate species in southern Italy, including roe deer (32). The potential pathogenic implications of *B. capreoli* infection in boars should be assessed in the future given that, although commonly asymptomatic in wildlife (6), cases of fatal babesiosis by this protozoan have been outlined in other wild ungulates, such as reindeer *Rangifer tarandus* (33) and Alpine chamois *Rupicapra rupicapra* (34, 35). Lastly, although not observed among boars in this study, the presence of suspected vectors of *B. vulpes* (i.e., *Ixodes hexagonus* and *Ixodes canisuga*) (36, 37) and *B. capreoli* (i.e., *Ixodes ricinus*) (38, 39) cannot be ruled out, considering that these tick species are commonly found on foxes (40) and hunting dogs (41) which live in sympatry with these ungulates. Indeed, due to the extensive time spent within sylvatic areas, hunting dogs show a higher prevalence of tick-borne pathogens compared to companion dogs (42). An example includes *B. vulpes* (43), capable to cause severe (4, 44) or fatal disease in dogs (45).

Despite the 18S rRNA gene is widely employed as a target for the molecular detection of piroplasmids (7, 14, 46, 47), the use of other genetic markers is recommended for species differentiation given the very high similarity of *Babesia* spp. sequences, such as *B. capreoli* and *B. divergens* which differ in just three positions (6, 30). Indeed, future

studies on a larger sample size, including other wild ungulate species, and multiple genetic targets are needed to investigate the occurrence of piroplasmids in southern Italy.

The spread of wild boar populations may enhance the chance of transmission for emerging tickborne pathogens, including piroplasmids. More research is required to clarify the role of these ungulates in the maintenance of *B. vulpes* and *B. capreoli* in other epidemiological scenarios.

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/nuccore; OQ520218, OQ520219, OQ520220, and OQ520222.

Ethics statement

The animal study was approved by the project "Ricerca corrente" (grant number: IZSME RC 05/16) by the Italian Ministry of Health. Written informed consent was not required for this study in accordance with national legislation and institutional requirements.

Author contributions

GS and VV conceptualized and designed the study. GS wrote the first draft of the manuscript. ND'A, CA, and HS wrote sections of the manuscript. AG, MGR, and FA performed molecular analyses. SR, SS, MO, and CDM were involved in sampling and database curation. GF and MGL managed project administration and resources. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Neglected zoonotic helminthiases in wild canids: new insights from South America

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The global threat of neglected tropical diseases (NTDs) constitutes a public health issue in underdeveloped countries. Zoonotic helminthiases are the most common human NTD agents in developing countries in sub-Saharan Africa, Asia, and the Americas, causing a global burden of disease that exceeds that of more recognized infectious diseases such as malaria and tuberculosis. Wild canids are well-known mammals that act as natural reservoirs of zoonotic-relevant helminthiasis worldwide, thus playing a pivotal role in their epidemiology and transmission to humans. Here we evaluate the occurrence of zoonotic gastrointestinal helminths in two Neotropical wild canid species from the Amazonian and Andean regions of Colombia, i.e., the bush dog (*Speothos venaticus*) and the crab-eating fox (*Cerdocyon thous*). We recovered tapeworm proglottids from bush dog fecal samples and identified them molecularly as the canine-specific lineage of *Dipylidium caninum* by using cytochrome c oxidase subunit I (*cox1*) gene sequences. Moreover, examination of a crab-eating fox during necropsy revealed the presence of non-embryonated eggs of the neglected nematode *Lagochilascaris cf. minor*, in addition to eggs and gravid proglottids of the cestode *Spirometra mansoni*. These findings represent the first report of zoonotic-relevant cestodes, i.e., *D. caninum* ("canine genotype"), *S. mansoni*, and the nematode *L. cf. minor*, in bush dogs and crab-eating foxes as final hosts. The occurrence of these zoonotic helminthiases in wild canid species calls for regular monitoring programs to better understand the epidemiology and transmission routes of neglected dipylidiasis, lagochilascariosis, and sparganosis in South America.

KEYWORDS

neglected, zoonosis, wild canids, Neotropics, dipylidiasis, lagochilascariosis, sparganosis

1. Introduction

Zoonoses account for approximately 60% of emerging human infectious diseases, and among these, up to 70% are wildlife-derived pathogens (1, 2). In addition, the global threat of neglected tropical diseases (NTDs) constitutes a public health issue in underdeveloped countries in sub-Saharan Africa, Asia, and the Americas. Among NTDs, zoonotic helminthiases are the most common human pathogens, causing a global burden of disease exceeding that of better-known infectious diseases such as malaria and tuberculosis. On a global scale, helminth infections account

for over 75% of disability-adjusted life years lost. However, many of them have fallen into oblivion as neglected diseases (3, 4). Wild canids are well-known natural reservoirs of zoonotic parasites (5–10), which include numerous helminth species, and thus play a pivotal role in the life cycle, epidemiology, and transmission routes of human infections (11–14). The forested tropical regions with high mammalian species richness are facing the emergence of zoonotic disease hotspots under ongoing land use changes, giving rise to an increased disease transmission risk at the human-animal interface (15, 16). Lower-latitude developing countries (e.g., Neotropical territories) have a concentration of emerging zoonotic pathogens, while scientific studies and surveillance efforts that focus on this issue remain scarce (17). Knowledge of zoonotic cestodes is limited to the genera *Dibothriocephalus* (diphyllobothriosis), *Hymenolepis,* and *Taenia*, leaving uncommon neglected cestode infections such as bertielliosis, dipylidiasis, echinococcosis, inermicapsiferosis, raillietinosis, mesocestoidiosis, and sparganosis, which are rarely reported clinically and underestimated even by specialists (4, 18, 19). Wild canids comprise a large group of carnivores that are distributed throughout the world, often living in close proximity to human populations (20–22). The Neotropics are home to a total of 10 wild canid species with varied behaviors, habitats, and forms (Table 1). The current study presents the findings on the gastrointestinal helminth parasite in two highly divergent free-ranging Neotropical wild canid (NWC) species: the elusive semiaquatic diurnal/crepuscular bush dog and the nocturnal ground-dwelling crab-eating fox. Furthermore, we examine the potential role that NWC may play as definitive hosts (DH) in the transmission and maintenance of neglected zoonotic helminthiases, providing new insights into this unresolved issue.

2. Materials and methods

2.1. Study areas and sample collection

Based on the Köppen–Geiger classification system, the Amazonian and Andean sampling areas were found in tropical rainforests and temperate, warm summers without dry climates, respectively (23). Animal sampling was focused on the northern regions of South America (Figure 1) and within the distribution range of bush dogs and crab-eating foxes in the Neotropical area. The current study included samples collected between 2019 and 2021 as part of a national wildlife conservation and monitoring program carried out by veterinarians/mammologists in Colombia. Additionally, animals found dead and collected by indigenous peoples and local communities were included in the study. Due to the evasive behavior of bush dogs, parasite specimens were collected from direct sampling sites on trails that were systematically monitored by trap cameras. The general features and morphometric characteristics of wild carnivore deposits were followed for fecal identification (24). Moreover, associated tracks and local traditional ecological knowledge were also used to sample these elusive individuals, as previously described (25, 26). Therefore, fecal samples were collected as fresh as possible. No bush dog carcasses were examined during the study period. However, scattered proglottids and cestode strobila segments were found partially dehydrated during the macroscopical examination of feces from two monitored bush dogs ($n = 2$) in the Amazonian municipality of Puerto Santander, Colombia. Parasite collection from crab-eating foxes was carried out during the necropsy of a dead animal ($n = 1$) in the Andean municipality of Ciudad Bolívar., Colombia, and in an environment where grass-fed cattle are raised. The head, thoracic, and abdominal cavities were extensively examined for the presence of ecto- and endoparasites. The entire gastrointestinal tract, heart, spleen, kidneys, and respiratory tract were removed and thoroughly inspected *in situ* for the presence of macroscopic parasites using a 40X-25 mm glass magnifier. An adult cestode specimen was carefully recovered from fresh feces collected from the gut lumen after a longitudinal intestinal incision. Fecal samples were then collected directly from the gastrointestinal tract and dry-preserved until examination.

After macroscopic observation of cestode strobila and free proglottids in the feces, all collected parasite specimens were handled using fine entomological tweezers, gently rinsed, washed three times with 0.9% pre-warmed phosphate-buffered saline (PBS), and subsequently preserved in ~96% EtOH until microscopic and molecular evaluation. Combined sedimentation-flotation and modified sodium acetate–acetic acid-formalin standardized parasitological techniques were used to analyze wild canid fecal samples (27). In addition, gravid proglottids obtained from the crab-eating fox tapeworm strobila were dissected and wet mounted on slides. This non-invasive method for fecal collection allowed for the recovery of adult cestode specimens without unnecessary manipulation, trapping, or disturbance of these free-ranging canids (25, 28).

TABLE 1 Extant wild canid species distributed in the neotropics.

Genus	Species	Common name	Classification risk [§]
Atelocynus	*microtis*	Short-eared dog	NT
Cerdocyon	*thous* [‡]	Crab-eating fox	LC
Chrysocyon	*brachyurus*	Maned wolf	NT
Speothos	*venaticus* [‡]	Bush dog	NT
Lycalopex	*vetulus*	Hoary fox	NT
Lycalopex	*sechura*	Sechuran fox	NT
Lycalopex	*gymnocercus*	Pampa's fox	LC
Lycalopex	*fulvipes*	Darwin's fox	EN
Lycalopex	*culpaeus*	Culpeo	LC
Lycalopex	*griseus*	Chilla	LC

[‡] Wild canid species included in this study. [§] Based on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. EN, endangered. NT, near threatened. LC, least concern.

2.2. Phenotypic evaluation of adult cestode specimens

General morphologic and morphometric taxonomic traits were observed, and parasite stage identification was conducted under microscopic analysis using an Olympus BX53™ semi-motorized light microscope (Olympus Corporation, Tokyo, Japan) at 400 and 1,000X magnification. The Olympus DP74™ digital camera was used to capture photomicrographs of eggs, adult strobila, and proglottids. Parasites were measured using the cellSens™ standard imaging software. Additionally, cestode proglottids were dehydrated in ethanol series (75, 80, 85, 90, 96, and 100%), transferred to a fixative solution

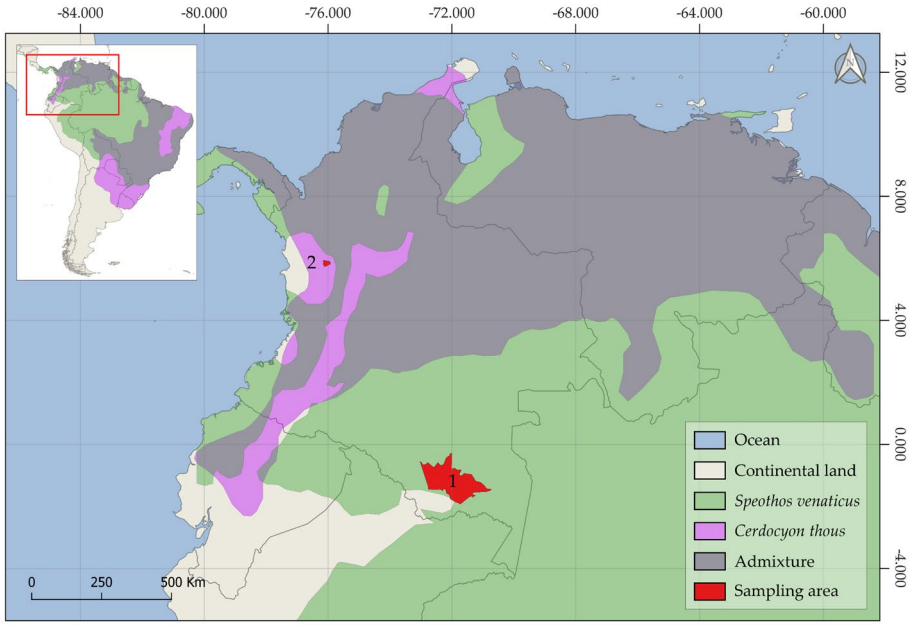

FIGURE 1
The geographic map depicts the historical distribution range of the bush dog (*Speothos venaticus*), the crab-eating fox (*Cerdocyon thous*), and the admixture zone where both species are found. The Amazonian (1) and Andean (2) sampling areas of this study are shown in red.

(i.e., formalin, 95% EtOH, glacial acetic acid, glycerine, and Milli-Q ultrapure distilled water; 10:25:5:10:50 parts, respectively), clarified with lactophenol, and stained with Semichon's acetocarmine. Finally, the proglottids were wet-mounted and Berlese's fluid-mounted on slides as described previously (29).

2.3. Molecular phylogenetics

The complete coding sequence of the cytochrome c oxidase subunit I (*cox1*) gene was amplified in two overlapping fragments with the primers *cox1*F and JB4.5, and JB3 and *cox1*R, respectively (30, 31) using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, USA) and the following cycling conditions: 35 cycles of 10 s at 98°C, 15 s at 50°C (*cox1*F + JB4.5) or 60°C (JB3 + *cox1*R), and 50 s at 72°C. PCR products were gel-checked, purified with Exonuclease I and FastAP alkaline phosphatase (Thermo Fisher Scientific, Waltham, USA), and directly Sanger-sequenced at SeqMe (Dobříš, Czech Republic). Contiguous gene sequences were assembled, visually checked, and trimmed to the *cox1* coding region in Geneious Prime 2020.0.5[1] and deposited in GenBank under accession numbers OR251823 and OR251823. The resulting sequences were aligned with previously published *cox1* data from *Dipylidium* specimens in addition to other closely related species using MAFFT's (32) L-INS-i translational align plugin of Geneious. The use of *Nippotaenia chaenogobii* (JQ2685509) and *Nippotaenia mogurndae* (ON640728) as outgroup taxa and the selection of relevant ingroup representatives were based on previous phylogenetic estimates, most notably by Waeschenbach et al. (33) and Guo et al. (34). The phylogenetic tree was estimated under the maximum likelihood criterion in IQ-TREE (35). The best-fitting model of nucleotide evolution was selected according to the corrected Akaike information criterion in IQ-TREE (36), and nodal supports were estimated by running 1,000 standard nonparametric bootstrap replicates and 10,000 repetitions of the SH-like approximated likelihood ratio test.

3. Results

3.1. Morphological and morphometric parasite identification

Morphological identification of the whitish, flat, barrel-shaped segments recovered from bush dog feces (Figure 2) was based on observation of the typical longer-than-wide shape, with each proglottid having two bilateral genital pores, one at the center of each lateral margin. The mean gravid proglottid measurements ($n = 10$) were 12.082 mm (SD ± 0.542 mm) in length and 3.996 mm (SD ± 0.344 mm) in width. The phenotypic evaluation corresponds well with *Dipylidium caninum* s.l. (Dipylidiidae). Thin-shelled capsules (ovigerous capsules) containing eggs were also noticed inside gravid proglottids (Figure 2D). Regarding the parasitological evaluation of the crab-eating fox, non-embryonated ascarid-type eggs (51.21 × 51.57 µm) with a thick eggshell and a coarsely pitted surface containing multiple excavations were observed (Figure 3A). The morphological traits of the egg correspond well to *Lagochilascaris minor,* previously described in South American wild carnivore definitive hosts (37, 38). Additionally, parasite stages (i.e., adults and eggs) of the diphyllobothriidean species *Spirometra mansoni* were detected. A weakly muscled, medium-sized, pink-colored cestode

1 http://www.geneious.com

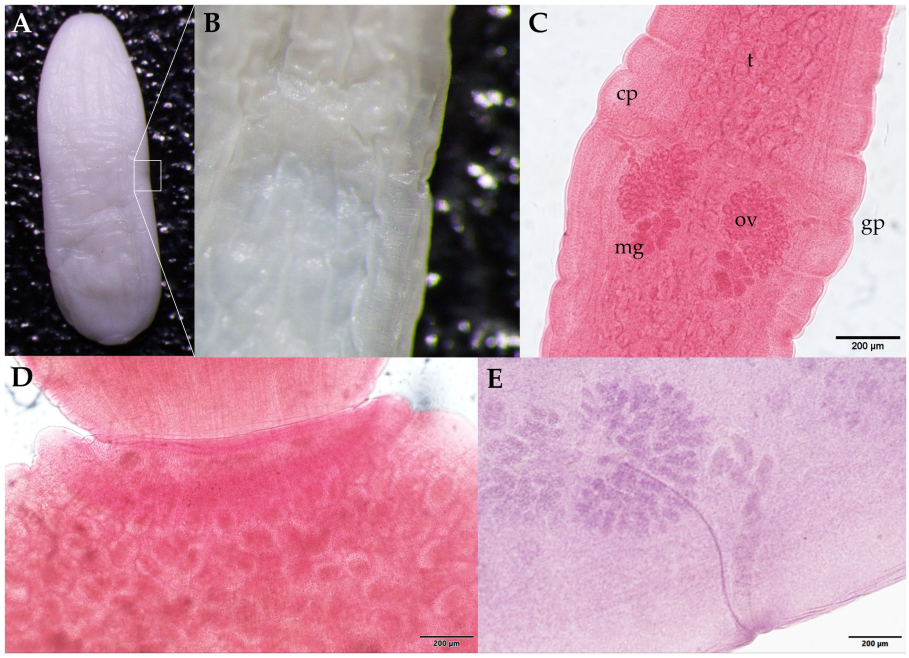

FIGURE 2
Proglottids of *Dipylidium caninum* s.l. (family Dipylidiidae) collected from the feces of the Amazonian bush dog (*Speothos venaticus*). **(A)** Wet mount unstained gravid proglottid. The white square indicates **(B)** a lateral magnified view of the genital pore. **(C)** Mature proglottids stained with Semichon's acetocarmine; two sets of symmetrically distributed genital organs are visible, with the testis parenchyma (t), cirrus pouch (cp), genital pore (gp), ovaries (ov), and the Mehlis glands (mg). **(D)** Close-up of the seed-shaped ovigerous proglottid end with round to oval egg capsules (packets) with an average length of 31–50 µm and a width of 27–48 µm ($n = 88$). **(E)** Photomicrograph of the parasite showing details of one of two sets of male and female reproductive organs. Scale bars: **(C–E)** 200 µm.

(89.73 cm in length) with a long, prominent neck was also recorded. External segmentation of the strobila was noted throughout the specimen. The cestode showed a well-developed spoon-shaped scolex without inrolling bothrial edges. The mature and gravid proglottids were serrated, and the eggs presented a clearly visible unique operculum and an oval shape with a pointed end (Figures 3B–E and Supplementary Video S1). The average proglottid measurements ($n = 483$) were 454.14 µm (SD ± 207.16 µm) in length and 1.78 mm (SD ± 0.73 mm) in width. The strobila segments of this parasite specimen were previously used for the molecular identification of *S. mansoni* reported by Brabec et al. (39).

3.2. Molecular characterization of *Dipylidium caninum*

Strobila segments isolated from two separately collected Andean bush dog fecal samples were molecularly characterized by *cox1* sequencing. Maximum likelihood phylogenetic analysis confirmed the species identification as *D. caninum*, placing both specimens at the base of a well-defined group composed exclusively of *D. caninum* representatives (Figure 4). The lineage of the *D. caninum* group consists of two genetically differentiated subgroups corresponding to the previously described canine- and feline-specific genotypes of *D. caninum* (40). Within these, five canine-specific genotype representatives formed a relatively basal, non-monophyletic, statistically unsupported assemblage of specimens, while the feline-specific genotype represented by two specimens (MG587892 and OK523385) formed a relatively derived, well-supported internal lineage. The Andean bush dog isolates are grouped basally within the canine-specific genotype representatives (Figure 4).

4. Discussion

In an increasingly globalized world, anthropogenic factors have intensified the human-wildlife interface, thus increasing the risk of disease spillover, reduction of biodiversity, and food web collapse (41–43). Among carnivores, only 54.3% of the global species' distribution range comprises high-quality habitats due to landscape fragmentation and loss of connectivity (44). Nowadays, surveillance of wildlife-derived infectious diseases is imperative to better understand the impact of disease on populations, eco-epidemiology, and biodiversity conservation (45–47). As already stated, synanthropic wild canids have also been reported as natural reservoirs of novel helminth parasites (48–50).

Parasitological surveys of bush dogs are still limited due to their elusive nature and crepuscular behavior, and thus they remain one of the lesser-known wild canid species. Nonetheless, the causative agent of chronic polycystic human echinococcosis (i.e., *Echinococcus vogeli*) was described for the first time in 1972 in a wild bush dog captured in South America (51, 52). Furthermore, other zoonotic-relevant parasites such as *Toxocara canis*, *Lagochilascaris* sp., and *Spirometra* sp. have been reported in wild bush dogs along with the occurrences of *Spirocerca lupi*, *Ancylostoma caninum*, *Taenia* sp., and the apicomplexan *Cystoisospora caninum* (53, 54). Additionally, parasites

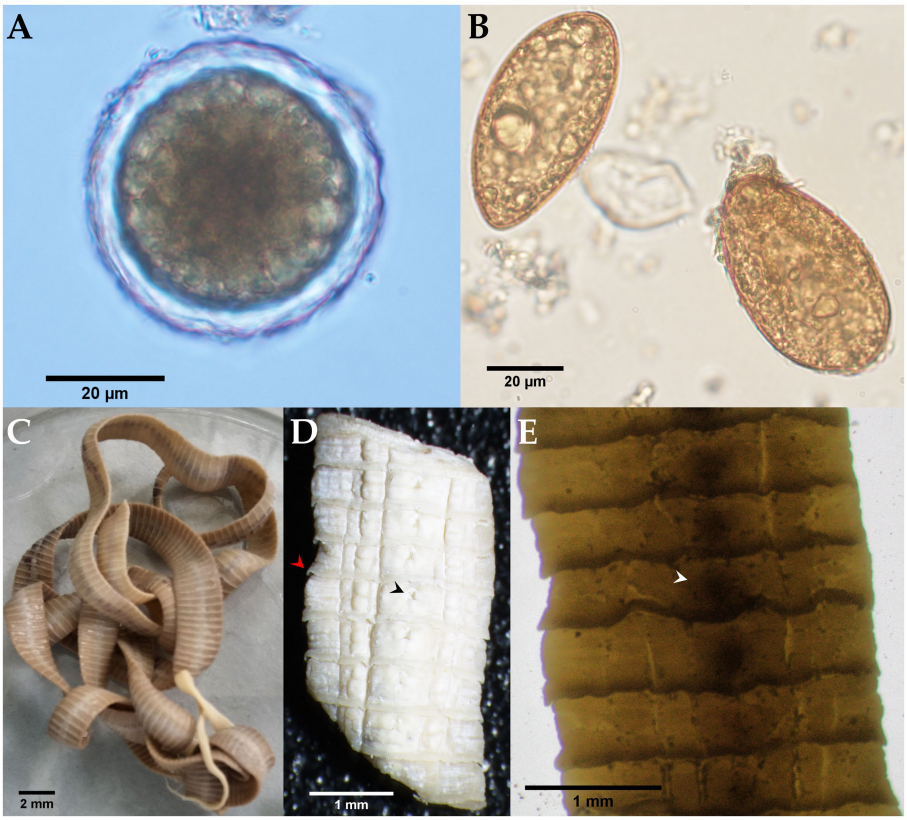

FIGURE 3
Microscopic and stereomicroscopic morphological examination of parasite stages found in the feces of a free-ranging crab-eating fox (*Cerdocyon thous*). **(A)** Non-embryonated egg of *Lagochilascaris cf. minor* (51.21 × 51.57 µm) with an evident 5.79 µm thick eggshell. **(B)** Yellowish-brown, cone-shaped operculated eggs of *Spirometra mansoni* (61.67 × 34.97 µm). **(C)** Adult *S. mansoni* (from the Andes) with a spoon-shaped scolex and characteristic pink color due to the presence of host vitamin B12. **(D)** Close-up photograph of serrated gravid proglottids (red arrowhead); a genital pore is indicated by the black arrowhead. **(E)** Whole-mounted strobilus segment showing the centrally located spiralled uterus (white arrowhead).

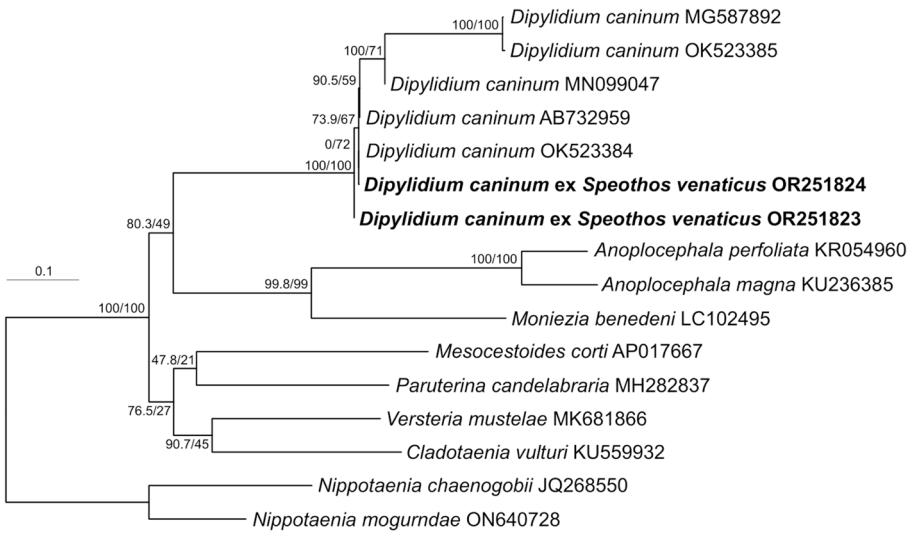

FIGURE 4
Phylogenetic position of *Dipylidium caninum* isolates obtained from two Amazonian bush dog hosts. Maximum likelihood tree from IQ-TREE based on nearly complete (1,563 bp) *cox1* gene sequences analyzed as a single partition using the TIM + F + R5 model. Nodal values show SH-like approximated likelihood ratio test values (10,000 replicates) and standard nonparametric bootstrap supports (1,000 repetitions). Newly characterized specimens are shown in bold. GenBank accessions are shown after taxon names. The branch length scale bar indicates the number of substitutions per site.

such as *Dioctophyme renale*, *Dirofilaria immitis*, *Neospora caninum*, *Rangelia vitalii*, the cyst-forming coccidia *Hammondia heydorni,* and the zoonotic parasites *Angiostrongylus cantonensis*, *Dipylidium caninum*, *Leishmania infantum* (syn. *L. chagasi*), *Toxoplasma gondii*, and various important ticks have been reported for the crab-eating fox (55–63). Thus, both NWC species may contribute to the environmental maintenance and transmission of human and domestic animal parasitoses. Given the lack of information regarding the occurrence and distribution of helminthiases of public health concern, such as dipylidiasis, lagochilascariosis, and sparganosis, the results presented here collectively provide new insights into the potential of NWC in the emergence and transmission of zoonotic infectious diseases. Here, we successfully perform the identification of neglected zoonotic helminths harbored by bush dogs and crab-eating foxes.

The worldwide occurrence of *D. caninum* s.l. in domestic dogs and cats is well documented, as is human dipylidiasis, particularly in young children (64–68). Humans become infected by accidental ingestion of *D. caninum*-cysticercoid-carrying intermediate hosts (IH), most commonly fleas or chewing lice (69, 70). Based on molecular techniques that allow for the differentiation of cryptic species and hidden genetic lineages, two genetically distinct lineages, i.e., the so-called *D. caninum* canine and *D. caninum* feline genotypes, have been proposed within the genus (40, 71). In contrast to human dipylidiasis, *D. caninum* infections rarely produce clinical manifestations in canids or felids. Nonetheless, animals that frequently carry *D. caninum*-infected IH contribute to human parasite transmission (72, 73). Wild carnivores such as dingoes (*Canis dingo*), golden jackals (*Canis aureus*), jaguars (*Panthera onca*), red foxes (*Vulpes vulpes*), and spotted hyenas (*Crocuta crocuta*) are wild natural reservoir hosts and thus essential for the maintenance of the parasite life cycle (72, 74–77). *Dipylidium caninum* infections in crab-eating fox populations have been suggested as a possible consequence of anthropogenic expansion into the natural habitats of wild hosts (60). A study in the rural high-mountain region of Colombia reported the occurrence of *D. caninum* with an estimated prevalence of 20% (SD ± 8.7%) in free-roaming and peri-domestic dog populations (78). In Colombia, the parasite has only previously been reported in humans and domestic hosts (79). Therefore, to the best of our knowledge, the results presented here expand the geographic distribution range of wildlife dipylidiasis to the Pan-Amazonian and northern Andean regions, providing the first host record for bush dogs. Additionally, we establish here that the analyzed cestode proglottids from bush dogs correspond to the *D. caninum* canine genotype, which occurs at a higher frequency in canids, has a shorter pre-patency, and has a longer life span than the *D. caninum* feline genotype (40).

The detection of the ascarid nematode genus *Lagochilascaris* raises public health concerns since human lagochilascariosis, mainly due to *L. minor,* is still an extremely neglected zoonotic disease of the Neotropics. The definitive hosts are carnivores (i.e., canids and felids) carrying intestinal adults that shed highly resistant ascarid-like eggs with a thick and rough eggshell (37, 80). Humans acquire lagochilascariosis through the ingestion of infected rodent IH (e.g., agoutis, mice, rats) containing third-stage larvae (L3), but there is also evidence that humans might become infected after ingestion of embryonated eggs of *Lagochilascaris* (81). So far, more than 100 human cases of lagochilascariosis have been recorded in the Americas (80). Three cases of human lagochilascariosis have been documented in the Caribbean/Pacific, and Amazonian regions of Colombia (82, 83). Regardless, the present study constitutes the first non-human report of this parasite in Colombia. Because the amount of DNA obtained from the isolated *Lagochilascaris cf. minor* eggs was extremely low and showed partial degradation, subsequent phylogenetic analysis was not feasible. Nevertheless, surveillance for human lagochilascariosis by local public health authorities should be recommended.

Globally distributed sparganosis is a neglected food- and waterborne zoonotic disease caused by infection with cestodes of the genus *Spirometra* (Diphyllobothriidea), which is frequently reported in numerous wildlife species (14, 18, 39, 84). Sparganosis manifests as muscular and subcutaneous larvae (spargana), but brain invasion has also been reported (85). The obligate heteroxenous parasite life cycle involves carnivores, where intestinal adults shed eggs that are subsequently released into the environment with feces. In the aqueous environment, the eggs hatch into coracidia, which are ingested by copepods as the first IH in which a procercoid larva develops. These larvae are infective to the second tetrapod IH (e.g., frogs, snakes, and birds), where maturation into plerocercoid larvae takes place (86). Humans become infected by eating raw IH flesh, using it in traditional poultices, or drinking water containing infected copepods (87). In South America, a total of 16 human cases of sparganosis have been reported, one of them in Colombia (87, 88). The cestode specimen of the crab-eating fox morphologically described here was molecularly identified as *Spirometra mansoni*, the first report of the species in South America (39). As sparganosis remains one of the least studied diseases, the morphological data presented here support the findings of Brabec et al. in 2022 (39), which effectively enlarged the distribution range of *S. mansoni* for the Neotropics, and call for further investigation of human sparganosis.

Here, we have provided evidence for important zoonotic helminth infections in highly divergent free-ranging NWC species. Consequently, investigation of the potential role of the elusive semiaquatic bush dog, the synanthropic/peri-domestic crab-eating fox, and other poorly studied NWCs in the transmission cycle of these parasites to humans seems essential. Additionally, future ectoparasite research on different lice and flea taxa (e.g., *Felicola subrostratus*, *Trichodectes canis*, Archaeopsyllinae, and Pulicinae subfamilies) infesting wild carnivore populations is urgently needed to identify the IH harboring *D. caninum* cysticercoids and thus enabling zoonotic transmission of dipylidiasis. The consequences of coinfections on epidemiology and host fitness require better knowledge of NWC-associated infectious agents to understand their role in the emergence of dipylidiasis, lagochilascariosis, and sparganosis (89). Based on these findings, we encourage further parasitologic investigations to be conducted among NWCs, specifically regarding their endo- and ectoparasites. In conclusion, an ongoing parasitological survey of wildlife is critical for implementing public health strategies to avoid zoonotic spillover in a pathogen-related surveillance network.

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/nuccore/OR251824, OR251824; and https://www.ncbi.nlm.nih.gov/nuccore/OR251823, OR251823.

Ethics statement

The animal study was approved by the Ethics Committee for Animal Experimentation (CEEA) of the Universidad de Antioquia, Colombia (AS No. 132) under collection permit No. 0524 of 2014 (IDB0321), procedures were conducted according to the Guidelines of the American Society of Mammologists for the use of wild mammals in research and education, and the EU Directive 2010/63/EU. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MU: conceptualization, investigation, writing-original draft preparation. MU and JB: methodology, software, and visualization. JB, JC-G, and CH: validation. JB and MU: formal analysis. CH and JC-G: resources and funding acquisition. JB, CH, and JC-G: data curation and writing – review and editing. JC-G and CH: supervision. All authors have read and agreed to the published version of 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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1235182/full#supplementary-material

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Analysis of complete mitogenomes and phylogenetic relationships of *Frontopsylla spadix* and *Neopsylla specialis*

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Fleas represent a group of paramount medical significance, subsisting on blood and acting as vectors for an array of naturally occurring diseases. These pathogens constitute essential elements within the plague biome, exerting deleterious effects on both human and livestock health. In this study, we successfully assembled and sequenced the whole mitochondrial genome of *Frontopsylla spadix* and *Neopsylla specialis* using long-range PCR and next-generation sequencing technologies. The mitogenomes of *F. spadix* and *N. specialis* both have 37 genes with full lengths of 15,085 bp and 16,820 bp, respectively. The topology of the phylogenetic tree elucidates that species *F. spadix* is clustered in a branch alongside other members of the family Leptopsyllidae, whereas species *N. specialis* is a sister taxon to *Dorcadia ioffi* and *Hystrichopsylla weida qinlingensis*. It also suggests that Pulicidae form a monophyletic clade, Ctenopthalmidae, Hystrichopsyllidae, Vermipsyllidae form a sister group to Ceratophyllidae/Leptopsyllidae group. The mitochondrial genomes of *F. spadix* and *N. specialis* were sequenced for the first time, which will contribute to a more comprehensive phylogenetic analysis of the Siphonaptera order. The foundation for subsequent systematic studies, and molecular biology of fleas was established.

KEYWORDS

***Frontopsylla spadix*, *Neopsylla specialis*, flea, mitochondrial genome, phylogenetic**

Introduction

Fleas (Order Siphonaptera) are small, wingless insects with laterally compressed bodies undergo holometaboliam and parasitize mammals and birds (1, 2). Over, 2500 species of fleas have been identified, with approximately 200 species capable of harboring epidemic bacteria (3, 4). These serve as vectors for a range of pathogens, including Rickettsia, Bartonella, bubonic plague, and Tularemia, thereby functioning as both disease-causing vectors and reservoir hosts (5, 6). As a result of changes in the environment and human behavior, the vector-host ecology has changed, increasing human exposure to flea vectors and the pathogens they transmit, and flea-borne diseases may re-emerge as epidemics (7). Fleas are an early warning indicator of the plague epidemic, which has important significance in medicine and veterinary medicine (8). Fleas and flea-borne diseases are increasingly threatening human and animal health and causing serious economic losses, so flea identification is of great practical importance for flea-borne disease prevention and control.

Both *Frontopsylla spadix* and *Neopsylla specialis* are found in the Siphonaptera order, belonging to the families Leptopsyllidae and Ctenophthalmidae, to the Amphipsyllinae and Neopsyllinae, and *Frontopsylla* and *Neopsylla*, respectively. Species *F. spadix* are parasitic on wild rodents, specifically *Apodemus chevrieri* and *Rattus flavipectus*, found in regions such as Yunnan, Gansu, and Tibet in China, and extending into Nepal. This geographic distribution is considered a conduit for the transference of plague from wild to domestic rodents (9). Species *N. specialis*, parasitizing mammals like *Apodemus chevrieri*, *Apodemus draco*, and *Apodemus latronum*, is in China and acts as a principal vector of the plague in Yunnan's natural foci, akin to species *F. spadix* (10). Morphological characteristics of *F. spadix* and *N. specialis* have been described previously, the identification resolution of traditional flea classification methods is low, which may have certain limitations (2).

Mitochondria are placed in cells that produce energy and have a separate set of genetic material called mitochondrial DNA (11). Mitochondrial DNA is one of the most commonly used molecular markers in systematics and is widely used in phylogenetic studies of different organisms because of its simple structure, maternal inheritance, and rapid evolutionary rate (12). The analysis of mitochondrial genome structure and sequence is helpful to clarify the classification, genetic evolution, and phylogenetic relationship of fleas more clearly (13). However, at present, the mitochondrial genome data of fleas is very limited, resulting in a huge obstacle to fleas and flea-borne diseases. Therefore, we need to continuously increase and improve the flea mitochondrial gene database to lay the foundation for flea taxonomy, population genetics, and phylogeny.

In this study, we provide the first complete description of the mitochondrial genomes of *F. spadix* and *N. specialis*, analyze mitogenome structures to address the lack of mitochondrial gene resources in fleas, and construct the phylogenetic relationships of known mitochondrial genomes in the order Siphonaptera, while providing molecular information for flea prevention and control.

Materials and methods

Sample collection and DNA extraction

Adult specimens of *F. spadix* (one female and one male) were collected in July 2020 from Luoping Mountain, eryuan City, Dali Bai Autonomous Prefecture, Yunnan Province, China (26°07′N, 99°85′E). Three females and one male adult specimens of *N. specialis* were found in June 2022 from Laojun Mountain, Lijiang City, Yunnan Province of China (26°53′N, 99°58′E). Species identification was conducted based on morphological characteristics with *F. spadix* and *N. specialis* samples extracted from an adult female using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) following the manufacturer's instructions.

PCR amplification

The study design included the development of two sets of overlapping long fragment PCR primers to amplify the mitochondrial genomes of *F. spadix* and *N. specialis*. This was conducted using *cox1* and *12S rRNA* genes of *Ctenophthalmus quadratus* (OQ023577) and *Leptopsylla segnis* (OQ023576), with primer design achieved through Primer 5.0 software, as delineated in Table 1. The PCR was performed in the 50 μl system, including 10 μl 5× PrimerSTAR GXL Buffer (Takara, Japan), 4 μl of each primer, 4 μl of dNTPs, 1 μl of PrimerSTAR GXL DNA Polymerase (Takara, Japan), 4 μl of DNA template and 23 μl of ddH_2O under the following reaction conditions: 92°C for 2 min for initial denaturation, followed by 35 cycles of denaturation at 92°C for 10 s, annealing at 68°C for 30 s and extension at 68°C for 10 min and the final 68°C extension time of 10 min. PCR amplification products were detected by electrophoresis on 1% agarose gels, purified, and sequenced by Sangon Biotech Company (Shanghai, China).

TABLE 1 PCR primers for sequencing the mitogenome of *F. spadix* and *N. specialis*.

Primes	Sequence (5′-3′)
FS1F	ATAGGAGCAGTATTCGCAATTATAGCC
FS1R	ACTATCAGGATAATCAGAGTAACGTCG
FS2F	CGTGGATTATCGATTACAGAACAGG
FS2R	GCAGCTGCGGTTATACAATTAA
NS1F	TGATTAGCAACTCTACACGGAAGAA
NS1R	AATGGAAATCAGTGAACGAATCCTG
NS2F	CCTTCCGGTACACCTACTTTGTTA
NS2R	CAAGGTGCAGTTAATGGTTTAGTAG

TABLE 2 Mitochondrial genome sequence information used in this paper.

Species	Family	Length (bp)	Accession number
Ceratophyllus anisus	Ceratophyllidae	15,875	OQ366407.1
Ceratophyllus wui	Ceratophyllidae	18,081	NC040301.1
Paradoxopsyllus castodis	Leptopsyllidae	15,375	OQ627398.1
Jellisonia amadoi	Ceratophyllidae	17,031	NC022710.1
Leptopsylla segnis	Leptopsyllidae	15,785	OQ023576.1
Frontopsylla spadix	Leptopsyllidae	15,085	OQ366408.1
Neopsylla specialis	Ctenophthalmidae	16,820	OQ366409.1
Hystrichopsylla weida qinlingensis	Hystrichopsyllidae	17,173	NC042380.1
Dorcadia ioffi	Vermipsyllidae	16,785	NC036066.1
Pulex irritans	Pulicidae	20,337	NC063709.1
Xenopsylla cheopis	Pulicidae	18,902	MW310242.1
Ctenocephalides canis	Pulicidae	15,609	ON109770.1
Ctenocephalides orienties	Pulicidae	22,189	NC073009.1
Ctenocephalides felis	Pulicidae	15,418	MK941844.1
Ctenocephalides felis	Pulicidae	20,873	MT594468.1
Casmara patrona	Oecophoridae	15,393	NC053695.1

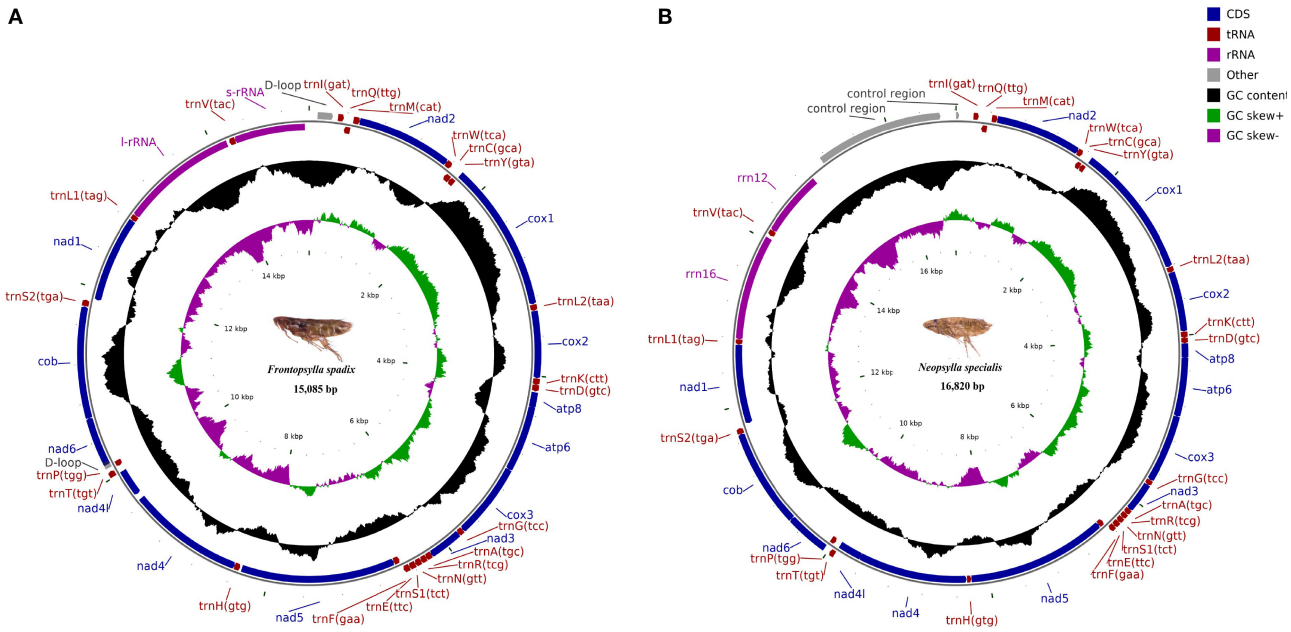

FIGURE 1
Map of the mitochondrial genome of *Frontopsylla spadix* **(A)** and *Neopsylla specialis* **(B)**. Genes in the inner circle plot are located on the reverse strand, and the rest of the genes are located on the forward strand.

Gene annotation

Sequencing employed next-generation sequencing technology (NGS) on the Illumina NovaSeq platform. Annotations were facilitated via the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py), with the A5-miseq v20150522 program utilized for the construction of the complete mitochondrial genome (14, 15). Alignment with closely related species in the NCBI database were performed to ascertain the location of protein-coding, tRNA, and rRNA genes. Predictive analyses of tRNA genes secondary structure were conducted on the tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) online platform (16), and the CGView Server (https://paulstothard.github.io/cgview/) was employed for mapping mitochondrial genome circles. Analytical tools included DNAStar V7.1 for nucleotide composition analysis and CodonW 1.4.2 the relative synonymous codon usage (RSCU) computation.

Phylogenetic analysis

The 13 protein-coding gene sequences from 15 flea species were independently aligned using MUSCLE nucleotide mode, and datasets were manually concatenated. Positions containing gaps and incomplete data were excluded through Bioedit v7.0.5.3 software. Phylogenetic relationships were analyzed using *Casmara patrona* as an outgroup (Table 2), and trees were constructed with MEGA 7.0 software and Mrbayes v.3.2.7 software. The ML tree was formulated using GTR+G+I as the optimal model based on the Akaike Information Criterion (AIC) (17), employing the maximum likelihood method across 1,000 bootstrap datasets. The BI tree underwent 10,000,000 generations, sampled every 1,000 generations. The evolutionary relationships among flea species were visually depicted using the software Figtree v1.4.2.

Results

Organization of mitochondrial genome

The mitochondrial genomes of *F. spadix* and *N. specialis*, which are typically closed double-stranded molecular structures, were uploaded to Genbank in TBL format and obtained accession numbers OQ366408 and OQ366409, respectively. The length of the mitochondrial genome was 15,085 bp and 16,820 bp, respectively (Figure 1), with differences in length mainly determined by the length of the control region. Most of the genes including 14 tRNAs and 9 PCGs are distributed on the positive strand, the same as the other fleas (18, 19). Both intergenic regions and overlapping domains are present within the mitochondrial genome (Table 3). The mitogenomes of *F. spadix* and *N. specialis* had a significant AT preference with AT content of 78.83% and 77.27%, respectively, and the base content was 37.99% (38.64%) A, 40.84% (38.63%) T, 12.85 (14.22%) C, and 8.31 (8.51%) G (Table 4).

Protein-coding genes

The PCGs of *F. spadix* and *N. specialis* were 11,144 bp and 11,142 bp long, accounting for 73.87% and 66.24% of the complete mitochondrial genome length, respectively. Of the 13 protein-coding genes (PCGs) of *F. spadix*, which encode a total of 3713 codons, the initiation codon is the standard codon ATN, with TAA

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TABLE 3 Summary of the mitogenome of *F. spadix* and *N. specialis*.

Gene	Strand	Position	Size(bp)	Initiation codon	Stop codon	Anticodon	Intergenic nucleotide
D-loop	N	0/1-36	0/36				303/35
trnI	N	304-366/205-267	63/63			GAT	16/42
trnQ	J	451-383/378-310	69/69			TTG	17/43
trnM	N	469-536/422-487	68/66			CAT	
nad2	N	537-1547/488-1498	1011/1011	ATT/ATT	TAA/ TAA		−2/−2
trnW	N	1546-1610/1497-1560	65/64			TCA	−1/−8
trnC	J	1676-1610/1613-1553	67/61			GCA	
trnY	J	1739-1677/1676-1614	63/63			GTA	−3/−3
cox1	N	1737-3272/1674-3209	1536/1536	ATC/ATC	TAA/ TAA		4/4
trnL2	N	3277-3340/3214-3277	64/64			TAA	1/1
cox2	N	3342-4022/3279-3959	681/681	ATG/ATG	TAA/ TAG		2/2
trnK	N	4025-4094/3962-4031	70/70			CTT	−1/−1
trnD	N	4094-4159/4031-4093	66/63			GTC	9/0
atp8	N	4169-4330/4094-4261	162/168	ATA/ATT	TAA/ TAA		−7/−7
atp6	N	4324-4998/4255-4926	675/672	ATG/ATG	TAA/ TAA		−1/−1
cox3	N	4998-5780/4926-5708	783/783	ATG/ATG	TAA/ TAA		
trnG	N	5781-5842/5709-5770	63/62			TCC	
nad3	N	5843-6193/5771-6121	351/351	ATT/ATT	TAG/ TAG		−2/−2
trnA	N	6192-6256/6120-6182	65/63			TGC	−2/0
trnR	N	6255-6318/6183-6245	64/63			TCG	
trnN	N	6319-6383/6246-6309	65/64			GTT	
trnS1	N	6384-6452/6310-6378	69/69			TCT	
trnE	N	6453-6518/6379-6442	66/64			TTC	−2/−2
trnF	J	6581-6517/6504-6441	65/64			GAA	0/−1
nad5	J	8315-6582/8221-6504	1734/1718	ATG/ATG	TAA/TA		1/1
trnH	J	8381-8317/8276-8223	65/54			GTG	−1/−5
nad4	J	9717-8381/9623-8282	1337/1342	ATG/ATG	TTA/T		−7/−7
nad4l	J	10004-9711/9910-9617	294/294	ATG/ATG	TAA/ TAA		2/2
trnT	N	10007-10071/9913-9977	65/65			TGT	
trnP	J	10134-10072/10040-9978	63/63			TGG	11/17
nad6	N	10146-10652/10049-10558	507/510	ATA/ATT	TAA/ TAA		−1/−1
cob	N	10652-11791/10558-11691	1140/1134	ATG/ATG	TAA/ TAA		3/2
trnS2	N	11795-11860/11693-11757	66/65			TGA	20/18
nad1	J	12813-11881/12717-11776	933/942	ATG/ATG	TAA/ TAA		1/1
trnL1	J	12876-12815/12780-12719	62/62			TAG	
rrnL	J	14157-12877/14042-12781	1281/1262				28/33
trnV	J	14252-14186/14142-14076	67/67			TAC	−1/−1
rrnS	J	15037-14252/14932-14142	786/791				47/206
D-loop	N	0/15139-16638	0/1500				0/181

TABLE 4 Composition and skewness of *F. spadix* and *N. specialis* mitogenome.

Region	A%	C%	G%	T%	A+T%	G+C%	AT Skew	GC Skew
Whole genome	37.99/38.64	12.85/14.22	8.31/8.51	40.84/38.63	78.83/77.27	21.16/22.73	−0.036/0.001	−0.215/−0.251
nad2	35.41/34.32	10.68/13.45	7.12/7.81	46.79/44.41	82.20/78.73	17.80/21.27	−0.138/−0.128	−0.200/−0.265
cox1	29.43/27.67	15.76/17.77	14.13/14.78	40.69/39.78	70.12/67.45	29.88/32.55	−0.161/−0.180	−0.055/−0.092
cox2	35.24/33.04	13.51/17.18	9.99/11.01	41.26/38.77	76.51/71.81	23.49/28.19	−0.079/−0.080	−0.150/−0.219
atp8	42.59/41.07	5.56/10.12	3.09/5.36	48.77/43.45	91.36/84.52	8.64/15.48	−0.068/−0.028	−0.286/−0.307
atp6	33.04/32.14	13.33/16.07	9.19/10.12	44.44/41.67	77.48/73.81	22.52/26.19	−0.147/−0.129	−0.184/−0.227
cox3	30.65/29.76	14.69/16.09	12.52/13.41	42.15/40.74	72.80/70.50	27.20/29.50	−0.158/−0.156	−0.080/−0.091
nad3	29.06/29.63	13.68/15.67	7.69/8.26	49.57/46.44	78.63/76.07	21.37/23.93	−0.261/−0.221	−0.280/−0.310
nad5	36.85/33.41	7.38/7.63	12.40/14.73	43.37/44.24	80.22/77.65	19.78/22.35	−0.081/−0.139	0.254/0.318
nad4	34.93/31.22	7.18/8.20	13.31/14.98	44.50/45.60	79.43/76.83	20.49/23.17	−0.120/−0.187	0.299/0.293
nad4l	38.78/31.97	3.06/5.44	12.24/12.93	45.92/49.66	84.69/81.63	15.31/18.37	−0.084/−0.217	0.600/0.408
nad6	34.91/37.45	10.06/11.37	5.52/6.86	49.51/44.31	84.42/81.76	15.58/18.24	−0.173/−0.084	−0.291/−0.248
cob	31.67/30.16	16.14/17.02	10.88/11.02	41.32/41.80	72.98/71.96	27.02/28.04	−0.132/−0.162	−0.195/−0.214
nad1	31.94/30.89	7.18/7.43	15.11/15.82	45.77/45.86	77.71/76.75	22.29/23.25	−0.180/−0.195	0.356/0.361
rrnl	43.17/39.14	5.31/6.26	11.71/13.31	39.81/41.28	82.98/80.43	17.02/19.57	0.040/−0.027	0.376/0.360
rrns	40.84/39.32	6.62/6.95	11.96/14.03	40.59/39.70	81.42/79.01	18.58/20.99	0.003/−0.005	0.287/0.337
trnI	39.68/38.10	7.94/7.94	12.70/12.70	39.68/41.27	79.37/79.37	20.63/20.63	0/−0.040	0.231/0.231
trnQ	40.58/37.68	4.35/4.35	11.59/13.04	43.48/44.93	84.06/82.61	15.94/17.39	−0.034/−0.088	0.454/0.500
trnM	38.24/36.36	19.12/19.70	10.29/10.61	32.35/33.33	70.59/69.70	29.41/30.30	0.083/0.239	−0.300/−0.300
trnW	44.62/42.19	12.31/10.94	7.69/9.38	35.38/37.50	80.00/79.69	20.00/20.31	0.116/0.059	−0.231/−0.077
trnC	49.25/39.34	5.97/9.84	10.45/16.39	34.33/34.33	83.58/73.77	16.42/26.23	0.179/0.067	0.277/0.250
trnY	39.68/41.27	9.52/6.35	15.87/15.87	34.92/36.51	74.60/77.78	25.40/22.22	0.064/0.061	0.250/0.428
trnL2	29.69/35.94	15.62/15.62	14.06/14.06	40.62/34.38	70.31/70.31	29.69/29.69	−0.155/0.022	−0.053/−0.053
trnK	34.29/35.71	15.71/15.71	15.71/15.71	34.29/32.86	68.58/68.58	31.42/31.42	0/0.042	0/0
trnD	46.97/41.27	6.06/9.52	7.58/11.11	39.39/38.10	86.36/79.37	13.64/20.63	0.088/0.040	0.111/0.077
trnG	40.32/41.94	8.06/9.68	9.68/9.68	41.94/38.71	82.26/80.65	17.74/19.35	−0.020/0.040	0.091/0
trnA	38.46/42.86	7.69/6.35	9.23/11.11	44.62/39.68	83.08/82.54	16.92/17.46	−0.074/0.039	0.091/0.273
trnR	39.06/39.68	14.06/11.11	9.38/9.52	37.50/39.68	76.56/79.37	23.44/20.63	0.001/0	−0.200/−0.077
trnN	44.62/48.44	7.69/10.94	9.23/10.94	38.46/29.69	83.08/78.12	16.92/21.88	0.074/0.240	0.091/0
trnS1	39.13/39.13	10.14/10.14	10.14/10.14	40.58/40.58	79.71/79.71	20.29/20.29	−0.018/−0.018	0/0
trnE	42.42/42.19	6.06/6.25	4.55/4.69	46.97/46.88	89.39/89.06	10.61/10.94	−0.051/−0.053	−0.142/−0.143
trnF	40.00/34.38	7.69/9.38	15.38/15.62	36.92/40.62	76.92/75.00	23.08/25.00	0.040/−0.083	0.333/0.250
trnH	40.00/35.19	3.08/3.70	13.85/18.52	43.08/42.59	83.08/77.78	16.92/22.22	−0.037/−0.095	0.637/0.667
trnT	40.00/40.00	7.69/7.69	9.23/9.23	43.08/43.08	83.08/83.08	16.92/16.92	−0.037/−0.037	0.091/0.091
trnP	39.68/38.10	4.76/4.76	14.29/15.87	41.27/41.27	80.95/79.37	19.05/20.63	−0.020/−0.040	0.500/0.539
trnS2	42.42/43.08	6.06/6.15	12.12/12.31	39.39/38.46	81.82/81.54	18.18/18.46	0.037/0.057	0.333/0.334
trnL1	40.32/38.71	6.45/6.45	12.90/12.90	40.32/41.94	80.65/80.65	19.35/19.35	0/−0.040	0.333/0.333
trnV	43.28/44.78	7.46/7.46	5.97/5.97	43.28/41.79	86.57/86.57	13.43/13.43	0/0.035	−0.111/−0.111
OH	0/45.31	0/9.38	0/3.78	0/41.54	0/86.85	0/13.15	0/0.043	0/−0.426

as the termination codon except for *NAD3* (TAG). *N. specialis* encodes a total of 3714 codons, with incomplete termination codons occurring in *NAD5* and *NAD4*, and *NAD3* with TAG as a stop codon. Leucine is the dominant amino acid and cysteine is the rarest amino acid (Figure 2). The mitochondrial genomes of *F. spadix* and *N. specialis* are mostly nonpolar amino acid groups with 1851 (49.85%) and 1933 (52.05%), respectively, and the remaining polar, basic, and acidic amino acid groups are 1209 (32.51%) and 1206 (32.47%), 235 (6.33%) and 230 (6.19%), 194 (5.22%) and 157 (4.23%).

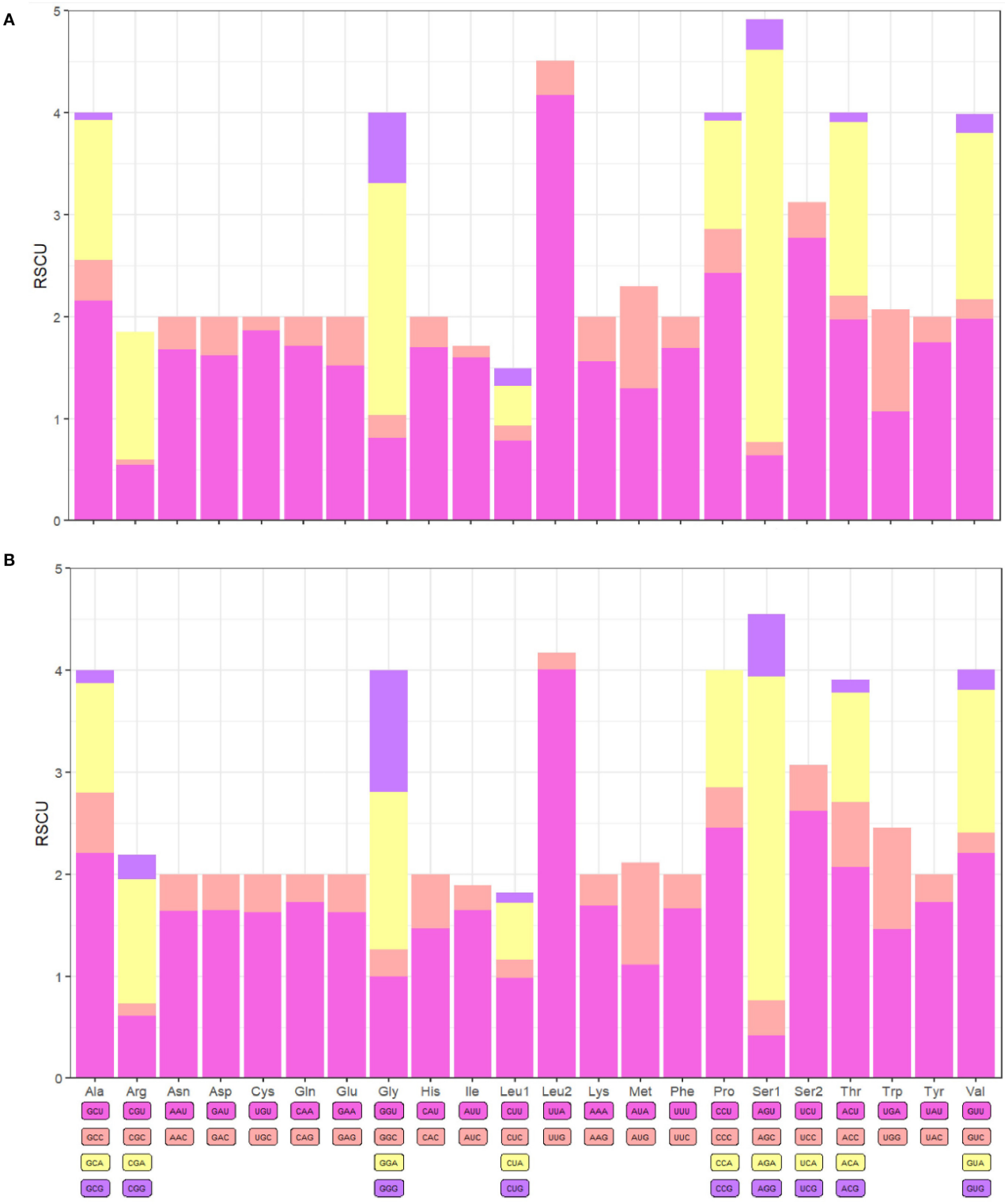

FIGURE 2
F. spadix **(A)** and *N. specialis* **(B)** protein-coding gene relative synonymous codon usage (RSCU).

Transfer RNA genes and ribosomal RNA genes

The mitogenomes of *F. spadix* and *N. specialis* have 14 tRNAs located in the positive strand with full lengths of 1439 bp and 1408 bp, respectively. Among the 22 tRNA genes of *F. spadix*, the length of tRNA genes ranged from 62 bp (trnL1) to 70 bp (trnK), with the shortest amino acid of 61 bp (trnC) in *N. specialis*. The mitochondrial genome was conjured according to the special genetic code so that all the 22 tRNA genes could be identified. G-U oscillating bases appear as a common mismatch in most tRNA genes to maintain tRNA secondary structure (20). The relationship between base mismatch and evolution needs further consideration. With a length of 7 bp, which is typical of arthropods, *ATP8* and *ATP6* overlap (21). The *16S rRNA* and *12S rRNA* of *F. spadix* and *N. specialis* are both located in the reverse strand, separated by *Valine*, with AT contents of 82.98% (80.43%) and 81.42% (79.01%), respectively (Table 4).

Phylogenetic analysis

We harnessed available flea genomic data from the NCBI database pertaining to fleas and amalgamated this with our successfully sequenced *F. spadix* and *N. specialis*, thus facilitating a refined exploration of the topology of flea phylogenetic relationships. Utilizing the maximum likelihood method, we constructed a phylogenetic tree anchored on the concatenated nucleotide sequences of 13 PCG genes, thereby providing an insightful perspective into flea evolutionary trajectories. The ML and BI trees show identical topologies. According to ML and BI analysis, the families Ctenopthalmidae, Hystrichopsyllidae, Vermipsyllidae, and Pulicidae form a monophyletic clade, while the family Ceratophyllidae and Leptopsyllidae are paraphyletic. A principal clade encompasses species of the family Pulicidae, crystallizing into a definitive monophyletic clade. In juxtaposition, Ctenopthalmidae, Hystrichopsyllidae, and Vermipsyllidae form a sister aggregation to the Ceratophyllidae and Leptopsyllidae group. Notably, *F. spadix* and *Leptopsylla segnis* belonging to the Leptopsyllidae family emerge as the most phylogenetically congruent entities, bolstered by robust node support values. *N. specialis* resides solitarily on a branch, constituting a strongly endorsed linkage with *Dorcadia ioffi* and *Hystrichopsylla weida qinlingensis* and fostering sister group affiliations (Figure 3).

Discussion

The endeavor to taxonomically identify and comprehend the ecological proclivities of fleas stands as an integral foundation in the mitigation and management of a plethora of naturally transpiring diseases. Fleas, being a medically salient insect group,

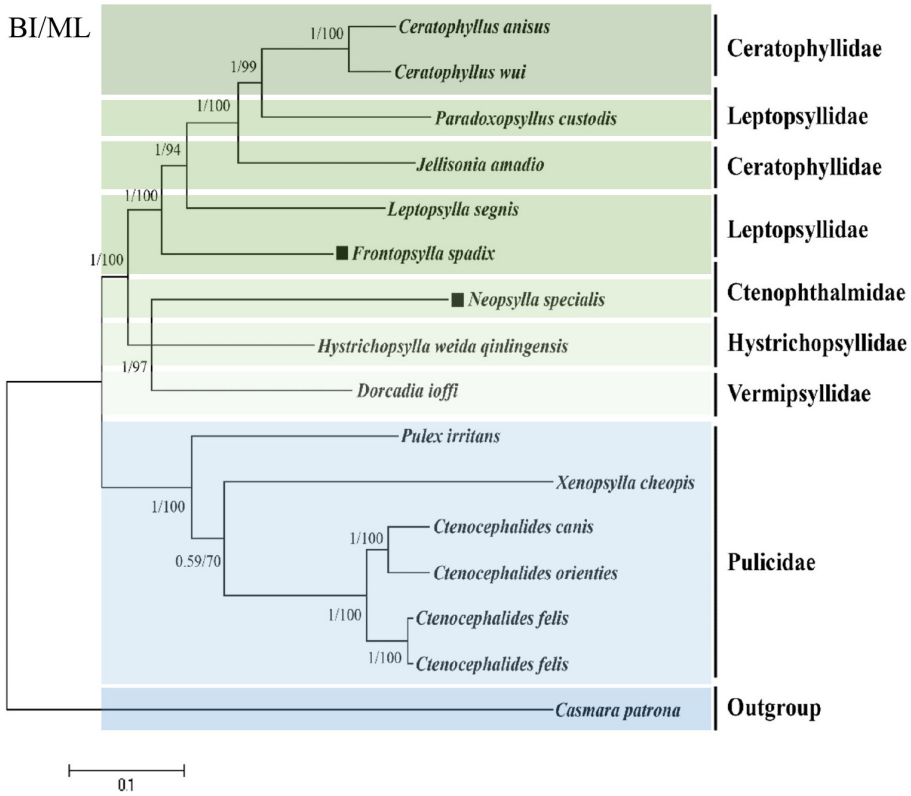

FIGURE 3
Phylogenetic analysis based on the nucleotide sequences of the 13 PCGs in the mitogenome. The number beside the nodes are posterior probabilities (BI) and bootstrap (ML). The black square markers represent the species in this study.

partake substantially in perpetuating major plague epidemics and preserving natural epidemiological origin (22). Their abundance and widespread dissemination render them instrumental in the animal-mediated propagation of diseases during epidemic occurrences. Species *F. spadix* and *N. specialis*, as principal flea vectors, are posited to serve as conduits for the transmission of wild rodent plagues into domestic rats, thereby instigating epidemics.

In an examination of mitogenomes of *F. spadix* and *N. specialis* a pronounced predilection for AT bases was discerned, with an AT content surpassing that of GC, a feature congruent with arthropods (23). An anomalous stop codon was detected in *N. specialis*, subsequently rectified to TAA by PolyA complementation to terminate translation (24). The control region, harboring initiation sites that govern the replication and transcription of the mitochondrial genome, evolves at a rate three to five times that of other regions, accounting for its employment in population genetics and origin evolution studies (25, 26). Variability in the number and location of non-coding regions is evidenced across species, with singular, dual, and triple D-Loops manifesting in different species. Within this study's purview, species *F. spadix* was devoid of D-Loop, while species *N. specialis* harbored two, spanning 1,536 base pairs.

The six families are split into two large clades, as shown by phylogenetic clustering, except the family Pulicidae, where the remaining five families cluster in the other clade, with Paradoxopsyllus custodis located in the Superfamily Ceratophylloidae as a member of the family Leptopsyllidae, which is a discovery that also indicates that expanded sequencing of mitochondrial genomic data is beneficial for more intensive phylogenetic studies of the species. However, individual mitochondrial genes are less informative than the whole mitogenome which may bias the reflection of phylogenetic relationships (27), and in order to make the phylogenetic relationships of the flea more convincing, we need to sequence the whole mitochondrial genome of the flea more frequently (11).

Accurate differentiation and identification of flea species are essential in the diagnosis of disease and basic and applied research on these important ectoparasites. The mitochondrial genome is frequently used in phylogenetic and phylogenetic studies of different ectoparasites at various taxonomic levels due to its matrilineal inheritance, lack of recombination, and rapid rate of evolution. The in-depth analysis of the mitochondrial genomes of *F. spadix* and *N. specialis* augments the data corpus, fortifying further phylogenetic inquiry within the Siphonaptera order. This enhances both the resolution at the family echelon and the informativeness of the phylogenetic tree. The entire mitochondrial genome sequence has also been demonstrated to proffer elevated phylogenetic precision, rendering it an apt molecular marker for elucidating the evolutionary interconnections amongst flea species. Nevertheless, the sequencing of additional flea mitochondrial genomes is requisite to facilitate a more systematized and encompassing analysis of flea evolutionary relationships.

Conclusion

In this study, the mitochondrial genomes of *F. spadix* and *N. specialis* were successfully sequenced based on the combination of long-range PCR technology and next-generation sequencing technology. In both *F. spadix* and *N. specialis*, the mitochondrial genomes are circular with the same genetic composition and arrangement as other fleas, which provides the basis for further understanding of the molecular evolution, and phylogeny of fleas, as well as providing useful molecular markers for studying the taxonomy and systematics of the flea species.

Data availability statement

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

Ethics statement

The animal study was approved the Laboratory Animal Management Committee of Dali University and First Affiliated Hospital of Chengdu Medical College. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL conceived the study and wrote the manuscript. BC, XL, SL, and DJ collected specimens and participated in experimental operations. XW, LY, and RL analyzed the experimental data. QZ, LW, and XY are responsible for the interpretation of experimental data, critical revision of important knowledge content, and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Diversity of *Anaplasma* and novel *Bartonella* species in *Lipoptena fortisetosa* collected from captive Eld's deer in Thailand

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Lipoptena insects are important ectoparasites of cervids and may affect humans that are incidentally bitten. The presence of zoonotic pathogen DNA, such as *Anaplasma*, and *Bartonella*, raises the importance of *Lipoptena* insects in veterinary and human medicine. Eld's deer (*Rucervus eldii thamin*), an endangered wild ruminant in Thailand, are bred and raised in the open zoo. The semi-wild zoo environment suggests ectoparasite infestation and potential risk for mechanical transmission of pathogens to visitors, zoo workers, or other animals. However, epidemiology knowledge of pathogens related to endangered wild ruminants in Thailand is limited. This study aims to determine the prevalence and diversity of *Anaplasma* and *Bartonella* in the *L. fortisetosa* collected from captive Eld's deer in Chon Buri, Thailand. Of the 91 *Lipoptena* DNA samples obtained, 42 (46.15%) and 25 (27.47%) were positive for *Anaplasma* and *Bartonella* by molecular detection, respectively. Further, 42 sequences of *Anaplasma* (4 nucleotide sequence types) showed 100% identity to those detected in other ruminants and blood-sucking ectoparasites. Twenty-five sequences of *Bartonella* (8 nucleotide sequence types) showed 97.35–99.11% identity to the novel *Bartonella* species from sika deer and keds in Japan. Phylogenetic trees revealed *Anaplasma* sequences were grouped with the clusters of *A. bovis* and other ruminant-related *Anaplasma*, while *Bartonella* sequences were clustered with the novel *Bartonella* species lineages C, D, and E, which originated from Japan. Interestingly, a new independent lineage of novel *Bartonella* species was found in obtained specimens. We report the first molecular detection of *Anaplasma* and *Bartonella* on *L. fortisetosa*, which could represent infectious status of captive Eld's deer in the zoo. Wild animals act as reservoirs for many pathogens, thus preventive measures in surrounding areas should be considered to prevent pathogen infection among animals or potential zoonotic infection among humans.

KEYWORDS

prevalence, *Anaplasma*, *Bartonella*, *Lipoptena fortisetosa*, Eld's deer, Thailand

1. Introduction

Deer keds of the genus *Lipoptena* spp. (Diptera: Hippoboscidae) are hematophagous insects that infest mammals (1). The insects become wingless after finding a suitable host and attach to a single host throughout their life span (2–4). Of over 30 species of *Lipoptena* insects worldwide, *L. fortisetosa* along with *L. cervi*, *L. depressa*, and *L. mazamae* are the most prevalent and threaten to wildlife, livestock, and pets (5–8). *Lipoptena fortisetosa* are found on sika deer (*Cervus nippon*), roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*) in many countries and incidentally found on dogs (2, 9–12). In addition, humans can be bitten by *Lipoptena* insects (13). Several molecular epidemiological studies show *L. fortisetosa* harbors DNA of various pathogens, including *Anaplasma phagocytophilum*, *Babesia* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella*-like endosymbionts, *Francisella tularensis*, *Mycoplasma* spp., *Rickettsia* spp., and *Theileria* spp. (14–16).

The genus *Anaplasma* includes intracellular gram-negative bacteria transmitted by ixodid ticks (17). Several *Anaplasma* spp., such as *A. marginale*, *A. centrale*, *A. ovis*, and *A. bovis*, are obligate bacteria parasitizing blood cells of many ruminants, while *A. platys* is mainly a pathogen of dogs (17). *Anaplasma phagocytophilum* is a pathogenic bacterium of a wide range of hosts, including humans and domestic and wild animals (18). In addition, *A. phagocytophilum* has been detected worldwide in wild ruminants and their ectoparasites (19–23). Although the role of wildlife in circulation of *Anaplasma* spp. is yet to be clearly defined, several species of wild ruminants are considered important reservoirs (24). In Thailand, studies found evidence of *A. platys* and *A. bovis* detection in *Dermacentor auratus* ticks collected from sambar deer (*Cervus unicolor*) (25). Because *D. auratus* ticks are found on humans in Thailand (26, 27), humans infected with *Anaplasma* bacteria via infected tick bites in addition to other deer ectoparasites is also possible.

Bartonella spp. are intra-erythrocytic gram-negative bacteria mainly transmitted among hosts by arthropod vectors, such as cat fleas (*Ctenocephalides felis*), body lice (*Pediculus humanus*), and sand flies (*Lutzomyia verrucarum*) (28–30). There are 45 *Bartonella* spp./subspp. Detected or isolated from various animals (31). *Bartonella schoenbuchensis*, *B. capreoli*, and *B. bovis* are detected in wild ruminants in several countries, which are strongly suspected to be transmitted by *Lipoptena* spp. (32–39). In Thailand, the novel *Bartonella* spp. was detected and isolated from captive Rusa deer (*Rusa timorensis*) blood samples (40). Since no ectoparasites are found on these deer, further studies are needed to determine whether ectoparasites transmit *Bartonella* among deer throughout Thailand. For zoonotic issues, human cases of bartonellosis caused by ruminant-related species, *B. schoenbuchensis* and *B. melophagi*, have been previously reported (41, 42). These findings highlight that although *Bartonella* bacterial infection in animals does not result in serious diseases, this wide range of infected animals could be a reservoir for potential zoonotic infection.

Khao Kheow Open Zoo is located within a wildlife sanctuary in Chon Buri province, eastern Thailand. Several endangered wildlife, as well as Eld's deer (*Rucervus eldii thamin*), are bred and raised in the open zoo to increase population numbers. Here, wild animals can freely roam the open zoo and sanctuary areas, increasing the possibility of pathogen transmission among wild animals. Recent evidence shows ruminant-related blood pathogens, including *Anaplasma*, *Babesia*, *Ehrlichia*, and *Theileria*, in various species of ticks in this surrounding environment (43). Moreover, Tiawsirisup et al. (44) reported the presence of *L. fortisetosa* on Eld's deer with *Theileria capreoli* and *T. cervi* in these insects in Thailand. Although DNA presence does not guarantee pathogen transmission, it may highlight the potential risk for mechanical transmission of pathogens to humans and healthy animals via bites of infected ectoparasites. Currently, knowledge surrounding epidemiology of pathogens related to endangered wild ruminants in Thailand is limited. This study aims to determine the prevalence and diversity of *Anaplasma* and *Bartonella* in the ectoparasite collected from captive Eld's deer. Our findings may be used to understand the current status of pathogens among Eld's deer and their ectoparasite, formulate animal welfare policies, and provide valuable information to prevent and control pathogens related to endangered wildlife species in the country.

2. Materials and methods

2.1. Background of *Lipoptena fortisetosa* specimens

From May to November 2021, 91 blood-sucking insects were collected from 12 Eld's deer at the wildlife animal hospital, Khao Kheow Open Zoo. The Eld's deer were admitted to the hospital for various reasons, such as disease diagnosis or regular health examination. Insect sample collection was done by veterinarians and zoo staff during an anesthetized stage of animals. Each specimen was kept in a microcentrifuge tube with RNA stabilization solution and transported to the Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University, for morphological identification by using a taxonomic key (45). All specimens were identified as *L. fortisetosa* (44). In addition, 38 males and 53 females were also defined during morphological identification.

DNA was extracted from each *Lipoptena* specimen using the IndiSpin Pathogen Kit (Indical Bioscience, Germany), according to the manufacturer's instructions. For molecular identification of *Lipoptena* specimens, we examined DNA samples using PCR assay with primers LCO1490 and HCO2198 (46). PCR mixture and condition were described by Tiawsirisup et al. (44) and the product size was 658 bp, which was confirmed using DNA sequencing. Using the nucleotide BLAST tool, all representative and validated sequences showed the closest similarity (94.28–94.45%) to *L. fortisetosa* (OL850869) from China. Moreover, *L. fortisetosa* can be classified into two clades: "clade I" based on sequences already deposited in the GenBank database and "clade II" based on sequences of *Lipoptena* specimens collected in Thailand (44).

2.2. *Anaplasma* and *Bartonella* detection

All DNA samples were used for *Anaplasma* and *Bartonella* detection using the PCR assay. Primers EHR16SD and EHR16SR were used to amplify a 345 bp segment of the 16S rRNA gene of Anaplasmataceae members (47). Primers BhCS781p and BhCS1137n were used to amplify a 380 bp segment of the citrate synthase gene (*gltA*) of *Bartonella* spp. (48). The PCR mixture was performed in a 25 µL reaction volume containing a DNA template, 10x PCR buffer

(KOD One, TOYOBO Co., Ltd., Japan), 10 μM of forward and reverse primers, and sterile distilled water. PCR conditions were adapted by following the manufacturer's instructions for PCR buffer and annealing temperatures were followed according to relevant studies (47, 48). DNA from *A. marginale* and *B. henselae* isolates (positive control) and distilled water (negative control) were used as controls for the PCR assay. The *Anaplasma* and *Bartonella* PCR-positive products from *Lipoptena* specimens were purified using a GenepHlow Gel/PCR cleanup kit (Geneaid Biotech Ltd., Taiwan) and sent for nucleotide sequencing (U2Bio Co., Ltd., South Korea).

2.3. Nucleotide sequence and statistical analyses

Forty-two sequences from *Anaplasma* PCR-positive and 25 sequences from *Bartonella* PCR-positive samples were analyzed for the closest similarity with reference nucleotide sequences in the GenBank database using the NCBI nucleotide BLAST tool. All sequences were validated, aligned, and compared for genetic similarity using MegAlign (DNASTAR, Inc., United States). The number of nucleotide sequence types (ntSTs) of *Anaplasma* and *Bartonella* sequences were analyzed using DnaSP version 6.12.03 (49).

We analyzed the best-fit models for constructing phylogenetic trees using the Find Best DNA/Protein Model in MEGA X. Phylogenetic trees were generated using MEGA X with the maximum likelihood (ML) algorithm on the Kimura 2-parameter model plus gamma distribution (K2 + G) for *Anaplasma* sequences and Tamura-Nei parameter model plus gamma distribution (TN93 + G) for *Bartonella* sequences applied bootstrap method with 1,000 replications. ntST networks were constructed using the Median-joining (MJ) network in PopART version 1.7 (50, 51).

Pathogen infection rates in different genders of *Lipoptena* specimens were calculated and compared using Fisher's exact test and $p < 0.05$ was considered statistically significant (GraphPad Prism 8.4.2 software, CA).

3. Results

3.1. *Anaplasma* and *Bartonella* detected in *Lipoptena* specimens

The PCR results showed that 46.15% (42/91) and 27.47% (25/91) of *L. fortisetosa* harbored *Anaplasma* and *Bartonella* DNA, respectively (Table 1). Based on the collecting date, *Lipoptena* specimens collected in June 2021 showed the highest prevalence of *Anaplasma* infection (66.67%; 24/36), while specimens collected in May 2021 showed the highest prevalence of *Bartonella* infection (39.28%; 11/28) and co-infection (28.57%; 8/28) (Table 1). However, no *Anaplasma* and *Bartonella* DNAs were detected from *Lipoptena* specimens collected in November 2021 (Table 1). *Anaplasma* infection rate in female specimens (47.16%; 25/53) was higher than in males (44.73%, 17/38; $p = 0.8346$). In addition, we also found a higher *Bartonella* infection rate in female specimens (33.96%, 18/53) than in males (18.42%, 7/38; $p = 0.1525$). Of the 91 specimens, 11 (12.08%) were co-infected with *Anaplasma* and *Bartonella* spp. (Table 1).

3.2. Genetic and BLAST analyses of *Anaplasma* and *Bartonella* detected in *Lipoptena* specimens

Among 67 validated sequences in this study, 42 sequences (primer cut; 305 bp) were from *Anaplasma* PCR-positive samples, while the other 25 were from *Bartonella* PCR-positive (primer cut; 337 bp) samples (Table 2). We aligned and compared the validated sequences of each pathogen, then grouped these into nucleotide sequence types (ntSTs) by using DnaSP version 6.12.03 (Table 2). The validated *Anaplasma* sequences were grouped into four ntSTs and representative sequence from each ntST was submitted to the GenBank database, including ntST1 (37 sequences; Acc. No. OQ692407), ntST2 (three sequences; Acc. No. OQ692408), ntST3 (one sequence; Acc. No. OQ692409), and ntST4 (one sequence; Acc. No. OQ692410) (Table 2). Among four ntSTs of the *Anaplasma* sequences, BLAST results showed ntST1 had 100% identity with various ruminant-related *Anaplasma*, including *A. capra* (ON872236) from horse in Iraq, *A. marginale* (OP851751) from cattle in India, *A. ovis* (OM282854) from sheep in Russia, and *Anaplasma* spp. (KY766240) from *Rhipicephalus microplus* tick in Thailand. The ntST2 shared 100% identity with *Anaplasma* spp. (AF497579) from the *Haemaphysalis lagrangei* tick in Thailand and *A. bovis* (OQ132528) from *H. hystricis* tick in China. The ntST3 and 4 shared 100% identity with the *Anaplasma* spp. (MH589424) from a mountain bongo in Kenya and *A. bovis* (KP062954) from a goat in China, respectively (Table 2).

Twenty-five sequences obtained from *Bartonella* PCR-positive samples were grouped into eight ntSTs and representative sequence from each ntST was submitted to the GenBank database, including ntST5 (10 sequences; Acc. No. OQ716819), ntST6 (one sequence; Acc. No. OQ716820), ntST7 (three sequences; Acc. No. OQ716821), ntST8 (six sequences; Acc. No. OQ716822), ntST9 (one sequence; Acc. No. OQ716823), ntST10 (two sequences; Acc. No. OQ716824), ntST11 (one sequence; Acc. No. OQ716825), and ntST12 (one sequence; Acc. No. OQ716826) (Table 2). The BLAST results showed ntST5 and 8 shared the highest similarity to *Bartonella* spp. (LP485116) from deer ked in Japan with 99.11 and 98.52%, respectively. The ntST6 and 10 had the highest similarity to *Bartonella* spp. (CP019781) from sika deer in Japan with 97.65%, while ntST7, 11, and 12 showed the highest similarity to *Bartonella* spp. (LC485115) from deer ked in Japan, with 98.22, 98.52, and 97.63%, respectively. Lastly, ntST9 showed the highest similarity to the uncultured bacterium (JX416234) with 97.35% from a bat fly in the USA (Table 2).

3.3. Phylogenetic analysis of *Anaplasma* and *Bartonella* detected in *Lipoptena* specimens

The phylogenetic tree of *Anaplasma* sequences showed samples in ntST2 and 4 clustered in the same clade as *A. bovis*, while samples in ntST1 and 3 grouped with clade of other ruminant-related *Anaplasma* spp. (Figure 1). Figure 2 shows the ntST network of 16S rRNA gene of *Anaplasma* spp. from a total of 12 ntSTs (68 sequences). ntST1 to ntST4 represented *Anaplasma* sequences obtained in this study. From the ntST network, ntST2 and 4 were classified into *A. bovis* group, which differed by one mutation step from ntST of the *A. bovis* clade (KY766234, MK028574, MH255937,

TABLE 1 Prevalence of *Anaplasma* and *Bartonella* spp. infection in *Lipoptena fortisetosa* detected by PCR.

Variables	Number of samples (*N* = 91)	Prevalence of infections (infected/tested samples)		
		Anaplasma spp.	*Bartonella* spp.	Co-infection (*Anaplasma* + *Bartonella*)
Collecting date				
May 2021	28	57.14% (16/28)	39.28% (11/28)	28.57% (8/28)
June 2021	36	66.67% (24/36)	27.78% (10/36)	8.33% (3/36)
August 2021	16	6.25% (1/16)	25.00% (4/16)	0% (0/0)
September 2021	3	33.33% (1/3)	0% (0/0)	0% (0/0)
November 2021	8	0% (0/0)	0% (0/0)	0% (0/0)
Total	91	46.15% (42/91)	27.47% (25/91)	12.08% (11/91)
Gender				
Male specimens	38	44.73% (17/38)	18.42% (7/38)	7.89% (3/38)
Female specimens	53	47.16% (25/53)	33.96% (18/53)	15.09% (8/53)

TABLE 2 Nucleotide sequence types (ntSTs), NCBI BLAST results, and accession number of the representative nucleotide sequences obtained in this study.

ntSTs	Number of sequences (*N* = 67)	Highest BLAST result		Submitted sequences (Acc. No.)
		Closely related species	% Identity	
***Anaplasma* sequences**				
1	37	*A. capra* (ON872236), *A. marginale* (OP851751), *A. ovis* (OM282854), and *Anaplasma* spp. (KY766240)	100	OQ692407
2	3	*Anaplasma* spp. (AF497579) and *A. bovis* (OQ132528)	100	OQ692408
3	1	*Anaplasma* spp. (MH589424)	100	OQ692409
4	1	*A. bovis* (KP062954)	100	OQ692410
***Bartonella* sequences**				
5	10	*Bartonella* spp. (LC485116)	99.11	OQ716819
6	1	*Bartonella* spp. (CP019781)	97.65	OQ716820
7	3	*Bartonella* spp. (LC485115)	98.22	OQ716821
8	6	*Bartonella* spp. (LC485116)	98.52	OQ716822
9	1	Uncultured bacterium (JX416234)	97.35	OQ716823
10	2	*Bartonella* spp. (CP019781)	97.65	OQ716824
11	1	*Bartonella* spp. (LC485115)	98.52	OQ716825
12	1	*Bartonella* spp. (LC485115)	97.63	OQ716826

KP314248, AB983376, and KP062958) found in ruminants and ticks in several countries (Figure 2). Furthermore, two mutation steps separated ntST2 from ntST4 (Figure 2). The samples in ntST1 were grouped with clade of other ruminant-related *Anaplasma* from GenBank; *Anaplasma* spp. from tick (KY766240), *A. capra* (ON872236), *A. marginale* (FJ226454, OP851751), *A. ovis* (KJ639880, OM282854; Figure 2). The samples in ntST3 were grouped with a sequence from mountain bongo in Kenya (MH589424), which differed by two (MN611757, MT371255, and MW899038) and three (OL690556) mutation steps from the clade of *A. phagocytophilum* (Figure 2).

Figure 3 shows phylogenetic tree of *Bartonella* sequences and ntST5 to 12 referred sequences obtained in this study. Except for the ntST9 that represents a novel *Bartonella* species, all obtained sequences were grouped into a distinct *Bartonella* phylogenic lineages C, D, and E, representing a novel *Bartonella* species (15, 39) (Figure 3). Samples in ntST6 and 10 belong to a distinct phylogenetic branch within lineage C, while lineage D is represented in the current study by samples in ntST7, 11, and 12 (Figure 3). Samples in ntST5 and 8 belong to the distinct phylogenetic lineage E (Figure 3). Notably, the phylogenic branch of ntST9 was separated from the clade of lineage C, representing a new independent lineage of a novel *Bartonella* species (Figure 3). The ntST network of *gltA* gene of *Bartonella* spp. from a total of 23 ntSTs (32 sequences) was showed in Figure 4. Based on the distinct phylogenic lineages of a novel *Bartonella* spp., the ntSTs of the *Bartonella* sequences obtained in this study could divided into three lineages: C (ntST6 and 10), D (ntST7, 11, and 12), and E (ntST5 and 8; Figure 4). Both ntST6 and 10 differed by eight mutation steps from clade of lineage C found from sika deer in

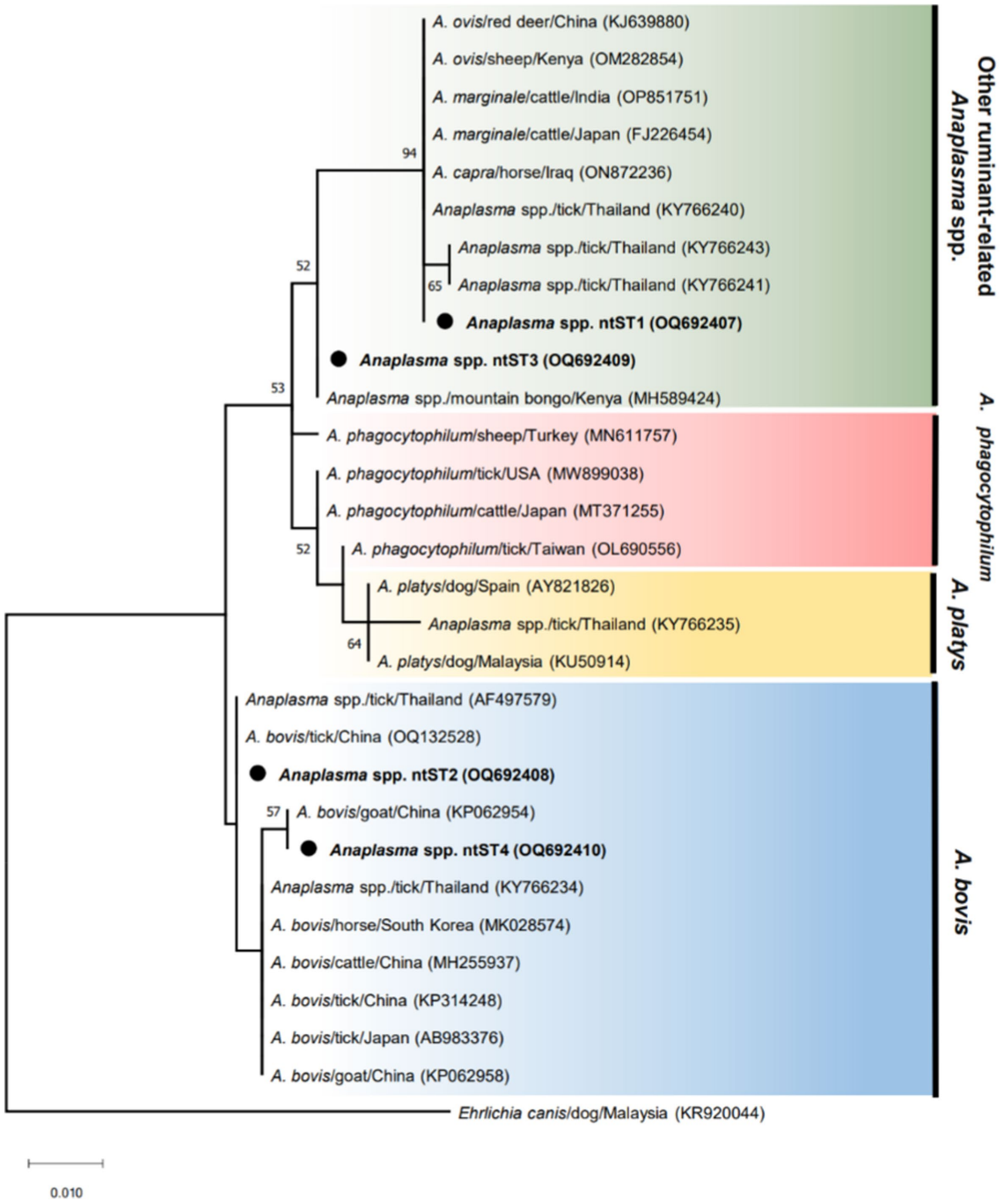

FIGURE 1
ML tree of 16 s rRNA gene of *Anaplasma* sequences (305 bp) computed with the K2 + G model. The phylogenetic relationships among sequences obtained in this study (black dot) and reference sequences from the GenBank database. *Ehrlichia* sequence isolated from dog (KR920044) represents as an out group.

Japan (CP019781 and AB703131; Figure 4). The ntST7, 11, and 12 were separated from the clade of lineage D found in *L. fortisetosa* collected in Japan (LC485115) by six, five, and eight mutation steps, respectively (Figure 4). The samples in ntST5 differed by three mutation steps from clade of lineage E found in *L. fortisetosa* collected in Japan (LC485116), while samples in ntST8 differed by five mutation steps (Figure 4). Furthermore, ntST9, respectively, differed from the designated novel *Bartonella* sequence lineages B, C, D, and E by 12, 10, 18, and 13 mutation steps, which suggests a new independent lineage of a novel *Bartonella* species (Figure 4).

4. Discussion

Lipoptena fortisetosa is a crucial ectoparasite infesting cervids worldwide. This insect is primarily found in sika deer (*Cervus nippon*)

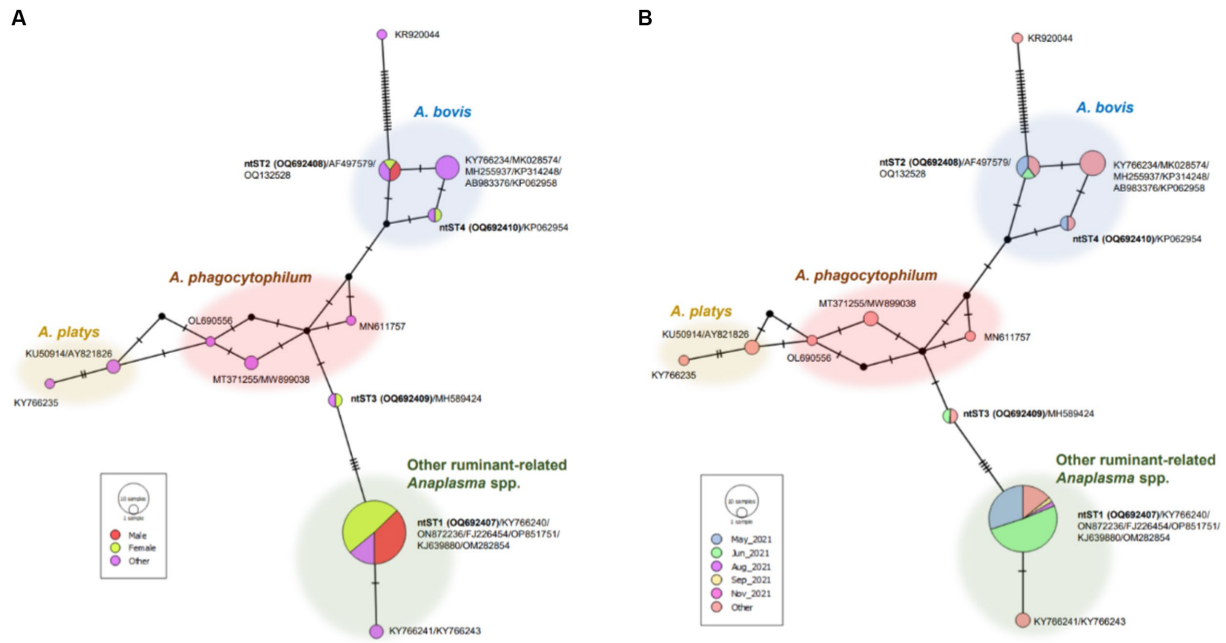

FIGURE 2
ntST network of 16S rRNA gene of *Anaplasma* spp. ntST1 to ntST4 represented sequences obtained in this study, while other sequences obtained from the GenBank database relating to the reference sequences shown in Figure 1. The size of circle represents the frequency of each ntST, whereas the color represents the gender of specimens **(A)** and collecting date **(B)**. "Other" refers to the reference sequences.

in Japan and has also been reported in Siberian roe deer (*Capreolus pygargus*) in Korea, Kazakhstan, and Russia (1, 9, 52). The distribution of *L. fortisetosa* in European countries has been hypothesized by climate change, introduction of alien cervid species into new areas, and adaptation of the insect to different hosts (11, 12). In Thailand, *L. fortisetosa* was first found on captive Eld's deer in Chon Buri, as previously reported (44). *Lipoptena* insects may cause anemia, skin irritation, itching, restlessness, and hair loss in animal hosts (53). However, there were no skin or other symptoms on infested Eld's deer in Thailand.

To our knowledge, this is the first report on *Anaplasma* and *Bartonella* detection in *L. fortisetosa* in Thailand. The prevalence of *Anaplasma* spp. detection in *L. fortisetosa* (46.15%) in the present study was higher than that reported in Poland (8.00%) (54). Although no previous evidence exists of *Anaplasma* harbored by this insect in the country, the presence of ticks, the primary *Anaplasma* vector, in the same area of wildlife habitat may enhance the possibility of bacterial infection in other blood-feeding ectoparasites, including *Lipoptena* insects. This possibility is supported by the results where ntST2 was identical to *Anaplasma* detected in *H. lagrangei* ticks in a previous study (43). In addition to *Anaplasma*, various pathogens, such as *Babesia*, *Ehrlichia*, *Theileria*, and *Wolbachia*, have also been detected in questing ticks in wildlife habitat in Chon Buri, Thailand (43). This finding highlights that, in the same surrounding area, various ectoparasites may harbor, or transmit the pathogen. Moreover, *Anaplasma* DNA is found in other species of *Lipoptena* insects, such as *L. cervi* and *L. depressa* (19, 23, 55).

Anaplasma DNA fragments from this study can be clustered with the clades of *A. bovis* and other ruminant-related *Anaplasma*. *A. bovis* has previously been detected in domestic goats (56), but no report on wildlife in Thailand. Interestingly, *A. bovis* DNA has been found in ticks collected from the Malayan sun bear (*Helarctos malayanus*), sambar deer (*Cervus unicolor*), and questing ticks dragged in wildlife habitat in Thailand (25, 43, 47). These findings suggest wild animals may act as a natural reservoir and their ectoparasites may be associated with *Anaplasma* infection of domestic ruminants. This possibility is supported by the present study showing ntST2 and 4 showed similar genetic material with *A. bovis* detected in goats in China (OQ132528 and KP062954, respectively). Other ruminant-related *Anaplasma*, including *A. marginale*, *A. ovis*, and *A. capra*, have been detected in various wild animals, suggesting a broad host range and genetic diversity (57–59). In the present study, using primers for the partial 16S rRNA gene of *Anaplasma* did not distinguish obtained sequence data from other ruminant-related *Anaplasma*. Amplification and sequencing of full-length 16S rRNA gene or other specific genes of *Anaplasma* would be necessary to clarify genetic characterization.

Besides biological transmission of *Anaplasma* by ticks, other potential vectors have been reported to mechanically transfer bacteria to animal hosts, including biting flies (60) and syringophilid mites (61). For *Lipoptena*, the insects detach their wings after finding suitable hosts and can only be transferred among hosts via direct contact. Since the insects acquire *Anaplasma*-infected blood meal on bacteremic hosts, it is possible to transmit bacteria horizontally to noninfected animals via direct contact (55). However, further experimental studies are needed to confirm direct evidence of the vector ability for *Anaplasma* bacteria transmission.

The prevalence of *Bartonella* detection in *L. fortisetosa* (29.67%) in this study was lower than those collected from free-living cervids in Japan (87.87%) and Poland (75.67%) (15, 62). Possible reasons for low prevalence of *Bartonella* detection are that Eld's deer are raised in captive areas in Thailand. The semi-wild conditions of deer may confer a lower infestation probability by pathogen-infected

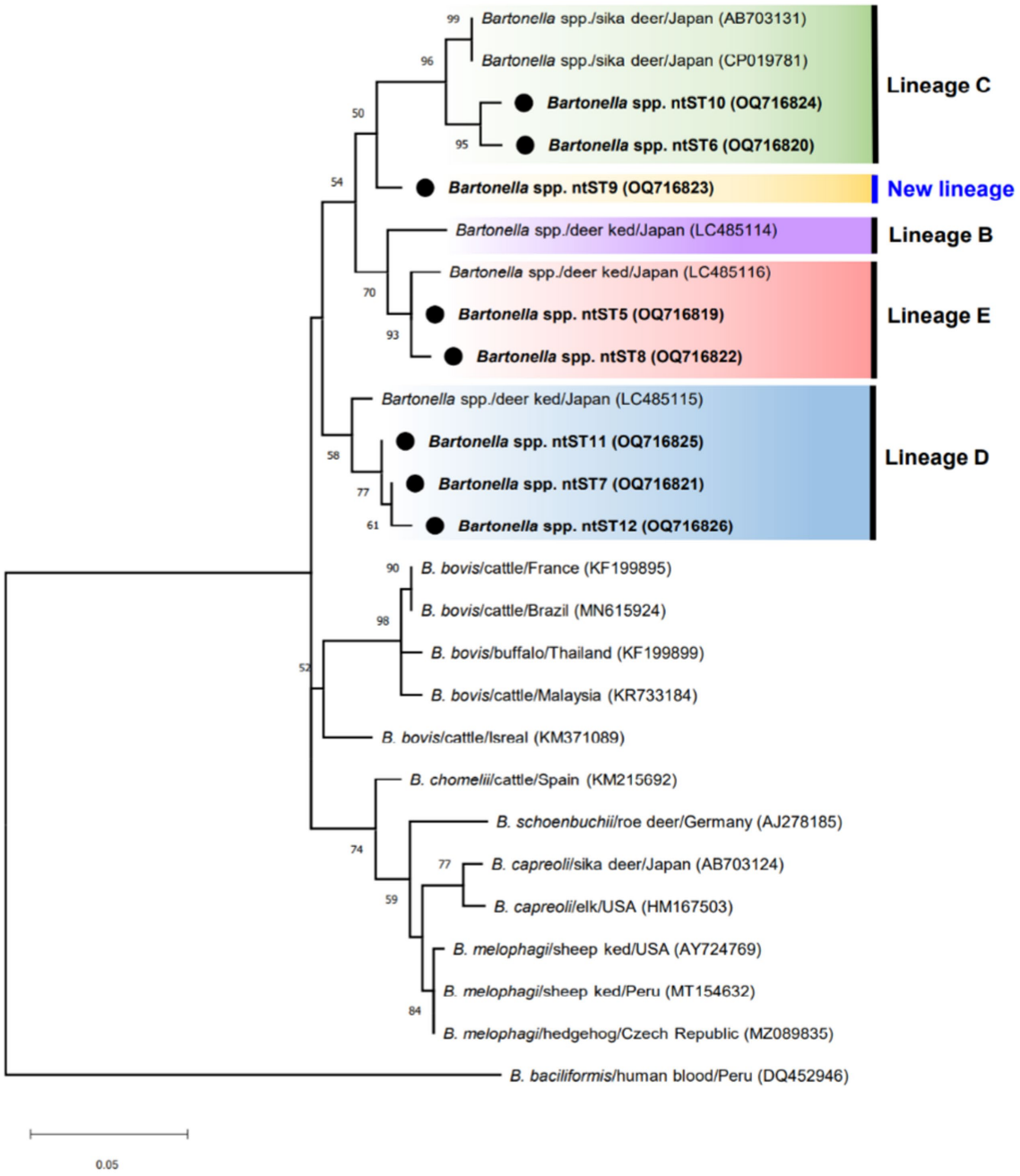

FIGURE 3
ML tree of *gltA* gene of *Bartonella* sequences (337 bp) computed with the TN93 + G model. The phylogenetic relationships among sequences obtained in this study (black dot) and ruminant-related *Bartonella* sequences from the GenBank database. Lineages B, C, D, and E were determined by Sato et al. (15, 39). *Bartonella bacilliformis* sequence isolated from human (KR920044) represents as an out group.

ectoparasites compared with free-living wild cervids in Japan and Poland. This possibility is supported by a previous study in Poland showing farm cervids had a lower prevalence of *A. phagocytophilum* infection than wild individuals (21). Myczka et al. (21) also mentioned the lack of *Anaplasma* detection in farmed cervids may be due to regular deworming, which strengthens their condition and makes them less susceptible to infection by the bacteria. Notably, novel *Bartonella* has been detected and isolated with low prevalence (3.60%) from captive Rusa deer in Thailand, including those being regularly dewormed (40). Secondly, despite prior tick presence in the same surrounding area (43), ticks may not be the essential vector for *Bartonella* transmission among cervids (15, 63). For this reason, we implied that since Eld's deer are infested by ticks and *Lipoptena* insects, *Bartonella* can still be detected, merely not in high prevalence. However, *Bartonella* detected in captive Eld's deer and ticks collected from surrounding areas should be analyzed to clarify these reasons.

Bartonella sequences obtained from this study can be grouped with novel *Bartonella* lineages C, D, and E, which originated from *L. fortisetosa* collected from deer in Japan (15, 39). In addition, the novel *Bartonella* lineage B, primarily derived from Japanese sika deer,

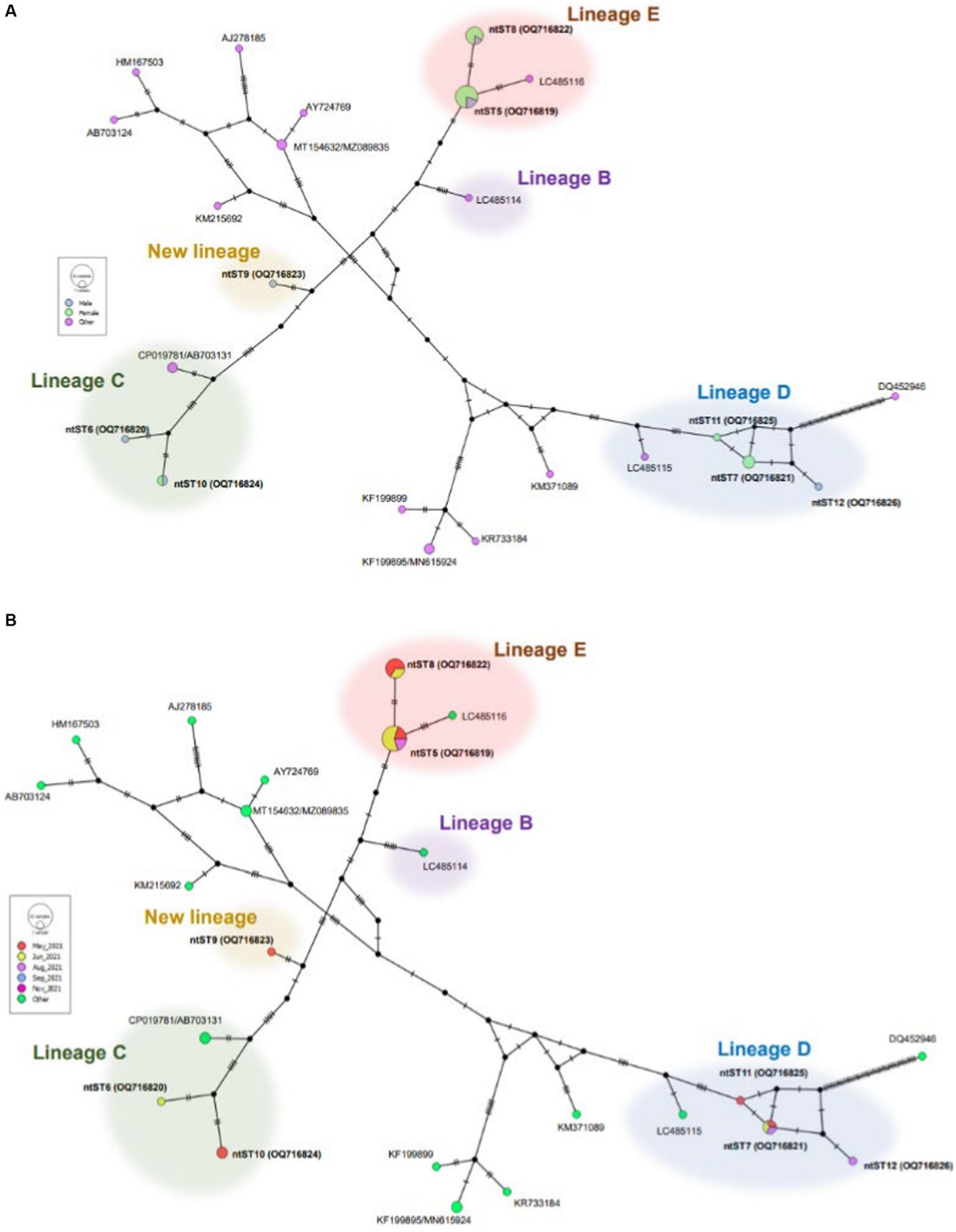

FIGURE 4
ntST network of *gltA* gene of *Bartonella* spp. ntST5 to ntST12 represented sequences obtained in this study, while other sequences obtained from the GenBank database relating to the reference sequences shown in Figure 3. The size of circle represents the frequency of each ntST, whereas the color represents the gender of specimens **(A)** and collecting date **(B)**. "Other" refers to the reference sequences. Lineages B, C, D, and E were determined by Sato et al. (15, 39).

has previously been found in *L. cervi* and *L. fortisetosa* collected from red deer in Poland (62, 64). These findings suggest novel *Bartonella* stains from wild ruminants in Japan may spread to other countries following *Lipoptena* vector introduction into these new areas. We also found a new independent lineage of novel *Bartonella* from collected *Lipoptena* insects. However, genetic characterization analysis is needed to determine whether these new lineages are *Lipoptena* insects-specific *Bartonella*. Obtained *Bartonella* sequences were clustered with ruminant-related *Bartonella*. In addition to the report of novel *Bartonella* isolated from captive Rusa deer in Thailand (40), further reports exist of *Bartonella* detected or isolated from domestic ruminants in the country. Bai et al. (65) revealed that *B. bovis* was isolated from water buffalo blood. In 2021, seroprevalence of antibodies against *B. henselae*, *B. vinsonii* subspp. *Berkhoffii*, and *B. tamiae* in water buffaloes has also been reported (66). These findings support the genetic diversity of *Bartonella* among ruminants in Thailand. Further molecular surveys of *Bartonella* in both wild and domestic ruminants and their ectoparasites are needed to clarify the role of bacterial infection among ruminants in the country.

The high prevalence of *Bartonella* DNA presence in *Lipoptena* insects raises the question that insects may play an essential role in *Bartonella* transmission among hosts (5, 15, 34, 37, 38). The evidence of *Bartonella* survival and propagation in *Lipoptena* specimens has been reported from previous studies using bacterial isolation from the insects and immunohistochemical analysis (15, 35). In addition, the bacterial DNA detected in both wingless *L. cervi* females and their offspring suggests the potential for vertical *Bartonella* transmission (34). However, both *in vitro* and *in vivo* studies are required to verify how vector competence of *Lipoptena* insects facilitates *Bartonella* transmission.

Co-infections occur in *Lipoptena* insects but pathogen diversity may vary by species, hosts, and geographic distribution. In the USA, 6.25% of *L. cervi* removed from white-tailed deer carried both *B. burgdorferi* s.l. and *A. phagocytophilum* DNA (19). Further, 50% of *L. fortisetosa* collected from Korean water deer harbored *Coxiella*, *T. ovis*, and *T. luwenshuni* DNA, but no *Rickettsia*, *Babesia*, *Bartonella*, *Borrelia*, or *Hepatozoon* were detected (67). In this study, 11 *Lipoptena* specimens (12.08%) harbored both *Anaplasma* and *Bartonella* DNA. We also found four specimens were additionally infected with *Theileria* spp. as previously reported by Tiawsirisup et al. (44). The prevalence of more than one pathogen in *Lipoptena* insects emphasizes their medical and veterinary importance.

In the present study, our findings provide the first molecular detection of *Anaplasma* and *Bartonella* on *L. fortisetosa* in Eld's deer in the country. Despite no blood samples from Eld's deer, pathogen DNA detected in insects could represent the health status of animal hosts. Further studies on molecular genetic characterization of related pathogens are needed to investigate correlations of vectors, hosts, and pathogens. In addition, visitors are allowed to have direct contact with animals through petting, feeding, or taking close photos with animals in the zoo. These activities can promote the risks of potential zoonotic infection. Preventive procedures, such as health monitoring, anti-parasite medication, and proper treatments for animals should be regularly conducted. Zoo staff should pay attention to regular hygiene measures before and after working with animals and surrounding areas, such as hand washing, wearing PPE, and foot bathing with an antiseptic solution. Finally, visitors should avoid direct contact with animals and be wary of insects or ectoparasites while visiting the zoo.

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/, OQ692407 https://www.ncbi.nlm.nih.gov/, OQ692408 https://www.ncbi.nlm.nih.gov/, OQ692409 https://www.ncbi.nlm.nih.gov/, OQ692410 https://www.ncbi.nlm.nih.gov/, OQ716819 https://www.ncbi.nlm.nih.gov/, OQ716820 https://www.ncbi.nlm.nih.gov/, OQ716821 https://www.ncbi.nlm.nih.gov/, OQ716822 https://www.ncbi.nlm.nih.gov/, OQ716823 https://www.ncbi.nlm.nih.gov/, OQ716824 https://www.ncbi.nlm.nih.gov/, OQ716825 https://www.ncbi.nlm.nih.gov/, OQ716826.

Ethics statement

The animal study was approved by the Chulalongkorn University Animal Care and Use Committee (Animal use protocol number: 2231011). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WW contributed data, performed the analysis, and wrote the manuscript. CS-i and KT collected the data and provided technical support. NY, CA, GR, NB, and NS collected samples and data. LB edited the manuscript. UM and PK provided professional support at the sample collecting sites. AS provided technical support. ST supervised the study, edited the manuscript, and corresponded. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Case report: Filarial infection of a parti-coloured bat: *Litomosa* sp. adult worms in abdominal cavity and microfilariae in bat semen

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Background: Filarial infections have been understudied in bats. Likewise, little is known about pathogens associated with the reproductive system in chiropterans. While semen quality is critical for reproductive success, semen-borne pathogens may contribute to reproductive failure.

Methods: For the first time we performed electroejaculation and used computer-assisted semen analysis to provide baseline data on semen quality in a parti-coloured bat (*Vespertilio murinus*).

Results: The semen quality values measured in the *V. murinus* male appeared high (semen concentration = 305.4×10^6/mL; progressive and motile sperm = 46.58 and 60.27%, respectively). As an incidental finding, however, microfilariae were observed in the bat semen examined. At necropsy, eight adult filarial worms, later genetically identified as *Litomosa* sp., were found in the peritoneal cavity, close to the stomach, of the same particoloured bat male dying as a result of dysmicrobia and haemorrhagic gastroenteritis in a wildlife rescue centre. Histopathology revealed microfilariae in the testicular connective tissue and the epididymal connective and fat tissues. A PCR assay targeting cytochrome c oxidase subunit 1 confirmed that adult worms from the peritoneal cavity and testicular microfilariae were of the same filarial species. Mildly engorged argasid mite larvae attached to the bat skin proved negative for filarial DNA and the adult filarial worms proved negative for endosymbiont *Wolbachia*.

Conclusion: While the standard filarial life cycle pattern involves a vertebrate definitive host and an invertebrate vector, represented by a blood-sucking ectoparasite, our finding suggests that microfilariae of this nematode species may also be semen-borne, with transmission intensity promoted by the polygynous mating system of vespertilionid bats in which an infected male mates with many females during the autumn swarming. Presence of microfilariae may be expected to decrease semen quality and transmission *via* this route may challenge the success of reproductive events in females after mating. Further investigation will

be necessary to better understand the bat-parasite interaction and the life cycle of this filarial worm.

KEYWORDS

Chiroptera, *Vespertilio murinus*, electroejaculation, semen quality parameters, semen-borne pathogens, filariasis, *Wolbachia*

1. Introduction

According to a conservative estimate, between 4,000 and 5,000 endoparasite species are believed to infect bats around the world (1). However, while hundreds of nematodes have been described in bats (2), their biology, life cycles and host–parasite specificity and interactions remain largely unknown (3). Onchocercid filarial nematodes, and especially the genera *Litomosa* and *Litomosoides*, occur relatively frequently in both Old World and Nearctic and Neotropical bats of the families Hipposideridae, Miniopteridae, Molossidae, Phyllostomidae, Pteropidae, Rhinolophidae and Vespertilionidae (4–13). Adult *Litomosa* and *Litomosoides* generally reside within the pleural and/or peritoneal cavities of bats; however, cerebral ventricles (14) or the pulmonary artery, the right ventricle of the heart and the portal vein (9), may also be favoured sites for some adult onchocercid filariae.

Aside from *Loa loa* (15), most filarial nematodes of medical and veterinary concern (16) also host the intracellular bacterial symbiont *Wolbachia*. Providing metabolites that filarial nematodes are incapable of synthesising, this endosymbiont is fundamental for key biochemical pathways required for growth and development of larvae and embryogenesis in female worms (17). *Wolbachia* also contributes to the immunopathology of filarial infections (18, 19).

To date, three onchocercid filarial nematodes have been recorded in parti-coloured bats, with *Litomosa ottavianii* (20) and *Litomosa vaucheri* (10) described in the parti-coloured bat (*Vespertilio murinus*) and similar *Litomosa* sp. described in its sibling species, the Asian parti-coloured bat (*Vespertilio sinensis*) (9). The distribution of *V. murinus* ranges over much of Europe to west Asia, Mongolia, northern China and the Russian Far East, where it overlaps with *V. sinensis* (21). Mainly found in cities in the Czech Republic, individuals with injuries are commonly found in high buildings and brought to rescue centres (22).

In October 2021, a *V. murinus* male was brought for examination and treatment to the wildlife rescue centre at the University of Veterinary Sciences in Brno, Czech Republic, after it was found in a block of flats unable to fly. As the animal was otherwise in good condition, we used the opportunity of general anaesthesia to collect a semen sample by electroejaculation. While evaluating the quality of semen collected using computer-assisted semen analysis, we recorded microfilariae as an incidental finding. Adult filarial worms were also later found in the peritoneal cavity of the same male, with histopathology revealing microfilariae in the testes. We then attempted to identify the filarial parasite genetically and test it for presence of the bacterial symbiont *Wolbachia*.

2. Materials and methods

2.1. Ethics statement

Experimental procedures within the project "Sperm and European bat mating systems" were approved by the Ethical Committee of the University of Veterinary Sciences Brno (document no. 9-2021) and the Ministry of the Environment of the Czech Republic (document no. MZP/2021/630/2084). Semen sample collection was based on a permit issued by the Agency for Nature Conservation and Landscape Protection of the Czech Republic (SR/0249/JM/2021-3). All team members were authorised to handle wild bats according to Czech Certificate of Competency (No. CZ01341; §17, Act No. 246/1992 Coll.).

2.2. Semen collection and examination

The male *V. murinus* treated at the wildlife rescue centre at the University of Veterinary Sciences in Brno had a left humerus fractured in diaphysis. The fracture site was open and infected and the distal part of the affected wing was necrotic due to disrupted blood supply; consequently, amputation was employed to save the bat's life. When animals have no chance of survival in the wild or a poor quality of life in captivity may be expected, euthanasia is considered an appropriate action. In this case, wing amputation leading to disability and permanent captivity of the injured bat was justified by keeping the animal for educational purposes. The surgery was performed under inhalation anaesthesia using isoflurane (Isoflurin 1,000 mg/g; Vetpharma Animal Health, S.L., Spain) delivered through a small mask from a Matrix Technologies VIP 3000 Veterinary Isoflurane Vaporiser (Midmark, United States) using an open non-rebreathing system. The bat was induced and maintained with 5 and 2% isoflurane, respectively, carried with 1.0 L/min oxygen.

As the animal was otherwise in good condition, we used the opportunity of general anaesthesia to collect a semen sample by electroejaculation, using an impulse electroejaculation generator (designed and developed by our team for semen collection from small mammals) connected to a 1 mm diameter rectal probe with electrodes 4 mm from the probe's tip. Lubricated with ultrasound gel, the probe was inserted approximately 9 mm into the bat's rectum and positioned against the prostate region. Electrical stimulation was then initiated with intensity ranging from 0.20 to 4 mA (50 Hz), as described elsewhere (23). A series of stimulations was alternated with periods of rest until erection was reached and a drop of semen was seen at the tip of the bat's penis. The ejaculate was then collected using a laboratory pipette and extended (dilution 1:10 v/v) immediately with a 37°C pre-warmed DMEM-F12 solution (Dulbecco's Modified Eagle Medium; Biosera, France).

Fresh bat semen characteristics were determined immediately after collection using a Sperm Class Analyser-Computer Assisted Sperm Analysis (SCA-CASA) system (Microptic s.l., Spain), with concentration and motility determination modules and a Nikon eclipse E200LED MV R camera equipped with a Nikon 10 × 0.25 Ph 1 BM WD 7.0 lens (Nikon Corporation, Japan) and an acA 1,300-200uc Basler c-Mount camera (Basler a.g., Germany). Semen concentration ($\times 10^6$/ml) and total motility parameters were measured in a pre-warmed 8 × 2 µL Leja SC 20-01-08-B-CE counting chamber (Cryo Tech s.r.o., Czech Republic).

2.3. Pathological examination

The bat's recovery from general anaesthesia and surgery was uneventful. The animal was housed in a bat box with soft mesh on the inner walls and cloth layers to provide roosting and hiding places along with a water dish for drinking. The bat accepted hand-offered mealworms twice daily from the day following surgery. After 3 weeks, however, the bat stopped feeding and died suddenly.

On necropsy, both the abdominal and thoracic cavities were visually inspected for gross lesions and presence of parasites. We also collected samples for histopathology (lungs, liver, kidney, testicle) and placed these in 10% buffered formalin. Paraffin embedded samples were then sectioned into 5 µm slices on a rotary microtome (RM2255, Leica Microsystems GmbH, Wetzlar, Germany), mounted onto a slide and stained using hematoxylin–eosin (Sigma-Aldrich, United States) as described earlier (24). The bat's skin was also examined for ectoparasites.

2.4. Identification of intestinal microbial agents

During necropsy, the gut contents were collected for DNA isolation with a commercially available E.Z.N.A.® Soil DNA Kit (Omega, United States), following the manufacturer's instructions. The isolated DNA sample was stored at −20°C, after which a 16S Barcoding Kit (Oxford Nanopore Ltd., United Kingdom) was used to amplify the entire 16S rRNA gene (25 cycles with primers) and prepare amplicons for sequencing. Barcoding was performed using a Bio-Rad Mini cycler (Bio-Rad, United States) and the amplicons purified in line with the manufacturer's instructions using AMPure XP Beads (Beckmann Coulter, United States). Sequencing was performed with a MinION nanopore sequencer using the reagents supplied with the 16S Barcoding Kit. Reads were filtered and those with a Phred score < 7 were removed from the analysis. A previously described pipeline was used for bioinformatic analysis (25), with individual reads taxonomically assigned using the RDP16S_v.18 database (26). Raw 16S sequences were deposited in the NCBI Sequence Read Archive (SRR25944610).

2.5. Molecular identification of the filarial parasite

DNA was extracted from adult worms, from the bat's testicles and from blood-sucking ectoparasites attached to the skin using the NucleoSpin® Microbial DNA extraction kit (Macherey-Nagel, Germany), following the methodology recommended in the manufacturer's instructions. The eluted DNA was then stored at −20°C until polymerase chain reaction (PCR) screening.

To identify parasitic filarial species in the samples, a PCR assay targeting the cytochrome c oxidase subunit 1 (COI) was performed, using the universal primers COIintF (5′-TGATTGGTGGTTTTGGTAA-3′) and COIintR (5′-ATAAGTACGAGTATCAATATC-3′) (27). The PCR mixture comprised 10 µL of Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fischer Scientific, United States), 0.5 µM of each primer and 2 µL of DNA, prepared to a final volume of 20 µL. Amplification was then performed using a TProfessional TRIO Thermocycler (Biometra, Germany) over a total of 40 cycles.

Amplification products were visualised on 2% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe, Germany). All samples yielding an amplicon of the appropriate size (689 bp) were purified using the Gel PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified PCR products were commercially sequenced (Macrogen Europe, The Netherlands) and the sequences obtained aligned in Geneious Prime software (Biomatters, New Zealand) and compared with available sequences in GenBank.

Sequences with mixed chromatograms were cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit (Thermo Fisher Scientific, United States). The plasmid DNA acquired was purified from the bacterial culture using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, United States) and sequenced using universal T7/SP6 primers.

2.6. *Wolbachia* screening

Two adult filarial worms from the bat's peritoneal cavity were used for DNA isolation and testing for the presence of the bacterial symbiont *Wolbachia*. We used the PCR protocol for *Wolbachia* detection described previously (28). The method is based on amplification of the 16S rRNA gene fragment (~1,100 bp) using the *Wolbachia*-specific primers: 16S 281F 5′- CTATAGCTGATCTGAGAGGAT-3′ and 16S 1372R 5′-YGCTTCGAGTGAAACCAATTC-3′. Reactions were performed in a 25 µL reaction mixture containing 12.5 µL of Super-Hot Master Mix 2x (Bioron GmbH, Germany), 0.5 µM of each primer and 1 µL of isolated DNA. PCR procedures included a first step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 60 s, 72°C for 90 s and a final step of 72°C for 10 min. The amplified DNA products (4 µL) were stained with SERVA DNA Stain G (SERVA Electrophoresis GmbH, Germany) and visualised by electrophoresis on 1.5% agarose gel.

3. Results

3.1. Bat semen quality

The semen sample, which was collected within a minute of electrical stimulation, had a total volume of 1 µL and was highly viscous. SCA-CASA revealed the following parameter values: semen concentration = 305.4 × 10^6/mL; progressive and motile sperm = 46.58 and 60.27%, respectively; rapid velocity = 45.21%; velocity and progressivity: rapid progressive = 24.66%, medium progressive = 21.92%, non-progressive = 13.70%, immotile = 39.73%;

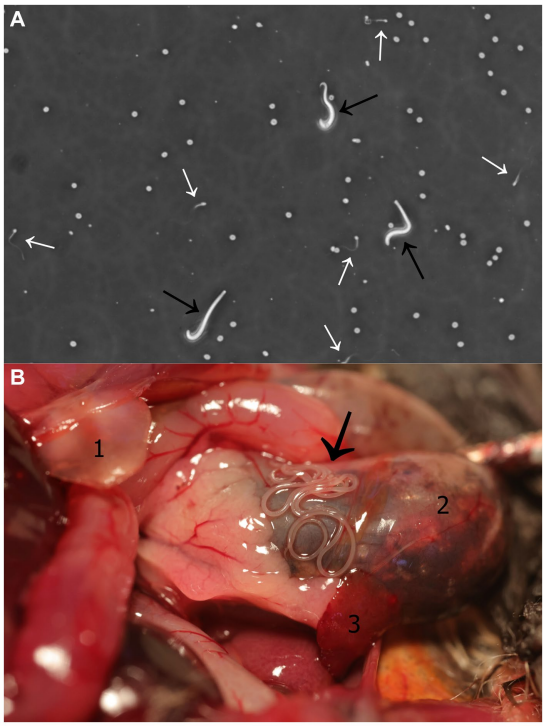

FIGURE 1
Litomosa sp. filarial infection. **(A)** Semen-borne microfilariae in a male parti-coloured bat (*Vespertilio murinus*). Image captured from video footage obtained during a Sperm Class Analyser - Computer Assisted Sperm Analysis. Microfilariae and spermatozoa are indicated with black and white arrows, respectively. **(B)** Filarial nematodes within the abdominal cavity of a male parti-coloured bat. Thread-like adult worms located along the stomach (black arrow). Anatomical structures seen in the figure: (1) *Cartilago xiphoidea*, (2) stomach, (3) spleen.

average sperm head area = 30.84 μm^2; speed: curve speed = 141.71 μm/s, average value = 136.90 μm/s, linear speed = 111.71 μm/s, straightness index = 71.94%, linearity index = 67.54%, oscillation index = 87.12%; beat frequency = 5.12 Hz; mucous penetration = 38.64%. In addition to spermatozoa, active microfilariae were also observed in the semen sample (Figure 1A; Supplementary Video S1).

3.2. Pathological examination

Gross pathology examination revealed haemorrhagic gastroenteritis and bloating. A total of eight adult filarial worms were found in the peritoneal cavity, close to the stomach (Figure 1B). Microscopic examination revealed microfilariae in the testicular connective tissue (Figures 2A,B) and epididymal connective and fat tissues (Figures 2C,D), with no inflammatory response. However, microfilariae were not observed in histopathological sections of the lungs, liver and kidneys, or the blood vessels within these organs. Three argasid mite larvae, mildly engorged with blood, were found attached to the skin of the bat (Figure 3).

3.3. Identification of intestinal microbial agents

A total of 87,368 reads were obtained after sequencing the library. During the bioinformatic analysis, singletons were filtered away and the remaining reads assigned to 17 operational taxonomic units (OTUs). At the phylum level, Proteobacteria were the most abundant taxon (68.94%), followed by Firmicutes (30.73%), while at the class level, most reads were assigned to *Gammaproteobacteria* (68.54%) and *Bacilli* (30.41%; Figure 4). Most *Gammaproteobacteria* belonged to the orders *Enterobacterales* (57.28%) and *Pasteurellales* (3.19%), while the majority of *Bacilli* were represented by Lactobacillales (27.84%), with 17.13% identified as members of the Morganellaceae family with *Morganella* (1.17%) the dominating genus, 11.57% assigned to the *Enterococcaceae* family with the *Enterococcus* genus (4.74%) predominant, 7.06% assigned to *Streptococcaceae* (5.92% *Lactococcus*), and 1.82% assigned to the *Pasteurellaceae* family (Figure 4).

3.4. Molecular identification of the filarial parasite and screening for *Wolbachia*

The PCR assay targeting COI in DNA extracted from adult worms produced two high quality sequences, while molecular clones of COI from the bat's testicular microfilariae produced eight high quality sequences. All sequences were deposited into the GenBank database under accession numbers OP796363 to OP796372. These sequences showed 91.08 to 91.38% genetic identity with the *Litomosa* sp. WB-2018 COI gene (sequence ID MH411205) from worms detected as filarial infection in the thoracic cavity of a Bulgarian Savi's pipistrelle bat (*Hypsugo savii*). The argasid mite larvae proved negative for filarial DNA, while the adult filarial worms from the bat's peritoneal cavity tested negative for presence of *Wolbachia*.

4. Discussion

4.1. Bat semen quality

To the best of our knowledge, this is the first report on semen quality for *V. murinus*. As we lack deeper knowledge about semen quality in most chiropteran species, and no study evaluating sperm quality of vespertilionid bats has ever been published, our data cannot be directly compared. However, the semen concentration and sperm motility values measured in our *V. murinus* male appear high compared with phyllostomid (23, 29, 30), vespertilionid and rhinolophid (31) and pteropid (32) bats.

Spermatozoa and seminal fluid are both standard parts of ejaculated semen, with the seminal fluid acting as a complex medium for the transport, protection and nourishment of the spermatozoa. However, the seminal fluid can also provide the same functions for semen-borne pathogens (33). For example, the seminal fluid of mammals is rich in fructose, the major source of energy metabolism for spermatozoa (34). In addition to glucose, however, filarial parasites are also able to utilise fructose as an energy source (35),

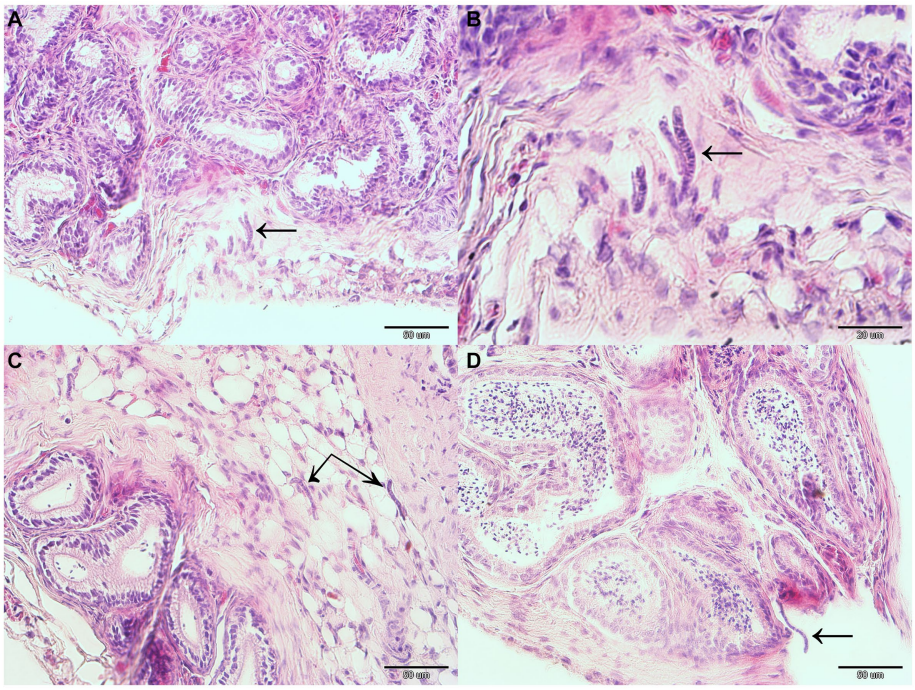

FIGURE 2
Microfilariae located within reproductive organ tissues of a male parti-coloured bat (*Vespertilio murinus*). Microfilariae (indicated with black arrows) can be seen in the testicular connective tissue **(A,B)** and epididymal connective and fat tissue **(C,D)**.

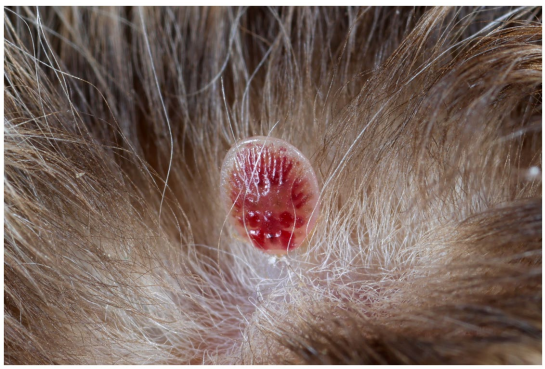

FIGURE 3
A mildly engorged argasid mite larva attached to the skin of a male parti-coloured bat (*Vespertilio murinus*).

suggesting that bat seminal fluid may facilitate filarial parasite transmission. Likewise, bat uterine fluids are high in fructose of both male and female contribution (36, 37), and these may contribute to the ability of hibernating female bats to store sperm for months as part of their delayed fertilisation reproduction strategy (38). We may hypothesise, therefore, that each mating in bats showing these reproductive physiology traits allows microfilariae to enter the uterus, where they can first infect the female and later, the developing embryo and/or foetus. Importantly, the sperm storage organs in male and female bats, i.e., the epididymis and uterus, are both immune-privileged sites (37, 39) in which both germ cells and the parasites may be protected from immune attack.

4.2. Pathological examination

Based on gross pathology, the primary cause of the bat's death was associated with haemorrhagic gastroenteritis and bloating. Outbreaks of haemorrhagic diarrhoea in groups of captive handicapped bats have been recognised in association with bacterial infection, with contaminated mealworms as a likely source of infection (22). Presence of adult filarial worms in the peritoneal cavity, however, induced no signs of disease, while the microfilariae observed in the testicular tissue during histopathology were not associated with any signs of inflammation, suggesting that immunological tolerance may be limiting the harm caused by the parasite (40). Unfortunately, there is limited information available on pathogenicity of filarial infections in bats. However, Rendón-Franco et al. (12) noticed signs of weakness, tachypnoea and patagial haemorrhages in two male Aztec fruit-eating bats (*Artibeus aztecus*) captured in Mexico, contrary to our findings. Histopathology of organs collected from these bats revealed lesions such as multifocal exudative pneumonia, neutrophilic inflammatory infiltration and oedema of the lungs associated with *Litomosoides* sp. microfilaraemia.

In vespertilionid bats, the testes descend into the scrotum and are covered by the tunica vaginalis, a pigmented sheet of peritoneum (41). During foetal development, the testis descend through the respective inguinal canals through an extension of the peritoneal cavity into the scrotum called the processus vaginalis. We hypothesise that microfilariae are able to invade the male bat's testes by migrating from the site of release by adult

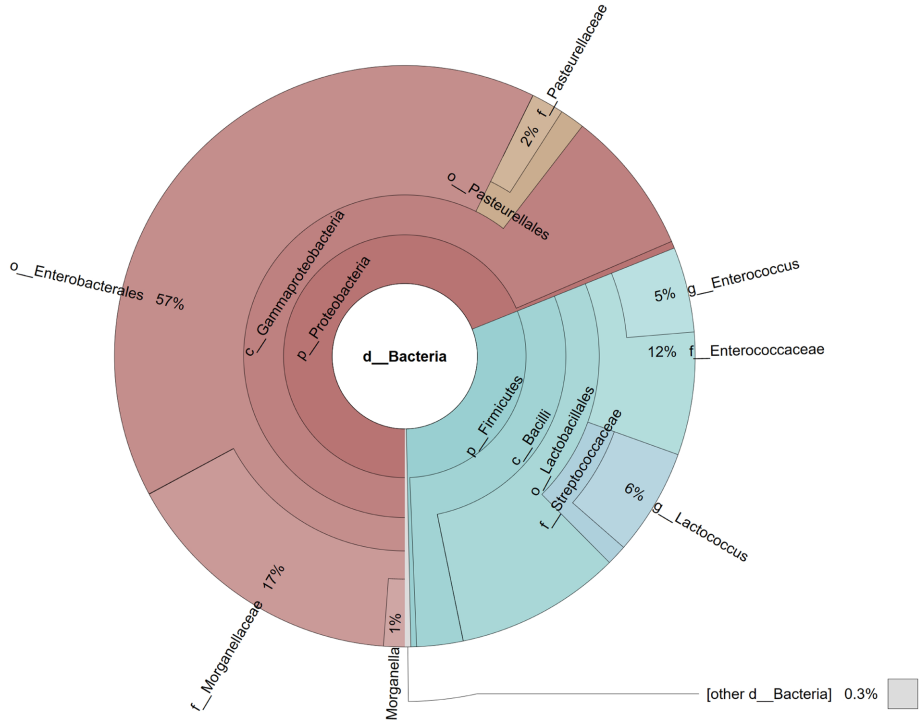

FIGURE 4
Pie chart illustrating the gut microbial community of a male parti-coloured bat (*Vespertilio murinus*) that died of dysmicrobia and haemorrhagic gastroenteritis.

females in the peritoneal cavity along this peritoneal tunnel route. An alternative migration route used by microfilariae to arrive at the processus vaginalis may be *via* the lymphatic vessels; observed, for example, in *Brugia malayi* infections (42). Interestingly, microfilariae of *Wuchereria bancrofti* have also been shown to infect testicular tissue (43, 44) and follicular fluid in humans (45).

4.3. Identification of intestinal microbial agents

The gut microbial community structure of insectivorous bats is highly specific, differing clearly from the microbiome of other mammalian species. Regarding the bacterial phylum, Proteobacteria and Firmicutes are most abundant, with the bacterial families *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Fusobacteriaceae* dominating (46). Microbiome analysis of the *V. murinus* in this study, which was suffering from haemorrhagic gastroenteritis, indicated a low diversity of bacterial taxa comprising just two classes, three orders and five families. The dominant family in this case were the Morganellaceae, mainly comprising the genus *Morganella*, which is normally a rather sporadic taxon in the chiropteran gut microbiome (47) and may have been the causative agent of the haemorrhagic gastroenteritis (48). A decrease in intestinal microbiome diversity is often associated with a state of dysbiosis. In humans, for example, an intestinal microbiome composition similar to that observed in our *V. murinus* bat has been referred to as 'low diversity dysbiosis' (49). Dysmicrobia, and subsequent haemorrhagic gastroenteritis, could result from stress, a significant problem for wild animals kept in captivity (50). Unbalanced and/or highly nutrient-rich diet could be another reason for dysmicrobia (51).

4.4. Molecular identification of the filarial parasite

A BLAST search of the parasites collected in the peritoneal cavity of our *V. murinus* generated results showing 91% genetic identity with a filarial parasite *Litomosa* sp. described in *H. savii*. While sequence divergencies of COI readily discriminate closely related species (52), there are unfortunately no COI sequences available in the GenBank database for *L. ottavianii*, *L. vaucheri* known from *V. murinus* and *Litomosa* sp. from *V. sinensis* (9, 10, 20). In fact, sequence of *Litomosa* sp. from *Hypsugo savii* is the only available COI sequence of genera *Litomosa* and *Litomosoides* obtained from European or Asian bats. While a PCR assay targeting COI confirmed that the adult worms from the bat's peritoneal cavity and testicular microfilariae were of the same filarial species, morphological characteristics of the worms from our *V. murinus* bat differed from those described previously (9, 10, 20), suggesting that the parasite is a novel filarial species (a full description of this new species will be included in a separate manuscript), together with the parasite's molecular phylogeny (Pikula et al., in preparation).

4.5. Screening the filarial parasite for *Wolbachia*

The adult filarial worms in the present study were all free of the endosymbiont *Wolbachia*, as were two bat filarial species (i.e., *Litomosoides yutajensis* and *Litomosa chiropterorum*) previously screened for *Wolbachia* (5, 53). It would appear, therefore, that presence of *Wolbachia* endosymbionts is not essential for filarial physiology (5). Considering the strict vertical transmission of endosymbiotic *Wolbachia* between females and their offspring, it would be expected that the phylogenies of filarial hosts and their symbionts match (27); however, phylogenetic analyses have indicated that some filarial lineages may have lost *Wolbachia* during their evolution (54, 55). Interestingly, *Wolbachia*-like DNA sequences have been found within the genomes of some *Wolbachia*-free filarial nematodes, suggesting ancient infection with the endosymbiont and horizontal genetic transfer, potentially explaining how these nematodes support their physiological needs (56).

4.6. The biology and life cycle of filarial nematodes

Microfilariae released by females at the site of infection spread within the host *via* blood and lymphatic circulation (57). While filarial infections are generally known to be vectored by different arthropods, just one macronyssid ectoparasite, the tropical rat mite (*Ornithonyssus bacoti*) has been experimentally confirmed as playing a role in the larval biology of a *Litomosoides* filaria of Costa Rican common fruit bats (*Artibeus jamaicensis*) (58). Vectors feeding on the host ingest microfilariae which then develop into infective L3 larvae. These larvae may then enter and infect a new host when the vector feeds on another bat. Further steps in the life cycle include migration of larvae to a specific site in the body, maturation, mating and production of microfilariae by adult females. Sometimes, microfilariae may be present in blood; however, no adult worms have been discovered during bat necropsy examinations, suggesting longer survival of microfilariae than adults in the host (58).

Argasid mite larvae attached to the *V. murinus* male in this study proved negative for filarial DNA; however, it is not known whether these ectoparasites serve as vectors for *Litomosa* microfilariae. Considering the host–parasite system dependent on a vector, it is possible that the social structure and roosting behaviour of bats could reduce opportunities for ectoparasite transmission (59) and infection with vector-borne filarial larvae because in *V. murinus* the males separate from females and move to different habitats/sites for most of the year, where they remain territorial and roost individually throughout the autumn mating period (21).

Temperate vespertilionid bats are monoestrous and mating tends to be polygynous and promiscuous (37). Hypothetically, sexual transmission may allow vector-borne filarial parasites to cycle in conditions where there is a reduced chance of reaching new hosts *via* transmission through ectoparasites. Indeed, intensity of transmission through this modified route may even be promoted by the bat's polygynous mating system, in which the infected male mates with many females during the autumn swarming. It remains unclear whether microfilariae sexually transmitted to females then continue their development. Most probably not, as microfilariae can continue further development only following ingestion by a proper vector (58). Even if they could not continue their development without the invertebrate vector, microfilariae may be able to get into the bloodstream of the female bat and continue the classic life cycle of filarial nematodes because microfilariae infecting females in large maternity colonies may have increased opportunities for ingestion by a proper vector, allowing it to develop further and enter a new host to mature in. This would be similar to the situation whereby canine puppies suffer transplacental infection with microfilariae of the heartworm *Dirofilaria immitis* (60). Likewise, microfilariae of *Wuchereria bancrofti* may cross the human placenta. As a result of *in utero* exposure, children born to infected mothers may be tolerant and more likely to become infected later in their life (61).

5. Conclusion

In the present case report, we provide the first description of semen quality of a parti-coloured bat and evidence for (a) adult filarial nematodes, genetically identified as *Litomosa* sp., residing in the peritoneal cavity of an adult male *V. murinus* bat, and (b) microfilariae of the filarial nematode within testicular tissues and in the bat's semen, suggesting that this nematode may also be a semen-borne pathogen. While comparative data is lacking, presence of microfilariae may be expected to decrease semen quality and transmission *via* this route may challenge the success of reproductive events in females after mating. Further investigation will be necessary to better understand this filarial species life cycle and gender differences in disease prevalence.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/sra; SRR25944610.

Ethics statement

The animal study was approved by Ethical Committee of the University of Veterinary Sciences Brno, Czechia. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. VP: Investigation, Methodology, Visualization, Writing – review & editing. HB: Formal analysis, Investigation, Writing – review & editing. MB: Investigation, Writing – review & editing. SB: Data curation, Formal analysis, Investigation, Writing – review & editing. RB: Investigation, Writing – review & editing. OD: Investigation, Methodology, Writing

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Conflict of interest

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An alien parasite in a changing world – *Ashworthius sidemi* has lost its traditional seasonal dynamics

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A non-native nematode *Ashworthius sidemi* has emerged in captive fallow deer in Central and Eastern Europe over the last decade. Although this parasite has been spreading in the wild outside it's native distributional range and colonising local European host species since the middle of the last century, limited information has been published on the seasonality of *A. sidemi* and its susceptibility to anthelmintics. To address this knowledge gap, we conducted a study to investigate seasonal dynamics of the non-native parasite in the current Central European climate conditions. We collected freshly voided faecal pellets at four-week intervals from February 2018 to February 2020 at a fallow deer reserve with a known history of *A. sidemi* presence. The faecal pellets obtained were pooled after each site visit ($n = 25$) and coprocultured to obtain the third stage larvae of trichostrongylid nematodes at monthly intervals. Total genomic DNA was extracted from the recovered larvae. Using real-time multiplex PCR, *A. sidemi* DNA was detected in 17 out of 25 larval samples (68% prevalence). During the monitoring period, the annual administration of ivermectin based premix (Cermix) took place in January 2018, 2019, and 2020, and additionally a mixture of rafoxanide and mebendazole (Rafendazol) was administered once in spring 2019. The probability of parasite presence was significantly influenced by the time since the drug administration ($p = 0.048$) and the mean temperature at the location ($p = 0.013$). Larval samples negative for *A. sidemi* were always identified shortly after the drug administration. However, rapid pasture contamination by the parasite eggs from two to three months after Cermix administration and within one month after Rafendazol administration suggest only a short-lived efficacy of both administered drugs. The abundance of *A. sidemi* DNA was positively affected by mean temperature ($p = 0.044$) and remained relatively stable throughout the monitoring period, with the highest peak in August 2018 and 2019. Pasture contamination with *A. sidemi* eggs occurred almost all year round, with the exception of the beginning of 2018, 2019, and 2020. These findings indicate adaptation of a non-native parasite to the current climatic conditions of the Czech Republic resulted in negligible seasonal patterns of parasite egg shedding.

KEYWORDS

invasive nematode, fallow deer, epidemiology, anthelmintic drug, temperature

1. Introduction

The fallow deer (*Dama dama*) is currently one of the most widespread cervids in the world, and despite being considered an allochtonous species in many ecosystems (1), its importance in agriculture is growing with the increasing interest in alternative sources of meat for human consumption. In Europe, the fallow deer is the second most farmed cervid after the red deer (2), and is now widely distributed both in hunting grounds and game reserves (3).

Like other ruminants, fallow deer is susceptible to infection with gastrointestinal (GI) nematodes. Although such infections are predominantly subclinical, they may have a significant impact on both the population dynamics of cervids and their individual fitness (4). The abomasal nematodes of the superfamily Trichostrongyloidea (trichostrongylids), especially *Ostertagia* spp. and *Spiculopteragia* spp. were traditionally considered to be the most important parasites in fallow deer (5–7); however, more recently there have been reports on emergence of an invasive nematode, *Ashworthius sidemi*, in captive fallow deer (8, 9). The alien parasite was introduced to Europe via sika deer in the late 19th century and subsequently disseminated among local cervid hosts, especially in Central and Eastern Europe (10–13). Currently, *A. sidemi* may be spread by migrating cervids and through further unintended human-mediated introductions (14, 15).

Transmission of *A. sidemi* occurs through accidental ingestion of infective larvae (L3) by a susceptible ruminant host when grazing. The endogenous development involves abomasal mucosa invasion and several larval molts to adult stages, which undergo sexual maturity and reproduction. Time elapsed between infection and shedding of nematode eggs may be delayed by arrested larval development (hypobiosis); the phenomenon occurs in response to unfavorable environmental and/or host conditions (16). Eggs are released with host faeces contaminating grazing pasture while their further fate, i.e., hatching, survival, development to the infective stage L3, and their movement on a pasture, is strongly influenced by environmental factors, especially humidity, and temperature (17). Trichostrongylid infections thus show seasonal patterns, which are an outcome of environmental, host, and parasite factors that are variable within a year. A comprehensive understanding of how the nematode population fluctuates throughout the season is crucial for planning effective and sustainable parasite control. While the seasonal dynamics of trichostrongylid infections in ruminant livestock have been thoroughly studied for many decades (18, 19), only limited information are available for cervids.

Certain aspects of *A. sidemi* seasonal dynamics were outlined based on necropsies of several wild ruminant species during a hunting season in Poland (11). The authors concluded that transmission of *A. sidemi* occurs from June to September, while during the winter and following spring, the non-native nematode survives only as L4 larvae and sexually immature individuals. Similar results have been previously published (20); only larvae and juveniles of *A. sidemi* were recovered from the abomasa of sika deer and Maral deer during autumn and winter in the Russian Far East. In contrast to the above mentioned studies, only negligible importance of hypobiosis has been detected in the Czech Republic (CR), indicating a possible modification of seasonal patterns in *A. sidemi* (15, 21). Seasonal dynamics of trichostrongylids can differ based on the climate conditions in different regions and may also shift over time due to global climate changes (22, 23). Seasonal patterns of *A. sidemi* egg laying remain unclear, as a longitudinal study has yet to be conducted.

Management practices, such as anthelmintic treatment, can disrupt the seasonal patterns of GI nematodes by suppressing egg output (24, 25). While the use of anthelmintic drugs in wild ruminants is somewhat controversial (26), cloven-hoofed animals at game reserves and especially at farms are often kept in overcrowded conditions and may suffer from parasitic infections to a similar extent as ruminant livestock. Application of anthelmintic treatments to such animals is therefore justified (27). Hunting legislation in the CR allows the administration of anthelmintics in the controlled conditions of game reserves and farms throughout the year. However, the common practice is to apply anthelmintic treatments to the animals during the winter season when natural food sources are limited, and drug efficacy is at its peak. Two anthelmintic drugs in the form of medicated feed are certified for this purpose in the CR – Rafendazol, a mixture of rafoxanide and mebendazole, and ivermectin-based premix Cermix. The high efficacy of both these drugs against GI nematodes has been previously demonstrated in various species of cloven-hoofed animals (28, 29). Long-term annual administration of Cermix to fallow deer resulted in a gradual suppression of both the prevalence and the faecal egg counts (FECs) of strongylid nematodes (27). However, the efficacy of any anthelmintics against *A. sidemi* infection has yet to be published.

Monitoring the fluctuation of GI nematode populations is traditionally based on FECs; however, the vast majority of trichostrongylids (including *A. sidemi*) cannot be reliably distinguished based just on morphological identification of nematode eggs (30). A molecular method was proposed earlier that enables reliable *A. sidemi* identification based on larval DNA isolates (31). This is achieved by amplifying specific sequences found in the ITS1 and ITS2 regions of ribosomal DNA using a simple PCR technique. Later, a real-time multiplex PCR method was optimized, allowing the detection of the parasite in co-infection with multiple nematode species (including *A. sidemi*) and semi-quantitative estimation of parasite burden (32).

The current study aims to fill the knowledge gap concerning the seasonality of a non-native nematode *A. sidemi* in cloven-hoofed animals in the current climatic conditions of Central Europe. We conducted a two-year study in a fallow deer reserve under intensive breeding conditions and routine management practices including regular administration of anthelmintics. Molecular techniques were used to monitor *A. sidemi* seasonal dynamics in host fallow deer during the two-year period from February 2018 to February 2020.

2. Materials and methods

2.1. Study site and animals

The seasonal dynamics of *A. sidemi* was monitored on a game reserve in the village of Budyně in the South Bohemian Region of the Czech Republic (49°8′ 48.39″ N, 14°4′ 11.96″ E). According to the Köppen–Geiger climate classification (33) the study location is represented by a warm-summer humid continental climate found in much of Central Europe. This climate is characterized by four distinct seasons with seasonal temperature differences of mild to warm summers, long cold winters, and reduced precipitation (34).

Meteorological data at the study location obtained from the Czech Hydrometeorological Institute are presented in Figure 1.

The game reserve occupies an area of approximately fifty hectares, mostly covered with perennial grassland and a narrow strip of sparse oak grove in the centre. A narrow stream runs through the reserve. A herd of approximately three hundred fallow deer in a balanced ratio of males and females were kept at the game reserve during the study period. Their diet consisted of year-round grazing, except during periods of continuous snow cover, supplemented by continuous feeding of concentrates. Additional feeding was carried out at a single common feeding site, where feed mixed with anthelmintics was also provided at timepoints throughout the grazing season.

2.2. Anthelmintic treatment

The anthelmintic drug Cermix (Biopharm – Research Institute of Biopharmacy and Veterinary Drugs, CR) containing ivermectin was administered annually in January 2018, 2019, and 2020 in the form of medicated feed. In addition, Rafendazol (Biopharm – Research Institute of Biopharmacy and Veterinary Drugs, CR), a mixture of rafoxanide and mebendazole, was applied once at the end of March 2019 by a game reserve owner. Dosage requirements for each anthelmintic were estimated according to the number of animals to reach the therapeutic dose recommended by the manufacturer, i.e., 0.2 mg of ivermectin and 10 mg of mebendazole per kilogram of live body weight. The application of anthelmintics was always preceded by a one-week adaptation to non-medicated concentrated feeds. Anthelmintics were administered on the feeding site in a mixture with concentrates at the prescribed ratio of one part drug to nine parts feed. The medicated mixture was administered according to the drug manufacturer's instructions.

2.3. Sample collection

Freshly voided faecal pellets of fallow deer were collected throughout the game reserve at four-week intervals from 15th of February 2018 to 15th of February 2020 resulting in a total of 25 site visits. During each visit, faecal pellets from 20 animals were collected opportunistically in an attempt to cover the entire area of the reserve and to avoid repeated sampling of individuals. The herd was monitored through binoculars from a sufficient distance to avoid stressing the animals, while the collection of faeces was carried out immediately after their movement to another part of the game reserve. Logistical constraints meant that it was not possible to collect faeces from individual animals using rectal sampling. Furthermore, due to the size of the herd, it was challenging to identify individuals accurately, thus we could not provide information on the sex of the individuals relative to the faecal pellets collected. However, it was possible to differentiate the faecal samples from adult deer and young deer, based on the size of pellets. Faecal pellets were collected exclusively from adult deer as this age group within the herd was more likely to exhibit anthelmintic suppresion of *A. sidemi* egg output based on the anthelmintic administration method. Faeces were collected as soon as possible after defecation to avoid damage of the GI nematode eggs by freezing or desiccation. Faecal pellets collected from 20 individual animals at each site visit were pooled into a single 1 kg

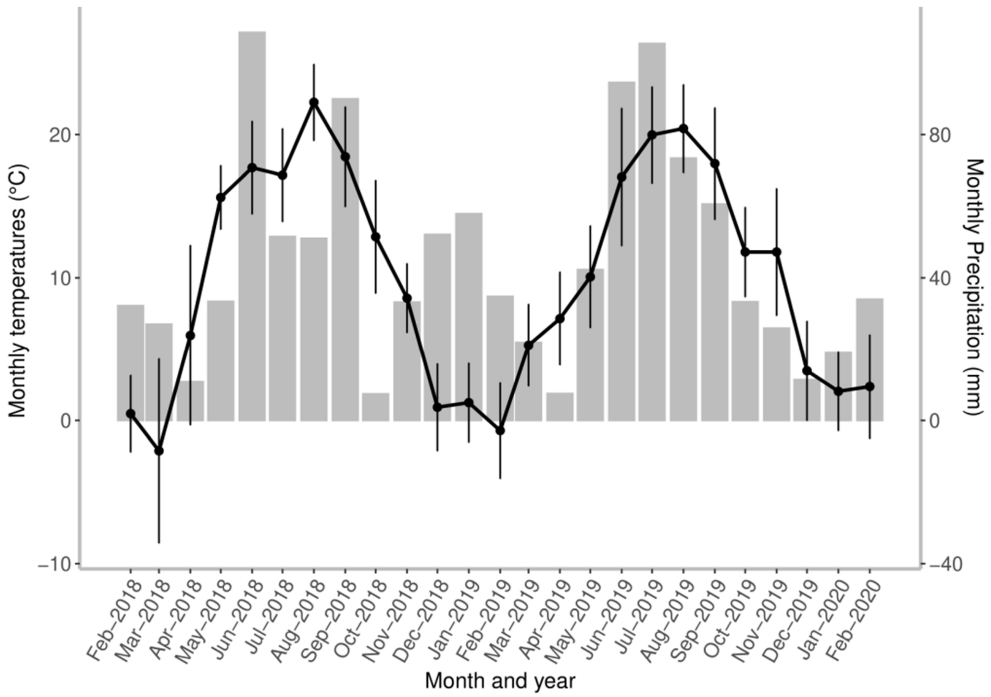

FIGURE 1
The course of environmental conditions shows local changes in mean monthly temperature (black points, standard deviation (SD) and total precipitation (grey bars) measured at the game reserve throughout the study period. All local weather information was obtained from the Czech Hydrometeorological Institute.

sample, stored in an open plastic box, and immediately transported to the laboratory for further processing. Clinical signs of disease were not observed in animals during the study period.

2.4. Parasitology

To examine the presence of *A. sidemi* in faecal samples, separate larval coprocultures were established from pooled faecal pellets collected at each sampling visit ($n = 25$) according to the methods of Hansen and Perry (35). Briefly, faecal material was homogenized, mixed with vermiculite and moistened, then placed in open microtene bags in an incubator for seven days at 27°C. During the incubation period, the coproculture was checked daily, homogenized by rubbing between the fingers to prevent mold formation, and moistened if necessary. At the end of incubation, the coproculture was transferred to the Baermann apparatus for the release of the L3s from the faecal material. After 24 h, the fluid containing the larvae was released into conical glass containers and decanted. The sediment formed by the larvae was subsequently purified by repeated Baermanization and stored at 4°C.

2.5. Molecular analysis

For each sample, total genomic DNA was extracted from L3s using the DNeasy Blood & Tissue commercial kit (QIAGEN, Hilden, Germany). Prior to DNA extraction, larvae were washed several times with sterile distilled water. The pooled L3s were then incubated at 56°C for 48 h in 180 µL ATL buffer and 20 µL proteinase K. Subsequent purification steps followed the manufacturer's protocol. The DNA extracts were immediately stored at −20°C until further processing.

Molecular detection of *A. sidemi* DNA presence and relative quantity within L3 pools was based on triplex real-time PCR using TaqMan technology and consisted of detection systems for (i) *A. sidemi* (targeting ITS1 sequence), (ii) a calibration standard, and (iii) an internal amplification control (IAC), which were adopted from previous studies (32, 36).

The reaction mixture contained: 1× Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA, United States), 250 nM of each primer, 100 nM of FAM probe, 100 nM Cy5 probe, 200 nM of HEX probe, 0.4 U of Antarctic Thermolabile UDG (New England Biolabs, Ipswich, MA, United States), 1×10^4 copies of IAC plasmid, 5 µL of template DNA and nuclease-free water to complete 20 µL volume.

All samples were run in duplicate on a fluorometric thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States) with the following conditions: incubation step at 25°C for 10 min (carryover prevention), initial denaturation at 95°C for 2 min followed by 40 cycles of amplification with 95°C for 15 s for denaturation, and 57°C for 45 s for combined annealing and extension, with the collection of fluorescence signal at the end of each cycle. The data was evaluated using the software Bio-Rad CFX Manager 3.0 (Bio-Rad Laboratories, Hercules, CA, United States).

The calibration curve was constructed using serially diluted plasmid standard (5×10^7, 5×10^6, 5×10^5, 5×10^4, and 5×10^3 copies per µL) and was used to extrapolate the amount of target DNA of *A. sidemi* in each sample. Detailed information on the optimization and design of detection systems, primer and probe sequences, and quantitative data evaluation is provided in the publication (32).

2.6. Data analysis

We considered two dependent variables: (i) the presence or absence of *A. sidemi* DNA (coded as 1 or 0) and (ii) the relative quantity of *A. sidemi* DNA. We tested the effect of external conditions and anthelmintic treatment on each variable. Data analysis consisted of two steps. First, the effect of these factors on the presence of the target *A. sidemi* DNA was tested separately for each explanatory variable using simple logistic binomial regression. Then, the relative level of DNA identified in the positive samples was modeled using quasi-Poisson generalized linear regression.

The explanatory variables included the average daily temperatures (°C) and total rainfall (mm) measured four weeks prior to each sample collection and factor of treatment (weeks post administration) expressed as the number of weeks elapsed between the last application of the anthelmintic drug and the sampling date. p values ≤ 0.05 were considered significant. All statistical tests were performed in R 4.1.3 (37). Data are presented in graphs created using the ggplot2 R package (38).

3. Results

The presence of *A. sidemi* DNA was molecularly confirmed in 17 out of 25 larval pooled samples (68%). The probability of target parasite DNA being detected in samples was positively ($p = 0.048$) affected by the time elapsed since anthelmintic administration (Table 1). The seasonal *A. sidemi* patterns detected within the study are presented in Figure 2. Samples collected during the first three visits, from mid-February to mid-April 2018 tested negative for *A. sidemi* DNA. The parasite was first detected fourteen weeks after the Cermix administration in May 2018. A similar pattern was observed in 2019; after the second application of Cermix in late January, the parasite was not observed for ten weeks, and the emergence of *A. sidemi* DNA was first detected in mid-April 2019, a month earlier than in the previous year. Furthermore, following the January application of Cermix in early 2020, the samples collected yielded negative results.

An interruption of the spring rise in target DNA level and a decrease to zero was observed in May 2019, two weeks after Rafendazol administration; however, over the next four weeks, there was a rapid recovery in measured DNA amount, and the amount of *A. sidemi* DNA detected in June 2019 exceeded the April 2019 values.

The probability of the presence of *A. sidemi* DNA was positively influenced by the mean temperature measured four weeks prior to sampling (Table 1). With the exception of April 2018 and May 2019, cooler temperatures below 5°C preceded the collection of all negative samples (Figure 2).

The abundance of target DNA detected in positive samples did not show high variation and was only slightly affected by the mean temperature prior to sampling (Table 1). Relative *A. sidemi* DNA quantities detected during the first season remained at a relatively consistent level from May to November 2018. The first noticeable decline in *A. sidemi* DNA relative quantity occurred between November and December 2018, but the parasite was still detected

TABLE 1 Model estimates of logistic regression and quasi-poisson generalised linear regression (GLM) testing influence of temperature, rainfall and anthelmintic treatment on presence of *A. sidemi* DNA in all samples.

Factor	Presence/absence (logistic regression)		DNA level (GLM)	
	Coefficient ± SD	*p*-value	Coefficient ± SD	*p*-value
Mean temperature	0.280 ± 0.112	0.013	0.021 ± 0.010	0.044
Rainfall	0.044 ± 0.024	0.076	0.001 ± 0.002	0.606
Wpa	2.467 ± 1.250	0.048	0.037 ± 0.021	0.092

Analyses of relative quantity of *A. sidemi* DNA was performed on positive samples only. SD – Standard Deviation, Wpa – weeks post anthelmintic administration.

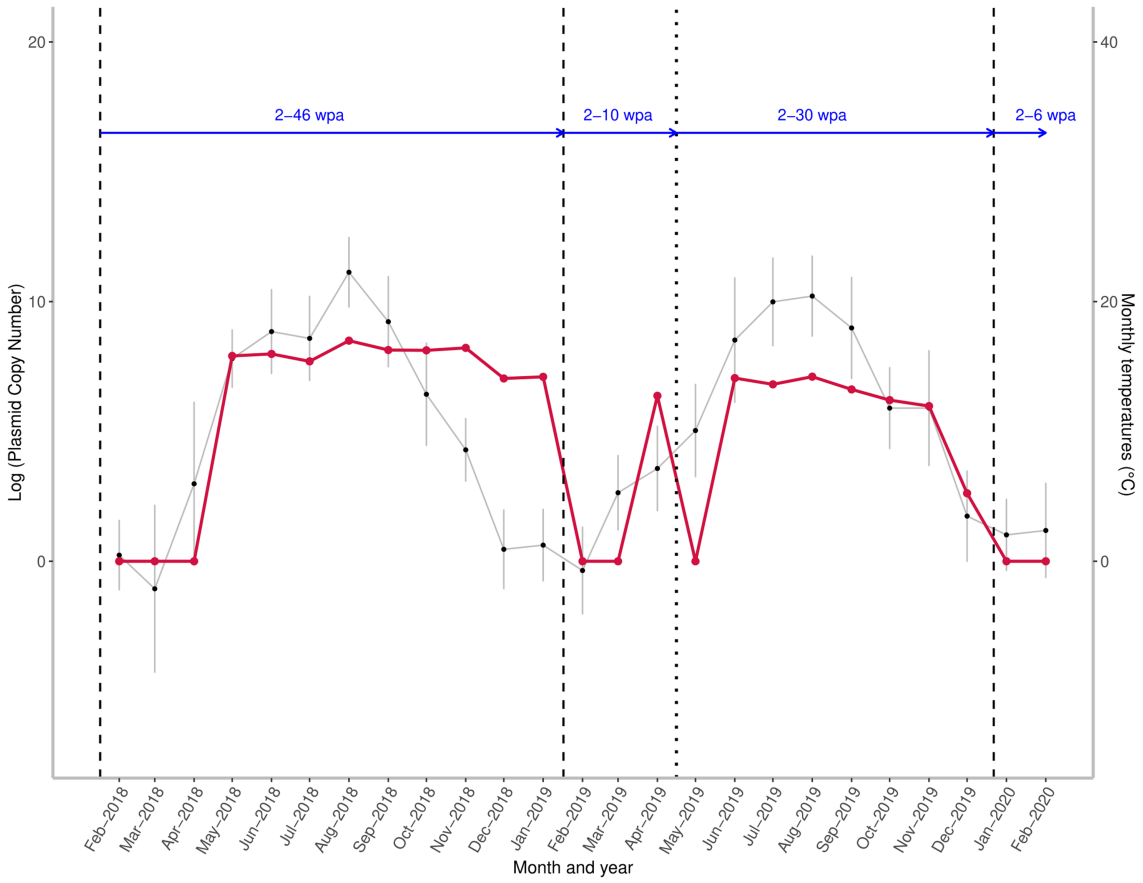

FIGURE 2
Seasonal variation in the quantity of the target A. sidemi DNA at a fallow deer game reserve between 2018 and 2020 relative to administration of anthelmintic and course of average monthly temperatures. Red line shows the fluctuation of parasite DNA levels expressed as the logarithm to base 10 (log) of the plasmid standard copy number. Vertical lines indicate the time of the Cermix (dashed) and Rafendazol (dotted) anthelmintic applications. Blue horizontal arrows represent the number of weeks elapsed since the last anthelmintic application (wpa – weeks post administration). Monthly average temperatures are denoted by black points.

until mid-January 2019. Dynamics of *A. sidemi* DNA in larval samples, and thus pasture contamination throughout 2019, showed a similar pattern to the previous year; after a moderate decline in July 2019, a peak was recorded in August 2019, followed by a gradual decline during the autumn and winter.

No significant impact of precipitation on either the presence or the abundance of parasite DNA was observed in this study (Table 1). The median of *A. sidemi* DNA values detected in the 2019 samples was significantly lower compared to the previous year ($p < 0.01$). The maximum values detected in the summer 2019 were approximately equal to the minimum values of the positive samples in 2018.

4. Discussion

We determined the presence and relative quantity of *A. sidemi* DNA from L3s coprocultured from faecal samples collected from fallow deer in a game reserve over a two year study (2018–2020) in the CR. The amount of detected *A. sidemi* DNA varied between monthly sampling events, with multiple zero values observed during the winter and spring seasons. The absence of *A. sidemi* DNA from coprocultured L3 consistently appeared no later than two weeks after anthelmintic administration with Cermix or Rafendazol, suggesting a potential decrease in egg production or removal of adult worms in response to

treatment. This finding was consistent with the reduction of strongylid FECs to zero values observed in mouflon from the second day after anthelmintic treatment with Cermix medicated mixture (29). Furthermore, a previous study investigating the seasonal dynamics of strongylids at a fallow deer farm in Poland, observed a temporary decrease in both prevalence and infection intensity after percutaneous administration of ivermectin; however, a resurgence of both characteristics occurred within the following two months (24). Similarly, the absence of the parasite during our survey persisted from one to three months following anthelmintic treatment.

The absence of *A. sidemi* DNA from coprocultured L3 during winter may reflect the natural seasonal patterns of the parasite. Previous post-mortem and coprological examinations of untreated cervids in various temperate regions have shown a lower prevalence and intensity of strongylid infection during the winter period (39, 40). Conversely, some studies have indicated higher prevalences and intensities of trichostrongylids in winter when supplementary feed is provided, suggesting the importance of winter feeding sites for transmission of parasites (41). Based on our results, we cannot definitively determine to what extent environmental conditions contributed to the observed winter reduction of *A. sidemi* DNA from coprocultured L3, as the probability of sample positivity may have been affected by both the time elapsed since anthelmintic treatment and the mean temperature. In the current study, we used published faecal culture protocols which were standardised at all sampling events (35). This technique is suitable for a wide range of GI nematode species; however, the survival rate and successful development of *A. sidemi* to the infective L3 larval stage in coproculture has not been examined. A molecular assay used in this study was previously found capable of identifying the parasite at input DNA levels as low as 5 pg (32). However, due to the assumed low infection intensity, we cannot exclude that the amount of DNA isolated from coprocultured L3 derived from faecal samples collected during the winter were not below the sensitivity threshold of the assay.

Differences in temperature at the study site may have caused variations in the emergence time of *A. sidemi* on pasture following anthelmintic treatment. In 2019, *A. sidemi* DNA was detected in mid-April (10 weeks post treatment application – wpa), whereas in 2018, it was not observed until May (14 wpa). This delay could be attributed to freezing temperatures persisting until March 2018. In contrast, the rapid reappearance of *A. sidemi* DNA four weeks after the administration of Rafendazol in May 2019 may have been the result of favorable conditions for larval development on pasture resulting in infection of grazing hosts, low efficacy of Rafendazol treatment or a combination of both. Potential sources of parasite infection in this herd may have been untreated individuals, especially juvenile hosts acting as a refugia for the parasite. Furthermore, the form of anthelmintic administration may lead to uneven dosage of drugs as the dominant individuals usually get first to the attractive medicated food, leaving weaker animals and especially the younger individuals, to consume remaining feed (42). According to Coop and Kyriazakis (43), the allocation of limited nutrient resources by the host prioritizes growth and reproduction over immunity to parasites. During pregnancy and lactation, both domestic and wild ruminants frequently exhibit periparturient rise (PPR), characterized by an increase of FECs (44, 45). Given that fawning on the monitored reserve occurred from May onwards, we cannot rule out reproducing females as a potentially significant source of *A. sidemi* during the spring and early summer. In August 2018 and, to a lesser extent, in 2019, we observed a slight peak in *A. sidemi* DNA levels, which approximately coincided with the weaning of early summer-born fawns. We did not collect data on juveniles in this study; however, this age category might also have an important role in pasture contamination later in the grazing season as they are exposed to high summer levels of infective larvae as naive hosts.

Contamination of the monitored pasture with *A. sidemi* is likely to have occurred rapidly rather than by a gradual process given the rise in *A. sidemi* DNA levels from coprocultured L3 from zero to near peak levels four weeks after anthelmintic application. This may have been influenced by high animal densities, which could facilitate the transmission of parasites (6). Similarly, rapid reinfection to levels equal to or higher than pre-treatment levels was observed in sheep within six weeks after drug administration (46).

Assessment of *A. sidemi* DNA levels from coprocultured L3 at multiple sampling events showed that the anthelmintic treatment had no significant impact on the long-term amount of DNA detected in the positive samples, suggesting a short-term effect of both anthelmintics used against the parasite. However, the levels of *A. sidemi* DNA detected in 2019 were significantly lower than those detected in 2018, despite environmental conditions being perceived as more favourable for larval development. Since there were no significant changes in the number of animals or herd structure (age or sex groups) between the monitored years, the repeated and frequent administration anthelmintics may have contributed to the decline of *A. sidemi* DNA detected over time. A previous study in a fallow deer reserve in the CR showed that repeated Cermix administration over seven years led to a gradual reduction in general strongylid infection intensity by 75% and complete elimination of *Haemonchus contortus* (27).

The findings from previous studies carried out in Poland and the Russian Far east indicate that the *A. sidemi* egg output and transmission are confined to the period from early summer to September. For example, there was a notable decline in adult numbers during autumn following post-mortem analysis, suggesting *A. sidemi* may preferentially survive the winter months as L4 larvae and juveniles (11, 20). In contrast, our results suggest that under the current Central European climate and management conditions at the study location, *A. sidemi* egg shedding may occur almost year-round from April to January. This finding is consistent with previous studies conducted in the CR that reported the occurrence of *A. sidemi* adults in wild ruminants and lack of hypobiosis during the winter (15, 21). A possible explanation for this shift could be recent higher temperatures that are more favorable for the parasite development than those prevailing during investigations of *A. sidemi* seasonal dynamics fifteen years ago or earlier. According to daily data collected from the European Climate Assessment & Dataset-ECA&D (47), the average minimum temperature in December at the location in our study was found to be 11.55°C higher compared to that of the Russian study in the Primorsky region between 1951 and 1955 (20) and on average 4.55°C higher than in the Polish study conducted between 1997 and 2001 (11). Furthermore, our study site experienced, on average, fewer frost days during December compared to the aforementioned studies. As modeled on the development cycle of the cervid trichostrongylid nematode *Ostertagia gruehneri*, a warmer climate may prolong the transmission of the parasite as mild temperatures facilitate both earlier and later larval development in

spring and autumn, respectively (48). The climatic conditions experienced by the larvae during exogenous development are also one of the factors determining whether they undergo hypobiosis. In the present study, we found a significantly positive relationship between the mean temperature and the non-zero values of the targeted *A. sidemi* DNA, which suggests that changes in contamination levels are likely influenced by the environmental factors that impact parasite development and transmission. On the other hand, the low variability of positive values within a year may indicate a lack of seasonality of the parasite. Changes toward a shift from traditional seasonality to uniform through-year distribution of trichostrongylid infections associated with a warming climate have been reported on sheep farms in Northern Ireland (22). More recently, a similar pattern of year-round egg production with little seasonality has been observed in farmed deer and wapiti in New Zealand (25).

In this study, we investigated seasonal changes in the egg production of an invasive parasite, *A. sidemi*, measured through detection and quantification of target DNA from coprocultured L3 following monthly faecal sampling of fallow deer on a game reserve in the CR. The presence and amount of *A. sidemi* DNA detected was examined relative to environmental factors and anthelmintic treatment, but seasonal parasite dynamics is a complex phenomenon with many factors contributing to its outcome. The seasonal pattern of parasite egg shedding can vary between host sexes due to different behavior and timing of investment into reproduction which subsequently alter immunity to parasites. At the same time, it can vary depending on the age or nutritional status of the host. Due to the unavailability of individual metadata on the sampled animals in our study, we could not assess whether the seasonal dynamics of the parasite differed by host sex, age class, or individual condition. Further assessment of the development and survival of *A. sidemi* life stages must also be an important consideration in the design of future investigations to improve predictions of seasonal dynamics, and thus infection risk attributed to this parasite in time and space. Although our results shed light on seasonal dynamics of *A. sidemi* in the CR, it is important to acknowledge limitations arising from the relatively small number of animals we sampled. Future studies with appropriately estimated sample sizes are needed to understand the epidemiology of ashworthiosis more comprehensively.

5. Conclusion

This study provides new insight into the epidemiology of a non-native nematode in the changing world. Our findings indicate almost negligible seasonal parasite egg shedding patterns in the current climatic conditions of the CR, contrasting with previous studies conducted more than a decade ago. We strongly recommend regular assessment of *A. sidemi* infection in cloven-hoofed animals in game reserves using reliable molecular tools and to take appropriate control measures to prevent the spread of this parasite to wild animal populations. This approach is especially desirable in animal species sensitive to this infection and those kept in conservation programmes. The control of *A. sidemi* is challenging once the parasite is well established among animals/at the locality. The use of anthelmintic drugs has been shown to suppress egg shedding and thus further pasture contamination significantly; however, treatment efficacy relative to the absence of *A. sidemi* DNA in coprocultured L3 in the current study was relatively short-lived. For an improved understanding of *A. sidemi* seasonal dynamics and subsequently development of effective parasite control strategies, it is necessary to investigate the contributions of all cohorts within the herd (including age groups and sexes) to the overall pasture contamination throughout the year.

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 requirement of ethical approval was waived by Institutional ethics and animal welfare committee of the Czech University of Life Sciences Prague for the studies involving animals because faecal samples were collected from the pasture without any direct contact with animals. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because the game reserve owner do not required written consent.

Author contributions

JM: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. LŠ: Data curation, Formal analysis, Methodology, Writing – original draft. CM: Conceptualization, Data curation, Writing – review & editing. JV: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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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.

Publisher's note

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Case-report: Massive infection by *Cysticercus longicollis* in a captive *Lemur catta* from Italy

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An adult male ring-tailed lemur (*Lemur catta*) from a biopark of northern Italy was submitted to necropsy. A multi-organ parasitic infection was macroscopically evident. Abundant sero-hemorrhagic fluid with larval parasites was present in all cavities. The microscopic evaluation of parasites and the molecular characterization revealed the presence of *Cysticercus longicollis* (the larval stage of *Taenia crassiceps*). Histology of liver, lungs, intestine and urinary bladder revealed several larval parasites surrounded by a severe lymphocytic infiltrate, fibrous tissue and hemorrhages. This is the first report of a ring-tailed lemur with an infection of *C. longicollis* in Italy. The source of infection is still not known however, the discovery of this parasite in a captive lemur poses more attention on the control of parasitic diseases implementing monitoring tests and biosecurity measures.

KEYWORDS

***Lemur catta*, *Taenia crassiceps*, zoo, ring-tailed lemur, Italy**

1. Introduction

In the past 20 years, the occurrence of parasitic diseases in captive *Lemur catta* has been largely reported. The ring-tailed lemur is an endangered non-human primate native to Madagascar and its natural habitat is still strictly related only to that country (1). However, this species easily adapts in zoos and wildlife parks worldwide, allowing many conservational programs to preserve this primate species. In this context, an appropriate medical monitoring of captive animals is a fundamental activity in zoo management. Special attention is given to parasitic diseases, in particular as a source of zoonotic threat. Less is known about parasites inhabiting lemurs as definitive hosts, and the only available information are just related to case-reports published in scientific literature (2–6). In only four case-reports are described severe infections in ring-tailed lemurs by *Echinoccous multilocularis* in Japan and France (7, 8) and by *Echinoccous equinus* and *Echinoccous ortleppi* in the UK (9, 10). However, recent findings suggest the presence of an emerging cestode parasite, i.e., *Taenia crassiceps* and its larval form *Cysticercus longicollis*, affecting ring-tailed lemurs in Europe with reported cases in Spain (11), Bosnia Erzegovina (12), Poland (13), and Serbia (14). Moreover, this parasite was also described in a black lemur (*Eulemur macaco macaco*) (15). *Taenia crassiceps* is a cestode parasite with an indirect life cycle, classically found in the northern hemisphere of the world (12). The definitive hosts are wild carnivores, in particular red foxes (*Vulpes vulpes*), artic foxes (*Vulpes lagopus*), and wolves (*Canis lupus*), but sometimes also domestic dogs (*Canis lupus familiaris*) (16). On the other hand, intermediate hosts are wild rodents, which are natural preys of the definitive hosts (16). In carnivores, *T. crassiceps* has a small intestinal localization and proglottids are released with feces (16). In this way, intermediate hosts can develop the larval stage

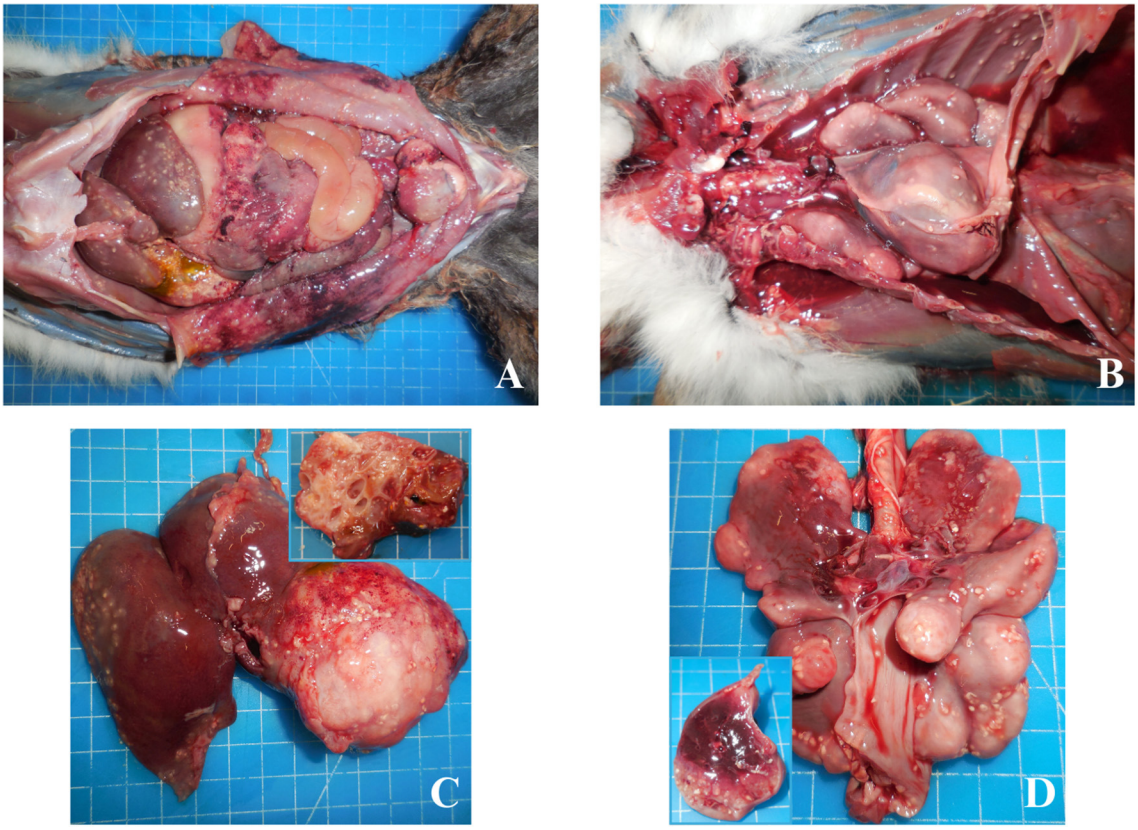

FIGURE 1
Lemur catta; adbomen, torax, liver and lungs. Abundant sero-hemorrhagic fluid with larval parasites was present in the abdomen **(A)** and thorax **(B)**. Enlarged liver with cyst **(C)**, composed of a multilocular structure (insert). Lungs with cystic lesions **(D)**, augmented consistency and with a multilocular structure (insert).

of the disease after the ingestion of proglottids. In rodents, normally *C. longicollis* has a subcutaneous localization and sometimes also within body cavities (16). In addition, *T. crassiceps* is also a zoonotic parasite and primates can become intermediate hosts trough the consumption of contaminated food or water (14). In human, the disease is mainly described in immunocompromised patients with subcutaneous and muscle cysticercosis (17). As already described, also non-human primates can be susceptible to *C. longicollis* infection suggesting an emerging zoonotic role for this parasite. In this context, the main aim of this case-report study is to describe the case presentation and diagnostic assessment of the first reported observation of *T. crassiceps* cysticercosis in a captive ring-tailed lemur (*L. catta*) in Italy.

2. Case description

In May 2022, an adult male ring-tailed lemur (*L. catta*) from a biopark of northern Italy was submitted to necropsy at the Department of Veterinary Sciences in Turin. The animal was living with other lemurs and was donated in 2012 from a zoo in Sicily. In the park, lemurs were living on an artificial island without any possibility of contact with domestic or wild canids. Before its death, the lemur was the only one in the group with a swollen abdomen, but with normal appetite and general condition. After induction and mask maintenance with isoflurane dose-effect, the animal was subject to clinical examination and abdominal echography revealing the presence of a severe abdominal ascites, with the presence of severe abdominal effusion and of a 5 cm diameter cyst in abdomen. Therefore, the abdominal fluid collected by ultrasound-guided cystocentesis was characterized by citric color and full of small white particles, which were examined at light microscopic confirming the presence of parasites infection. The day after, the animal was subjected to an explorative laparotomy with the aim of removing the cyst and performing an abdominal lavage. However, due to the too severe clinical presentation with massive parasitic infection the animal was euthanized. The remaining lemurs of the group were subjected to clinical examination and echography excluding other infested cases, nevertheless all animals were still treated with a therapeutic protocol of albendazole (10 mg/kg once a day for 3 consecutive days and repeated with same protocol after 2 weeks).

3. Diagnostic assessment

At necropsy, a multi-organ parasitic infection was macroscopically evident. Abundant sero-hemorrhagic fluid with larval parasites was present both in abdomen (Figure 1A) and thorax (Figure 1B). The liver, the main organ presenting lesions,

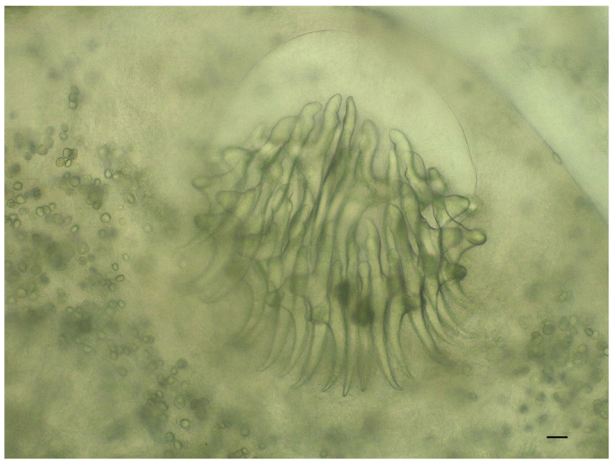

FIGURE 2
Taenia crassiceps. Scolex with hooks of the cysticercus, isolated from liver (200×, bar 500 μm).

was enlarged with a 5 cm diameter cyst in the parenchyma with a multilocular structure, filled with citrine fluid and larval parasites (Figure 1C). Cystic lesions were bilaterally also found in the lungs, with variable dimensions from 1 to 3 cm, augmented consistency and with a multilocular structure (Figure 1D). The presence of peritonitis, pleuritis and pericarditis was also recorded, together with the presence of disseminated larval parasites in all serosae. Kidneys and the remaining organs had no evident macroscopic lesions. Samples for histological, parasitological, and biomolecular investigations were taken and appropriately stored. Liver, kidney, seminal vesicles, urinary bladder, intercostal skeletal muscle, lung, heart and spleen were collected and fixed in 4% buffered formalin. The abdominal wall, spleen, kidney, lung, liver and seminal vesicles were also collected and immediately frozen at −20°C for microbiological and parasitological investigations. In addition, feces were collected from rectum for parasitological investigations.

The microscopic observation of parasites was consistent with *T. crassiceps* cysticerci (Figure 2). In particular, in line with literature (18–20), we emphasize: (i) the high number of ovoid to elongate cysticerci, several hundred in number; (ii) the variable size of cysticerci, with the largest ones ranging between 2–7 mm in length and 1–3 mm in width; (iii) in some of the larger cysticerci, the occurrence of endogenous and/or exogenous budding at the end opposite the invaginated scolex; (iv) the number of rostellar hooks, in the range of 30–34/scolex, including a similar number of small and large hooks; (v) the length of fully developed hooks, in the order of 150 and 120 μm for large and small hooks, respectively, and their shape, with the blade appearing much longer than the handle (approximately the double).

DNA was extracted from two cysticerci using DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's tissue protocol; the lysis step was carried out at 56°C overnight with Proteinase K. The PCR protocol described by Bowles et al., targeting the cytochrome C oxidase subunit I mitochondrial (mtDNA cox1) gene was conducted on extracted DNA (21). The PCR mixture contained 2.5 μl of template DNA (5–20 ng/μl), 0.5 mM of each primer (JB3 TTTTTTGGGCATCCTGA GGTTTAT and JB4.5 TAAAGAAAGAACATAATGAAAATG), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Platinum Taq DNA polymerase, 10 × PCR buffer and RNase-free water to a total volume of 25 μl. The amplification was performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, CA, USA) with the following cycling profile: a denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 40 s, 55°C for 30 s and 72°C for 30 s and final extension 72°C for 5 min. Positive results were observed with an agarose gel electrophoresis. Subsequently, amplicons were sequenced to confirm parasite species. Amplification products were purified with Exo-Sap (USB Europe, Staufen, Germany) treatment according to the manufacturer's recommendations. Forward and reverse sequencing reactions were performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA). Sequenced fragments were purified by DyeEX (Qiagen, Hilden, Germany) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA). Forward and reverse sequences were manually assembled into consensus sequences using the Alignment Explorer within MEGA X (22).The nucleotide sequences were analyzed using the BLASTN sequence similarity search at the NCBI database. The sequencing of the amplified products resulted in two sequences of 433 bp each, with 100% identity with published sequences *T. crassiceps* (accession number KY321321). Sequences generated in the present study were submitted in GenBank under accession numbers OR350515 and OR350516. Fecal samples for the case and for all the other lemurs in the same environment were analyzed with flotation technique for parasitological diagnosis (23). Samples tested negative for any intestinal parasites.

Histology of liver revealed several larval parasites surrounded by a severe lymphocytic infiltrate, fibrous tissue, hemosiderosis and hemorrhages (Figures 3A, B). Lungs (Figures 3C, D) presented inflammatory lesions similar to those observed in the liver. Moreover, moderate emphysema, oedema and focal necrosis were observed. Intestine was hyperemic with focal hemorrhages in the muscular layer and inflammatory lesions associated to parasites, similar to the abovementioned organs, were also observed. Pleura and peritoneum were thickened, and severe lymphocytic infiltrates were also present. In the urinary bladder severe congestion and perivascular lymphocytic infiltrate with fibrous tissue and hemorrhages were observed. Spleen presented severe lymphocytic depletion and diffuse subcapsular hemorrhages. The intercostal skeletal muscle was characterized by parasitic cysts surrounded by a severe lymphocytic infiltrate, fibrous tissue and hemorrhages both in the epimysium and within the muscular fibers. Kidney and heart did not present any histological lesions.

Finally, microbiological investigations have been conducted by an external laboratory. The presence of the main non-human primate pathogens has been investigated in organs collected during necropsy. In particular, Simian immunodeficiency virus (SIV), Simian Type D Retrovirus (SRV) and Herpesvirus of exotic animals (HVES), but also of *Neospora caninum* and *Toxoplasma gondii* have been investigated by PCR or sequencing (for HVES) according to

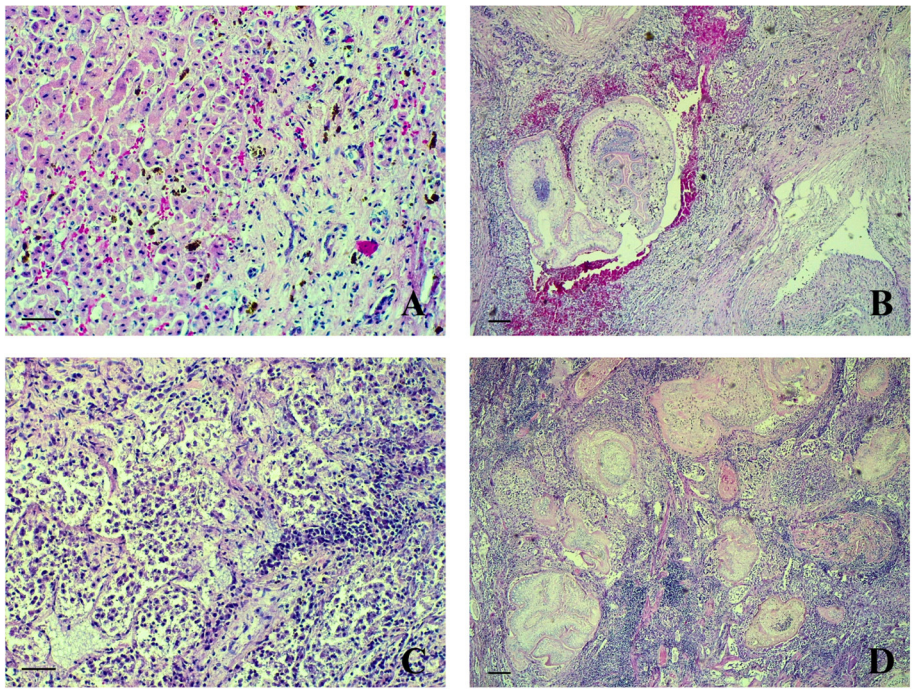

FIGURE 3
Lemur catta, liver, lungs. Lymphocytic infiltrate, fibrous tissue and hemorrhages in the liver **(A)** and lungs **(C)** (HE, 200×, bar 500 μm). Larval parasites in the parenchyma of liver **(B)** and lungs **(D)** (HE, 40×, bar 1,000 μm).

the external laboratory routine methods. Moreover, bacteriological culture was performed through non-selective conditions according to the external laboratory routine methods. All microbiological tests resulted negative for the research in all collected organs of the abovementioned pathogens.

4. Discussion

According to previous scientific literature, this is the first report of a ring-tailed lemur with an infection of *C. longicollis* in Italy. Case presentation and related findings were similar to those already reported in other European countries (Spain, Bosnia Herzegovina, Poland and Serbia) (11–14). Therefore, this study represents the fifth published case report of a *T. crassiceps* cysticercosis in a captive ring-tailed lemur (*L. catta*) worldwide.

Considering that lemurs were living on an artificial island without any possible contact with domestic or wild canids, the most likely route of infection of this atypical intermediate host is contaminated water or feed. Nevertheless, the proliferative nature of *T. crassipes* cysticercosis deserves caution in ruling out other possible sources, as suspected in humans (17). Although the source of infection is still unknown, the discovery of this parasite in a captive lemur poses more attention on the control of parasitic diseases by implementing monitoring tests and biosecurity measures.

In the present case, the lemur did not present a subcutaneous infection, which is typical of intermediate and paratenic hosts. In fact, macroscopic cutaneous lesions were not observed, as reported in previous case reports (11, 13, 14). The massive infection found involving all body cavities and organs is similar to the case report of Alić et al. (12). These findings may suggest a correlation with an immunocompromised status, as described in humans (24, 25), and also in carnivores (20), where patients were diagnosed with an immune deficiency syndrome or HIV positive. Even if an immune deficiency of the lemur during life could not be excluded, the subject tested negative for bacteriological and virological (SIV and SRV) diseases. Further studies have to be conducted to evaluate the invasive and aggressive nature of this parasite in lemurs.

The negative results at parasitological investigation prove that the lemur was not eliminating the parasite through feces confirming its role as intermediate host.

5. Conclusions

The prevalence of *C. longicollis* in *L. catta* was never investigated worldwide, moreover, the prevalence of the parasite is not known also in intermediate hosts belonging to wild rodent species. In recent years, epidemiological investigation has been conducted in Italy on wild wolves and red foxes reporting some positive results for *T. crassiceps* in these species and confirming the presence and dissemination of the parasite in the area (26, 27).

In conclusion, further studies are need for a better understanding of *T. crassiceps* prevalence in definite and intermediate hosts in Italy. In addition, epidemiological studies evaluating the role of rodents as intermediate hosts of the parasite and their relationship with captive animals are needed. In this way, a better clarification of the epidemiological aspects of *T.*

crassiceps will allow an optimal implementation of prophylactic and biosecurity measures in zoos and wildlife parks.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/genbank/, OR350515-OR350516.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the lemur was submitted to necropsy after its death as a regular procedure of the zoo. This action did not require an ethical approval.

Author contributions

MC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. SR: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. LR: Methodology, Writing – review & editing. SP: Methodology, Resources, Writing – review & editing. FS: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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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.

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Molecular survey and phylogenetic analysis of *Bartonella* sp., *Coxiella* sp., and hemoplamas in pudu (*Pudu puda*) from Chile: first report of *Bartonella henselae* in a wild ungulate species

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Introduction: Recent evidence shows a high diversity of infectious agents in wildlife that represent a threat to human, domestic, and wild animal health. In Chile, wild populations of the most common cervid species, pudu (*Pudu puda*), have been reported as hosts for novel pathogens such as *Mycoplasma ovis*-like and a novel ecotype of *Anaplasma phagocytophilum*. A better understanding of the epidemiology of this group and other intracellular bacteria that might have cervids as hosts would enlighten their population relevance. This study aimed to determine the occurrence and genetic diversity of *Bartonella* spp., hemotropic mycoplasmas, and *Coxiella burnetii* in pudus from Chile.

Methods: The DNA was extracted from the blood samples of 69 wild free-ranging and 30 captive pudus from Chile. A combination of real-time (nouG gene for *Bartonella* and IS1111 element for *C. burnetii*) and conventional PCR (16S rRNA for hemotropic *Mycoplasma* spp. and rpoB, gltA, and ITS for *Bartonella* spp.) was used for pathogen screening and molecular characterization.

Results: DNA of *Bartonella* spp. was detected in 10.1% [95% CI (5.2–18.2%)] samples, hemotropic *Mycoplasma* spp. in 1.7% [95% CI (0.08–10.1%)], and *C. burnetii* in 1.0% [95% CI (0.05–6.3%)] samples. Two sequenced samples were identified as *Mycoplasma ovis*-like, and one free-ranging pudu was positive for *C. burnetii*. While one captive and two free-ranging pudus were positive for *Bartonella henselae*, one wild pudu was co-positive for *B. henselae* and *Bartonella* sp., similar to *Bartonellae* identified in ruminants.

Discussion: To the best of our knowledge, this is the first report of *B. henselae* in wild ungulate species, and *C. burnetii* and *Bartonella* spp. in wild ungulate

species in South America. Further research will be necessary to evaluate the potential role of pudu as reservoirs of infection and identify the sources for disease transmission among humans and wild and domestic animals.

KEYWORDS

zoonotic diseases, wildlife host, endangered species, *Coxiella burnetii*, hemoplasmas

1 Introduction

The recognition of the role of wildlife as reservoirs of pathogens that threaten the health of humans and/or livestock species has increased in the past several decades; accordingly, the relevance of infectious agents in the wildlife conservation field has also amplified (1, 2). As expected, there are differences between regions of the world. In South America, for example, there is a lack of scientific publications on infectious diseases in wildlife when compared with more developed countries (3–6).

Bartonella spp., *Coxiella burnetii*, and hemotropic *Mycoplasma* spp. are intracellular bacteria that infect a wide range of animals (7–9) and humans. Hemotropic mycoplasmas (hemoplasmas) are obligate epi-erythrocytic, cell wall-deficient bacteria that usually generate hemolytic anemia in numerous animal species. Routes of transmission are not fully elucidated, but aggressive interactions and possibly fleas and ticks might be involved. The pathogenic potential of hemotropic mycoplasmas, as a cause of human disease, has not been clearly defined; the public health implications derived from these emerging zoonotic pathogens are underestimated (10). *Bartonella* is composed of gram-negative fastidious, facultative intracellular microorganisms transmitted by fleas and other vectors that provoke a long-lasting bacteremia in the mammal host. The zoonotic potential of these bacteria is well described, and the term bartonellosis has been implemented to refer to human diseases (11). *Coxiella burnetii* is a zoonotic, strictly intracellular gram-negative bacterium that infects a wide range of animals. In its sylvatic cycle, it can be transmitted by ticks. In humans, it is considered the causal agent of query fever (Q-fever), and the Centers for Disease Control and Prevention (CDC) has classified this microorganism as a potential bioterrorism agent (12).

There is increased evidence that wildlife species are also susceptible (13–15) and have the potential to be zoonotic (7, 16, 17). In Chile, several domestic and wildlife species have been identified as potential hosts for several hemoplasmas (18–22) and *Bartonella* spp. (23–28). Information on *Coxiella burnetii* is much more limited, with only one report with molecular evidence in bats from Chile (26). Despite being commonly reported in domestic and wild ruminants in Europe and North America (13, 29, 30), there are no studies for the detection of *Bartonella* spp. and *C. burnetii* in these taxa in Chile, and only until recently has it been possible to identify hemoplasmas in domestic camelids, llamas (*Lama glama*), and alpacas (*Vicugna pacos*) (31). Finally, the native pudu (*Pudu puda*) has been identified as the potential host species of several hemoplasmas (10), including *Mycoplasma ovis*-like, in the Chilean template forest.

Pudu is the most common cervid in Argentina and Chile and is considered threatened in both countries (32, 33), as shown in CITES Appendix I. In Chile, pudus inhabit temperate forests heavily affected by anthropic factors such as deforestation, housing construction, free-ranging dogs, and livestock (34). Additionally, a high diversity of infectious agents that could be a threat to their health status has recently been identified (10, 35–38). Recently, pudus were identified as potential reservoir hosts for the bovine viral diarrhea virus, which is a cause of major disease in cattle (39). This study aimed to determine the occurrence and genetic diversity of *Bartonella* spp., hemotropic mycoplasmas, and *C. burnetii* in free-ranging and captive pudus from Chile.

2 Materials and methods

2.1 Animal sampling

Blood samples from frozen banks in rescue centers and zoos/breeding centers were used. The frozen bank samples were opportunistically collected from 69 free-ranging pudus between 2016 and 2022 on admission day from two wildlife rehabilitation centers in the template forest ecosystem of southern Chile in Los Lagos District, one (USS: Cerefas, Universidad San Sebastian) located in the continental area and the other (Ch. S: Chiloe Silvestre NGO) in Chiloe island. Additionally, blood samples from 30 captive pudus were collected between 2017 and 2021 during preventive medicine procedures in two facilities, one located in the Mediterranean ecosystem of Central Chile in the Metropolitan District and the other in Los Lagos District, and do not have contact between centers. Blood samples were obtained by venipuncture of the jugular vein using an evacuated tube system (Vacutainer, Beckon, Dickson and Company, Franklin Lakes, New Jersey, USA) and stored at −20°C within 6 h of collection. For extensive sampling details, refer to the study mentioned in the reference (10).

2.2 Molecular detection and phylogenetic analysis

2.2.1 DNA extraction/purification

The 99 frozen EDTA-blood samples were thawed at room temperature and vortexed at the UACh Veterinary Clinical Pathology Laboratory, Valdivia, Chile. DNA extraction from 200 µl of blood was performed using an E.Z.N.Z. Tissue DNA Kit (E.Z.N.A. Omega BioTek®, Norcross, GA, U.S.A.), according to the manufacturer's instructions, to obtain a concentration

between 20 and 50 ng/μl of purified DNA. Concentration and purity of DNA were measured (NanoDrop ND-1000, Thermo Scientific©, Waltham, MA, U.S.A.). The 260/280 nm absorbance ratio (OD_{260}/OD_{280}) provided an estimate of sample purity, accepting a ratio of 1.8 ± 0.2 as pure. DNA was stored at −20°C before performing PCR assays.

2.2.2 Endogenous control conventional (c) PCR

DNA samples were subjected to qPCR targeting the irbp gene (interphotoreceptor retinoid-binding protein) using the primers IRBP-CF_FWD (5′-TCCAACACCACCACTGAGATCTGGAC-3′) and IRBP-CF-REV (5′-GTGAGGAAGAAATCGGACTGGCC-3′), with the aim to check DNA template integrity and discard the presence of PCR inhibitors, as previously described (40). All cPCRs were performed with nuclease-free water as a negative control in a T100TM Thermal Cycler (Bio-Rad).

2.2.3 Quantitative real-time PCR for *Bartonella* spp. screening

To detect and quantify *Bartonella* spp., the DNA of all irbp cPCR-positive samples were subsequently subjected to an initial screening by quantitative real-time PCR (qPCR) targeting the *nuoG* gene of *Bartonella* spp. (83 bp), using primers (F-Bart [5′-CAATCTTCTTTTGCTTCACC-3′] and R-Bart [5′-TCAGGGCTTTATGTGAATAC-3′], hydrolysis probe (TexasRed-5′- TTYGTCATTTGAACACG-3′[BHQ2a-Q]3′) as previously described (41). qPCR amplifications were conducted in Hard-Shell PCR plates (Bio-Rad©, CA, USA) using Thermal Cycler CFX96 Touch Real Time (Bio-Rad, CA, USA). Amplification efficiency (E) was calculated from the standard curve slope in each run using the following formula: (E = 10–1/slope). Copy numbers were estimated using 10-fold serial dilutions of gBlock® (Integrated DNA Technologies, Coralville, IA, U.S.A.), encoding the *nuoG B. henselae* sequence (insert containing 83 bp). *Bartonella henselae* genomic DNA from a cat tested in a previous study was used as a positive control (42). All PCR runs were performed with nuclease-free water (Promega®, Madison, WI, USA) as a negative control. Replicates showing a Cq difference higher than 0.5 were retested.

2.2.4 Conventional (c) PCR for *Bartonella* spp. characterization

All positive *Bartonella* spp. *nuoG*-qPCR positive samples were subjected to cPCR amplification of a fragment of three loci [*gltA* (43), *rpoB* (44), and ITS (45)] by cPCR with the aim to molecularly characterize *Bartonella* spp. cPCR amplification reactions were performed in a T100 Bio-Rad thermocycler (Bio-Rad©, Hercules, CA, U.S.A.), and the details of the amplification conditions are presented in Table 1. *Bartonella henselae* genomic DNA from a cat tested in a previous study was used as a positive control (42).

2.2.5 Quantitative real-time PCR for Coxiella burnetii screening

The screening real-time qPCR targeted a 295-bp fragment of the multicopy insertion element IS1111 and is used for sensitive detection of *C. burnetii* in biological samples (46) (Table 1). Primers Cox-F (GTC TTA AGG TGG GCT GCG TG) and Cox-R (CCC CGA ATC TCA TTG ATC AGC) and hydrolysis probe Cox-TM (FAM-AGC GAA CCA TTG GTA TCG GAC GTT–TAMRA–TAT GG) were used. Standard curves were constructed using 10-fold serial dilutions (2.0×10^7 to 2.0×10^0) of a gBlock® (Integrated DNA Technologies, Coralville, IA, USA), encoding a 295-bp fragment of the IS1111 element of *C. burnetii* (Integrated DNA Technologies, Coralville, IA, USA). Amplification efficiency (E) was calculated from the standard curve slope in each run using the following formula (E = 10–1/slope). *Coxiella burnetii* genomic DNA from a cow was used as a positive control. All PCR runs were performed with nuclease-free water (Thermo Scientific©, Waltham, MA, USA) as a negative control. Replicates showing a Cq difference higher than 0.5 were retested.

2.2.6 Conventional (c) PCR for hemotropic *Mycoplasma* spp.

All positive samples in the irbp cPCR were subjected to a cPCR protocol targeting the 16S rRNA hemotropic *Mycoplasma* spp. gene (620 bp), using HemMycop16S-322s and HemMycop16S-938as primers (Table 1), according to a previously described protocol (7). All cPCR runs were performed with nuclease-free water (Thermo Scientific©) as a negative control, and a cat sample known to be infected by *M. haemofelis* was used as a positive control. This protocol was used for screening and later sequencing for molecular characterization of detected hemoplasmas.

2.2.7 Electrophoresis

Conventional PCR products were separated by 1.5% agarose gel electrophoresis (LE Agarose Seakem®, Lonza) and stained with SYBR© safe DNA gel stain (Thermo Scientific©). The DNA products with the expected size were purified and sequenced.

2.2.8 Purification and sequencing

Only positive samples presenting strong band intensity (*Bartonella* spp. and hemotropic *Mycoplasma* spp.) were purified by enzymatic reaction using ExoSAP- IT™ PCR Product Cleanup Reagent (Thermo Scientific©, Carlsbad, CA, U.S.A.), following the manufacturer's instructions. Purified DNA was sent to MACROGEN (Seoul, Korea) for sequencing by the Sanger method in an automatic sequencer (A.B.I Prism 310 genetic analyzer; Applied Biosystem ©/PerkinElmer) for species identification. Forward and reverse sequences were analyzed in Geneious 7.1 (https://www.geneious.com), to obtain consensus sequences. Identity percentages were obtained using BLASTn (47).

2.3 Phylogenetic analysis

Before constructing the phylogenetic inference, sequences belonging to different samples, but representing the same bacterial species, were aligned with Geneious 7.1 (https://www.geneious.com) using the MAFFT alignment

TABLE 1 Summary information of the conventional and Real time PCR primer sets, amplification conditions and their amplicon sizes used in the present study.

Target	Primers	Amplification cycles	Amplicon size (pb)	Reference
Endogenous control				
Interphotoreceptor Retinol-Binding Protein (IRBP)	IRBP-CF_FWD (5′-TCCAACACCACCACTGAGATCTGGAC-3′) IRBP-CF-REV (5′-GTGAGGAAGAAATCGGACTGGCC-3′)	95°C × 4min 94°C × 30s 52°C × 30s 72°C × 1 min 72°C × 5 min 35cycles	227	(40)
Screening real time PCR				
Nicotinamide adenine dinucleotide dehydrogenase gamma subunit (NUOG) gene of *Bartonella* spp.	F-Bart (5′-CAATCTTCT TTTGCTTCACC-3′) R-Bart (5′-TCAGGGCTTTAT GTGAATAC-3′) Hydrolysis probe: TexasRed-5′-TTYGTCATTTGAACA CG-3′[BHQ2a-Q]3′	95°C × 3 min 95°C × 10 min 52.8°C × 30s 40 cycles	83	(41)
Multicopy insertion sequence (Is111) of *Coxiella Burnetii*	Cox-F: (5′-GTCTTAAGGTGGGCTGCGTG-3′) Cox-R: (5′-CCCCGAATCTCATTGATCAGC3′) Hydrolysis probe: FAM-5′-AGCGAACCATTGGTATCGGACGTT- 3′TAMRA-TAT GG	50°C × 2 min 95°C × 10 min 95°C × 15s 60°C × 30s 45 cycles	295	(46)
Conventional PCR molecular characterization				
Citrate synthase (*GLTA*) gene of *Bartonella* Spp.	CS443f (5′-GCTATGTCTGCATTCTATCA -3′) CS1210r (5′-GATCYTCAATCATTTCTTTCCA -3′)	94°C × 2 min 94°C × 30s 48°C × 1 min 72°C × 1 min 72°C × 5 min 45 cycles	767	(43)
Intergenic tegion 16s-23s rRNA (ITS) of *Bartonella* Spp.	325s (5′-CTTCAGATGATGATCCCAAGCCTTYTG GCG -3′) 1100as (5′- GAACCGACGACCCCCTGCTTGCAAAGC A-3′)	95°C × 5 min 94°C × 15s 66°C × 15s 72°C × 15s 72°C × 1 min 55 cycles	453- 717	(45)
β subunit of rna polymerase (*RPOB*) of *Bartonella* spp.	rpoBF (5′-GCACGATTYGCATCATCATTTTCC-3′) rpoBR (5′-CGCATTATGGTCGTATTTGTCC-3′)	95°C × 5 min 94°C × 45s 52°C × 45s 72°C × 45s 72°c × 7 min 40 cycles	333	(44)
16s *rRNA* gene of Haemotropic *Mycoplasma* spp.	HemMyco16S-322s: GCCCATATTCCTACGGGAAGCAGCAGT HemMyco16S-938as: CTCCACCACTTGTTCAGGTCCCCGTC	95°C × 5 min 94°C × 15s 68°C × 15s 72°C × 18s 72°C × 30s 55 cycles	620	(45)

method (48) and subsequently analyzed for detection of polymorphism and haplotype identification using DnaSP v5 software (49).

The sequences of the present study were aligned with other sequences from the database (GenBank) through the MAFFT program (Multiple Alignment by Fast Fourier Transform) (48) incorporated in Geneious 7.1 software (https://www.geneious.com). Then, multiple alignments were analyzed using BMGE (Block Mapping and Gathering with Entropy) software to remove ambiguously aligned regions (50).

For the phylogenetic analysis, the best evolutionary model was selected according to the Bayesian Information Criterion (BIC) for each one of the codon positions (partition) for the encoded genes (*gltA* and *rpoB*) (51). Thus, the best evolutionary models for *Bartonella* spp. *gltA* were K3P+G4 (partition 1), TIM3+F+G4 (partition 2), and TNe+G4 (partition 3). For *Bartonella* spp. *rpoB*, the best models were TPM3u+F+G4 (partition1), TNe+G4 (partition 2), and TIM3e+G4 (partition 3). For the non-coding genes (ITS), the best evolutionary model was selected according to the Akaike information criterion (AIC) (52). The best model for *Bartonella* spp. ITS was TPM2u+F+G4. For ITS, the best evolutionary model selection was assessed using Model Finder (53). Finally, all trees were inferred with a bootstrapping of 1,000 by the maximum likelihood (ML) method with IQ-TREE (54). To enroot the trees, the outgroups were the following for the *Bartonella* spp. trees: *Ochrobactrum anthropii* (*gltA*, ITS, *rpoB*), *Brucella abortus* (*gltA*, *rpoB*), and *Brucella melitensis* (ITS). *Mycoplasma pneumoniae* was used as an outgroup for the construction of the 16S rRNA hemoplasma tree.

3 Results

3.1 *Bartonella* spp. qPCR results

All DNA samples (median and standard deviation (SD) of DNA concentration = 31.5 ± 56.2 ng/uL; mean and SD 260/280 ratio = 1.3 ± 0.35) were positive for the *irbp* gene.

Molecular occurrence of *Bartonella* spp. DNA in pudu detected by qPCR (mean and SD of reactions' efficiency = 100 ± 5.04%; r2 = 0.99 ± 0.005; slope = −3.32 ± 0.11; Y-intercept = 39.26 ± 1.09) was 10.1% (10/99) [95% CI (5.2–18.2%)]. Only three samples had consistent Cq, and the quantification of *Bartonella* spp. was 18.5 ± 14.02 *nuoG*-copies/µl (mean ± standard deviation, SD).

Representative sequences of *Bartonella* spp. *gltA*, ITS, and *rpoB* genes were deposited in GenBank (55) under the accession numbers OQ162290, OQ137267, and OQ162291. Within sequences that represented the same haplotype, only one representative sequence (with a higher size) was deposited in GenBank and used for phylogenetic analysis.

3.1.1 *Bartonella* spp. cPCR results

Bartonella spp. DNA was successfully amplified by cPCR in 60% (6/10) of qPCR-positive samples, and six sequences were obtained [4 *rpoB* (samples: #6235, #5144, #8184, and 902020), 1 *gltA* (sample #6235), 1 ITS (sample #6235)] from four pudus [one captive (902,020) and three free-ranging (6,235, 5,144, and 8,184)]. The *rpoB* sequences were 100% similar to each other and showed 98.2% similarity with *B. henselae* from cats in Brazil (MN107418), 99.7% identity with *B. henselae* from a cat from Paraguay (MW514669), and 100% identity with *B. henselae* from *Urva auropunctata* from St. Kitts (MW728257). The *gltA* sequence showed 95.05% identity with uncultured *Bartonella* sp. from a cattle tail louse from Israel (KJ522487), and the ITS sequence showed 93.1% identity with *Bartonella* sp. from deer ked (DQ485307). As such, wild pudu #6235 was co-positive to *B. henselae* and *Bartonella* sp., similar to Bartonellae identified in ruminants.

3.1.2 *Bartonella* spp. phylogenetic analysis

The *rpoB* sequences of the present study were allocated in the same taxa, sharing a clade with *B. henselae* Houston 1 (AF171070), *B. henselae* from a cat from Paraguay (MW514660), and *B. henselae* from *Urva auropunctata* from St. Kitts and Nevis (MW728257) (Figure 1). The rpoB diversity analyses are represented on Table 2.

The *gltA* phylogenetic reconstruction evidenced that the sequence of the present study was allocated to the same clade with *Bartonella* sp. from a Cervus from Japan (CP019781), *Bartonella* sp. from a cattle tail louse from Israel (KJ522487), and *B. capreoli*, *B. schoenbuchensis*, and *B. chomeli* (Figure 2).

Finally, the ITS sequence was closely positioned to *Bartonella* sp. sequence from a deer-ked (DQ485307), *B. schoenbuchensis* (CP019789, HG77197), *B. chomeli* (KM215718), *B. melophagi* (JF834886), and *B. bovis* (KF218234, KR733201) (Figure 3).

3.2 *Coxiella burnetii* qPCR results

Molecular occurrence of *C. burnetii* DNA in pudu detected by qPCR (mean and SD of reactions' efficiency = 100.6 ± 5.08%; r2 = 1.0 ± 0.005; slope = −3.31 ± 0.12; Y-intercept = 37.38 ± 0.88) was 1.0% (1/99) [95% CI (0.05–6.3%)].

3.2.1 Hemotropic *Mycoplasma* cPCR results

Molecular occurrence of hemotropic *Mycoplasma* spp. in pudu by cPCR was 1.7% (1/60) [95% CI (0.08–10.1%)]. The sequence of the 16S rRNA fragment showed 100% BLASTn identity with *Mycoplasma ovis*-like amplified previously from Chilean pudu (MW532816) (Figure 4).

4 Discussion

This is the first study to document the presence of DNA of *B. henselae* in a wild ungulate species and *Bartonella* spp. and *C. burnetii* in wild ungulate species in South America. The circulation of *Mycoplasma ovis*-like in free-ranging pudu in Chile is also confirmed (10). The presence of these intracellular bacteria in free-living pudu could suggest an increase in the interaction between domestic species and their ectoparasites and these native species in their natural habitats. Unlike studies in wildlife in other regions (56–58), no co-infection with the three evaluated pathogens was found in pudus. However, one pudu was possibly co-infected with more than one *Bartonella* species, since *B. henselae* and *Bartonella* sp., similar to Bartonellae identified in ruminants, were detected. The co-occurrence of different *Bartonella* species in the bloodstream of reservoir animals such as pudus was earlier described in cats and rodents (59–61), and it illustrates the outstanding tolerance of these hosts to harbor mixed *Bartonella* infections. This could be mediated by an arthropod vector via multiplication and interaction of different *Bartonella* genetic variants in their digestive tract, with subsequent simultaneous transmission to the mammal host (62, 63). Culture and further molecular characterization of the isolates (64) should be attempted with these samples in future to confirm the co-positivity with multiple species of *Bartonella*.

Coxiella burnetii is an important bacterial zoonotic pathogen that can cause Q fever in humans. The bacterium has the potential to cause large-scale outbreaks due to its low infectious dose, environmental resistance, and ability to spread airborne through aerosolization of the pathogen, and is a potential biological threat classified as a "Select Agent" in the USA. *Coxiella burnetii* has a worldwide geographical distribution, apart from Antarctica and New Zealand, and has a wide and diverse host range. The pathogen primarily affects sheep, goats, and cattle, which are considered their primary reservoirs and sources for human outbreaks (65). The livestock species can be infected with *C. burnetii* and appear healthy, and people often become exposed by breathing in dust contaminated with animal feces, urine, and birth products. Wild ungulate species have been reported commonly exposed to *C. burnetii* infection in Europe and North America (66), including eight cervid species, but this report in pudu represents the first in deer from the Southern Hemisphere (65). In

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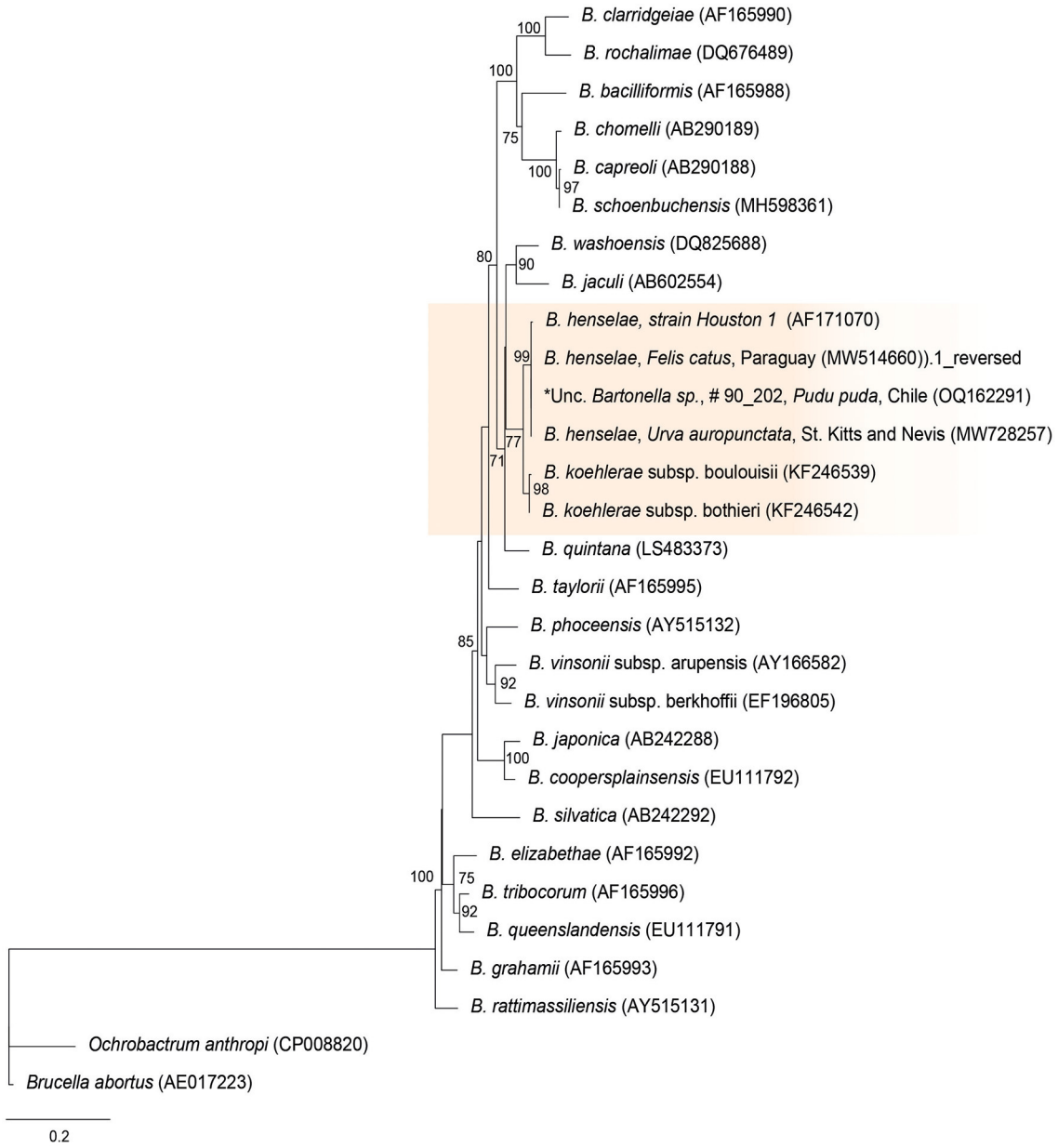

FIGURE 1
Maximum likelihood phylogenies for a subset of *Bartonella* spp. inferred using an alignment (1,152 bp) of the gene encoding the β subunit of RNA polymerase (*rpoB*). Calculated substitution model was TPM3u+F+G4 (partition 1), TNe+G4 (partition 2), and TIM3e+G4 (partition 3). Best models were chosen using the Bayesian information criterion (BIC).

TABLE 2 **Polymorphism and genetic diversity of *rpoB Bartonella* species sequences identified in pudu from Chile.**

Gene	bp	N	VS	GC%	H	Hd (mean ± SD)	Π (mean ± SD)	K
rpoB	4	219	0	0.42	1	0	0	0

N, number of sequences analyzed; VS, number of variable sites; GC%, C + G content; h. number of haplotypes; hd, diversity of haplotypes; S.D., standard deviation; π, nucleotide diversity (per site); K, nucleotide difference number.

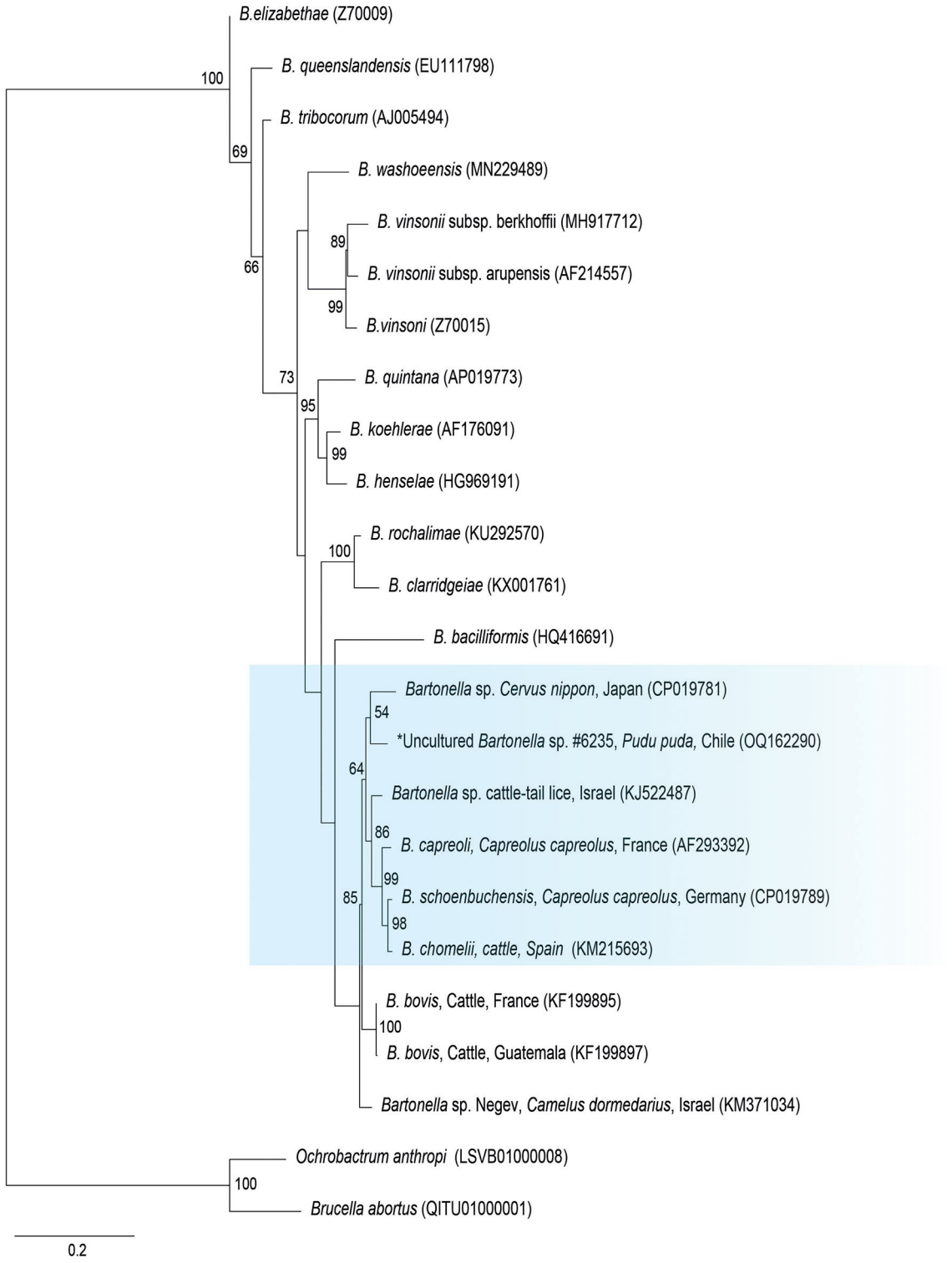

FIGURE 2
Maximum likelihood phylogenies for a subset of *Bartonella* spp. inferred using an alignment (1,290 bp) of the gene encoding citrate synthase (*gltA*). Calculated substitution model was K3P+G4 (partition 1), TIM3+F+G4 (partition 2), and TNe+G4 (partition 3). Best models were chosen using the Bayesian information criterion (BIC).

the Basque region in Spain, the prevalence has been categorized as stable throughout time. Therefore, the roe deer (*Capreolus capreolus*) plays a role in the sylvatic cycle of Q fever (67). In South America, there is no evidence of *C. burnetii* DNA in blood samples of wild boar (*Sus scrofa*), marsh deer (*Blastocerus dichotomus*), brown brocket deer (*Mazama gouazoubira*), small red brocket deer (*Mazama bororo*), red brocket deer (*Mazama americana*), and pampas deer (*Ozotocerus bezoarticus*) (12, 68).

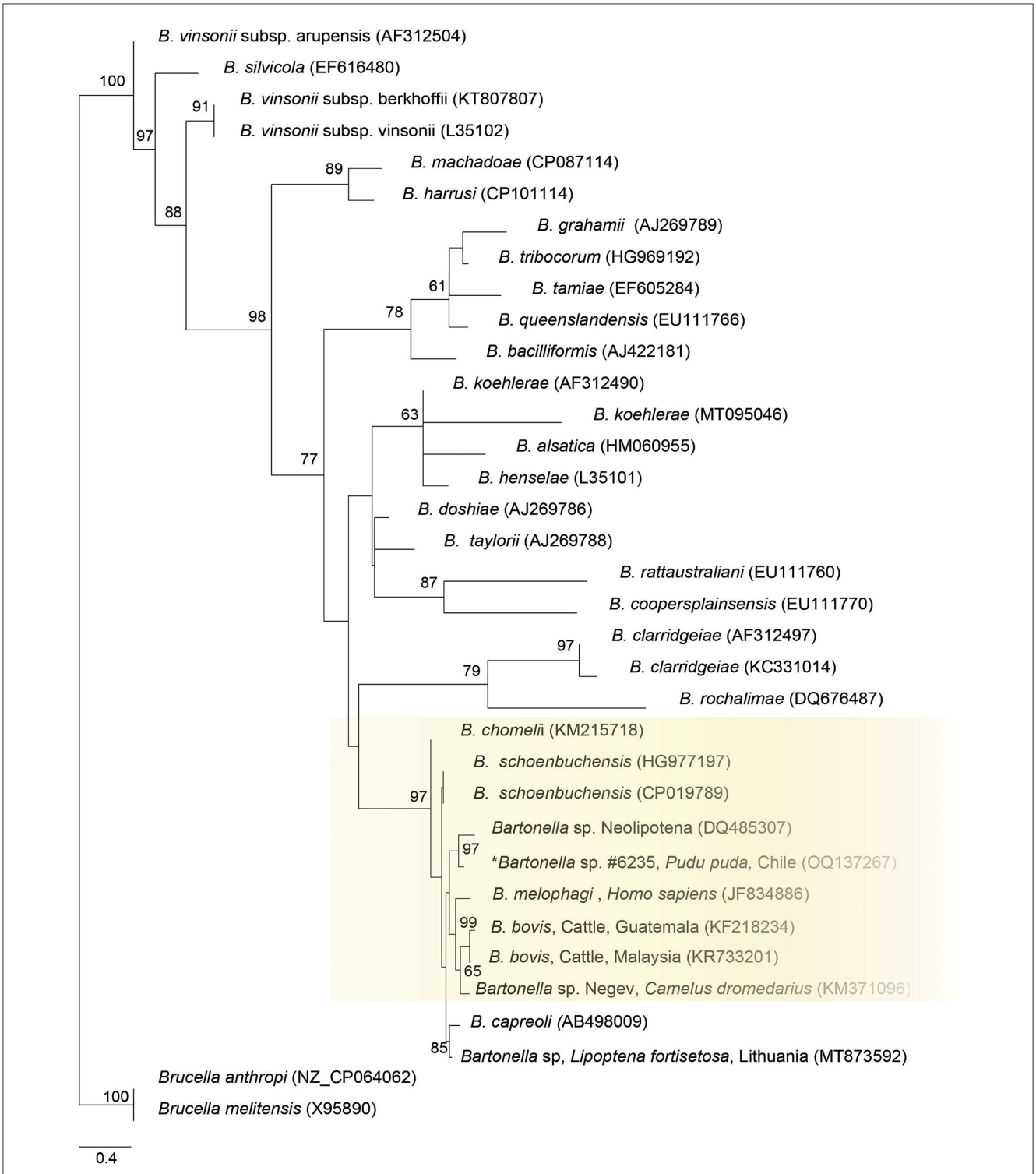

FIGURE 3
Maximum likelihood phylogenies for a subset of *Bartonella* spp. inferred using an alignment (522 bp) of the internal transcribed spacer (ITS). Calculated substitution model was TPM2u+F+G4. Best models were chosen using the Akaike information criterion (AIC).

A recent study (12) found that 5.32% of the sampled deer was seropositive for *C. burnetii* by an indirect immunofluorescence assay (IFA) for IgG antibodies (anti-phase I); to date, it is the only evidence of exposure to this pathogen in deer in the region.

In Chile, DNA findings of *C. burnetii* have been reported in samples of animal origin only in bats and bulk tank milk from cows (26, 69). The last human Q fever outbreak in Chile was declared in 2017 in the Los Lagos District, the same region where molecular evidence was found in pudu in our study (70). This district is a part

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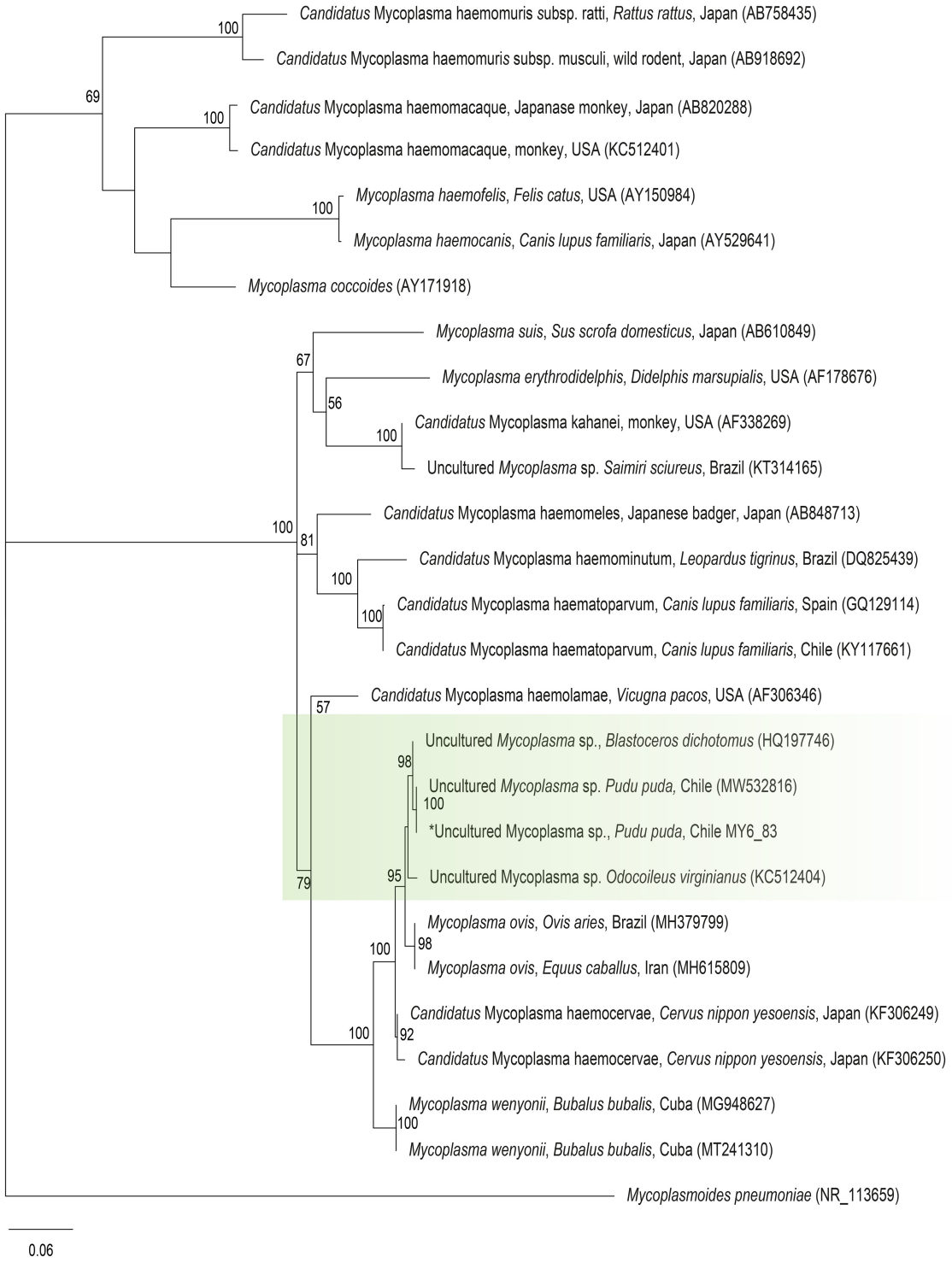

FIGURE 4
Maximum likelihood phylogenies for a subset of *Mycoplasma* spp. inferred using an alignment (620 bp) of the 16S rRNA gene. Calculated substitution model was GTR+F+G4. Best models were chosen using the Akaike information criterion (AIC).

of the southern macrozone where seropositivity for humans (6%) was significantly higher than in other regions of the country (70). It is likely that the source of infection for pudu is of anthropogenic origin (livestock), or from exotic deer species, red deer, and/or fallow deer, which have been reported in the area (71) and have been commonly reported infected by *C. burnetii* in Europe (60, 65), or from rodent species previously found to be a source of livestock coxiellosis (72). Other serological or molecular studies in dogs and

Darwin fox (*Lycalopex fulvipes*) in the southern macrozone found no evidence of *C. burnetii* infection (18, 73, 74). The finding of only one pudu being positive for the bacterium and the low prevalence of *C. burnetii* in Chile make serological and molecular screening necessary for a much larger number of pudu samples from the Los Lagos region, to evaluate their potential role as a host of infection for transmission to animals and humans. Additionally, the reports of infectious abortions in captive pudus in Chile (39) added to the evidence that *C. burnetii* has been involved in reproductive loss in captive exotic ungulates, mainly in bovid species (65), making it necessary to include in the differential diagnosis of possible causes of abortion in pudu. Moreover, future studies should attempt to molecularly characterize *via* sequencing the *C. bunetii* found in pudus from Chile.

In this study, *B. henselae*, an emerging zoonotic pathogen that causes scratch disease in humans and whose transmission mainly involves domestic cats as the main reservoir and cat fleas (*Ctenocephalides felis*) as the main vector (75), is described for the first time in wild ungulate species. Otherwise, to a lesser extent, it is reported in other mammals, bovines (76, 77) and rodents among them (78–81), suggesting that they have a permissive cycle in nature, being detected in several ecological niches (hosts and vectors) (77). Thus, this finding could indicate the circulation of *B. henselae* in an infected vector, favoring *B. henselae* transmission among domestic and wild mammals. Nonetheless, further epidemiological and genotyping studies are necessary to confirm this hypothesis. The DNA of *Bartonella* bacteria has been widely described in cervid species from Europe (30, 58, 82–84), North America (29, 85–87), and Asia (88, 89), usually with a higher prevalence (between 4.9 and 77.7%) than reported in our study. In South America, there are reports of *Bartonella* spp. in vector species of wild ungulates (68, 90) but not in their blood samples (68). In Chile, during the last decade, there have been reports of the presence of *Bartonella* spp. in cats, dogs, minks, and bats (23–26, 28, 91, 92). *Bartonella henselae* in pudu was similar to *B. henselae* reported in small Indian mongooses in the Caribbean (93) and cats from Brazil (94) and Paraguay (27). *Bartonella* sp., related to those reported infecting ruminants, such as *B. schoenbuchensis* [CP019789, HG977197 (95)], *B. chomeli* (KM215718) (96), *B. melophagi* (JF834886) (97), *B. bovis*, and *B. capreoli*, was also detected in a pudu in this study. More screening will be necessary to confirm the role of pudu in the epidemiology of this infectious agent and its impact on animal health.

Hemoplasma bacteria have been extensively studied in wild and domestic carnivores in Chile during the last decade. Darwin foxes (*Lycalopex fulvipes*) present a high prevalence of *M. haemocanis* causing enzootic and asymptomatic infections (18, 19) that could be a source of infection for pudu since both share the same habitat within the Los Lagos region. Hemotropic *Mycoplasma* spp. have been recently reported in llamas (12.8%) and alpacas (6.3%) (*Candidatus Mycoplasma haemolamae*) (31) and *Mycoplasma ovis*-like in free-living pudu in southern Chile (14%) (10). Molecular screening of hemotropic *Mycoplasma* spp. in sheep, livestock, and native (huemul) and exotic ungulates (wild boars, red deer) in the Los Lagos District is recommended to understand the epidemiology of these infectious agents and the possible role of pudu as a host. It is also recommended to evaluate the pathogenicity of *Mycoplasma ovis*-like in pudu.

For the first time in pudu, the finding of *B. henselae* and *C. burnetii*, both zoonotic pathogens, could be relevant to public health. Both *B. henselae* and *C. burnetii* are pathogens related to occupational diseases, with evidence of health risks for those working with infected species through occupational exposure in rehabilitation centers, breeding centers, and zoological parks (98, 99), representing an important factor to consider in medical and management practices with this animal species.

5 Conclusion

This study expands the knowledge of bacteria with zoonotic potential carried by pudu. *Mycoplasma ovis*-like was confirmed in pudus, while *Bartonella* spp., *Bartonella henselae*, and *C. burnetii* were described for the first time in South American ungulates. The results of this study suggest an anthropic impact on wildlife species with domestic species interacting epidemiologically with pudus in their natural habitats. Further research will be necessary to evaluate the potential role of pudu as a host and reservoir of infection, and identify the sources for disease transmission among humans and wild and domestic animals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/nuccore; OQ162290, OQ137267, OQ162291.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because we only use samples from Frozen Banks of the rehabilitation centers and zoos. We don't have involved in any managements animals procedure.

Author contributions

EH-H: conceptualization, supervision, funding acquisition, investigation, resources, data curation, writing—original draft preparation, and writing—reviewing and editing. PS-G: methodology, investigation, resources, data curation, writing—original draft preparation, and writing—reviewing and editing. JC, CV, FV, and IK: resources and data curation. SC and CO: resources. DM-A: writing—original draft preparation, and writing—reviewing and editing. MO and NC: methodology and investigation. AG: investigation and writing—reviewing and editing. AM: conceptualization, supervision, funding acquisition, methodology, investigation, writing—original draft preparation,

and writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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New species of *Delicata* (Molineidae: Anoplostrongylinae) parasite of *Cabassous tatouay* (Desmarest, 1804) from the Atlantic Forest, Rio de Janeiro, Brazil

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A new species of nematode parasite of the genus *Delicata* (Molineidae: Anoplostrongylinae) is described from the small intestine of a road-killed Greater Naked-tailed Armadillo *Cabassous tatouay* (Cingulata: Chlamyphoridae) on the BR-040 highway in Rio de Janeiro state, Brazil. The genus *Delicata* includes 13 species of parasitizing armadillos and anteaters distributed in Brazil, Argentina, and Trinidad and Tobago. The present species is distinguished from almost all species of *Delicata* by the longest length of the body, except for *D. khalili* and *D. appendiculata*. However, these can be distinguished from each other by the length of the spicules. The species that closely resembles, *Delicata tatouay* n. sp. is *D. speciosa*, but it can be distinguished by a robust branch from rays 2 and 3, rays 4 larger, and rays 8 longer compared to those of the new species. The new species is the only one with a tail, characterized by a terminal spine with rattlesnake tail-like transversal striations.

KEYWORDS

armadillo, biodiversity, Nematoda, road-killed, Xenarthra

Introduction

Currently, 13 species are assigned to *Delicata* Travassos, 1935, infecting the small intestine of armadillos: *Dasypus novemcinctus* Linnaeus, 1758; *Dasypus hybridus* (Desmarest, 1804); *Cabassous unicinctus* (Linnaeus, 1758); *Euphractus sexcinctus* (Linnaeus, 1758); and the Southern anteater *Tamandua tetradactyla* (Linnaeus, 1758) (1–3), distributed in Brazil, Argentina, and Trinidad and Tobago (1, 3).

The greater naked-tailed armadillo, *Cabassous tatouay* (Desmarest, 1804), can be found inhabiting Uruguay, northeastern Argentina, eastern Paraguay, and south, central, and northeastern Brazil (4). It is the largest species of the genus, measuring approximately 48 cm (head-body length) and weighing approximately 4.8 kg (5, 6). In Brazil, it occurs in the Atlantic Forest, Cerrado, Caatinga, Pampas, and Pantanal near the transition to the Cerrado

savanna (4, 7). They are solitary and insectivorous (8, 9), feeding on terrestrial ants and termites (10, 11). The species uses both forested and open areas but prefers forested habitats (5, 12).

This is a poorly known species of armadillo, with relatively few records in Brazilian museums (13). In the IUCN Red List, it is listed as the least concern (12), whereas in the Brazilian Red Book, it is considered data deficient (14). The main threats to the species are probably deforestation and fire, as well as hunting and persecution (14).

The use of carcasses of wild road-killed vertebrates for scientific purposes has provided discoveries for science (15–17). Considering that the greater naked-tailed armadillo is poorly known, the use of samples from these animals is an opportunity to contribute to scientific development, including helminthology, given that there is almost no information regarding helminths from this host (18).

During a parasitological survey in the small intestine of one *C. tatouay* road-killed on the BR040 highway in Rio de Janeiro state, Brazil, a new species of the nematode *Delicata* was collected and described herein.

Materials and methods

One road-killed adult *C. tatouay* was collected in April 2011 on the federal BR-040 highway, 38 km, in Areal municipality, as part of the project "Caminhos da Fauna." The project "Caminhos da Fauna" started in 2006, is still in progress, and comprises the pioneering study in the monitoring of road-killed vertebrates in the state of Rio de Janeiro. The database used in the study comes from the monitoring of the road-killed vertebrate along a 180.4 km stretch of the BR-040 (from 125.2 km in the municipality of Duque de Caxias, state of Rio de Janeiro, to 773.5 km in the municipality of Juiz de Fora, state of Minas Gerais).

Carcass collections are included in the SISBIO License Number: 30727-9. The animal carcasses used in this study meet and are in accordance with operation license No. 1187/2013 and authorization for capture, collection, and transport of biological material – Abio (first renewal and third rectifier) 514/2014.

The abdominal and thoracic cavities of the host specimen were opened, and the organs were placed separately in Petri dishes, washed in saline solution (0.9% sodium chloride), and dissected under a stereomicroscope to remove the small helminths. Collected nematodes were conserved in 70° ethanol. Ten specimens were clarified in a 50% alcohol/glycerin solution, mounted as temporary slides, and examined under a Zeiss Standard 20 light microscope. Drawings for morphologic and morphometric analyses were made with the aid of a camera lucida, and the images were obtained with a digital camera (Olympus DP-12) and a light microscope (Olympus BX-51). Transversal sections on the anterior, middle, and posterior parts of the body of males and females were made in order to study the synlophe. The nematodes were identified following Anderson et al. (19), Travassos (2), and Durette-Desset (20). The measurements are given in micrometers unless otherwise indicated. Means are followed by the range between brackets. The holotype, allotype, and paratypes were deposited in the helminthological collection of the Oswaldo Cruz Institute (CHIOC) in Rio de Janeiro.

Results

General: Small, slender, coiled body, with sexual dimorphism (female larger than male); presence of a cephalic vesicle. Rounded mouth opening in apical view, surrounded by two amphids, six external labial papillae. Excretory pore situated between 42 and 60% in relation to esophagus length (Figure 1A). Deirids situated anterior to excretory pore.

Synlophe (studied in one male and one female): ridges appear longitudinally along the body, beginning posterior to the cephalic vesicle in both sexes. It is not observed at the proximal region of the caudal bursa in males and at the posterior extremity in females. Synlophe with 11 ridges in females and 12 in males at the level of the esophagus (Figures 2A,D); 12 ridges at mid-body in both sexes (Figures 2B,E); 12 ridges anterior to the anus in females and anterior to the caudal bursa in males (Figures 2C,F). Ridges at mid-body are slightly unequal in size in both males and females, with smaller ridges oriented from the ventral right axis and to the ventral left and from the dorsal right quadrant to the dorsal left.

Male (based on one holotype and nine paratypes): length 5.97 mm (5.31–6.94 mm) and width 81 (72–107); cephalic vesicle 81 (73–83) long and 31 (30–39) wide; nerve ring, deirids, and excretory pore 212 (187–241), 234 (207–268), and 276 (253–293) from the apex; esophagus 500 (425–687) long; presence of prebursal ray 1 slightly pedunculated. Trilobate caudal bursa, right lobe slightly longer than left (Figures 1F, 3D). Rays 2 and 3 bifurcated at the second third of the trunk, with distal extremities almost reaching the bursal margin and directed ventrally. Rays 4, 5, and 6 emerging together at the base of the trunk. Ray 4 is smaller than other rays and bifurcate at the second third of the trunk. Rays 5 longer reach the bursal margin. Rays 5 and 6 bifurcate at the middle of the trunk, both distal extremities directed dorsally. All lateral rays present cuticular ornamentation. Patterns of the caudal bursa 2-1-2. Ray 8 emerging at the first third of the dorsal trunk, extending the level of the distal end of the dorsal ray but not reaching the bursa edge. Dorsal ray bifurcates at the distal extremity into 2 branches, ray 9 arising first, rays 10 divided into two branches (Figure 1H). Genital cone well developed with two membrane projections presenting papillae 7 in each extremity (Figure 3E). Papillae zero not observed. Spicules ornamented and complex in shape, wrapped in a thin sheath. Spicules are divided into two processes at the first third and show a lanceolate shape at the distal part. Spicules slightly dissimilar, left spicule 134 (126–146) and right spicule 144 (134–160) long (Figures 1G, 3C). Gubernaculum present 36 (29–45) long and 16 (12–19) wide (Figure 3F).

Female (based in one holotype and nine paratypes): length 7.73 mm (6.81–9.40) and width at middle body 91 (70–121); cephalic vesicle 80 (63–89) long and 37 (31–41) wide; nerve ring, deirids, and excretory pore 215 (190–239), 225 (217–316), and 286 (250–370), and from the apex, respectively (Figures 1A, 3A); esophagus length 534 (531–704); Amphidelphic, vulva situated at 1.480 (1.318–1.703) from caudal extremity with expansion digitiform (Figures 1C,D), vagina vera 44 (33–54). Anterior branch of ovejector with vestibule 62 (52–75), sphincter 40 (33–57) long and 48 (39–51) wide, infundibulum 136 (81–163) long, and uterus 1.253 (604–1.744) filled with 28 (0–61) eggs. Posterior branch of ovejector with vestibule 64 (53–78), sphincter 40 (33–54) long and 47 (39–52) wide, infundibulum 123 (76–152) long, and uterus 820 (449–1.052) with 20 (0–49) eggs. Eggs 56 (52–59) long and 33 (30–37) wide (Figure 1I). Tail 112 (107–150) long (Figures 1E, 3B). Caudal spine digitiform with fine transverse striations 20 (15–49) long (Figure 1B). Presence of phasmids 28 (43–61) from the posterior extremity.

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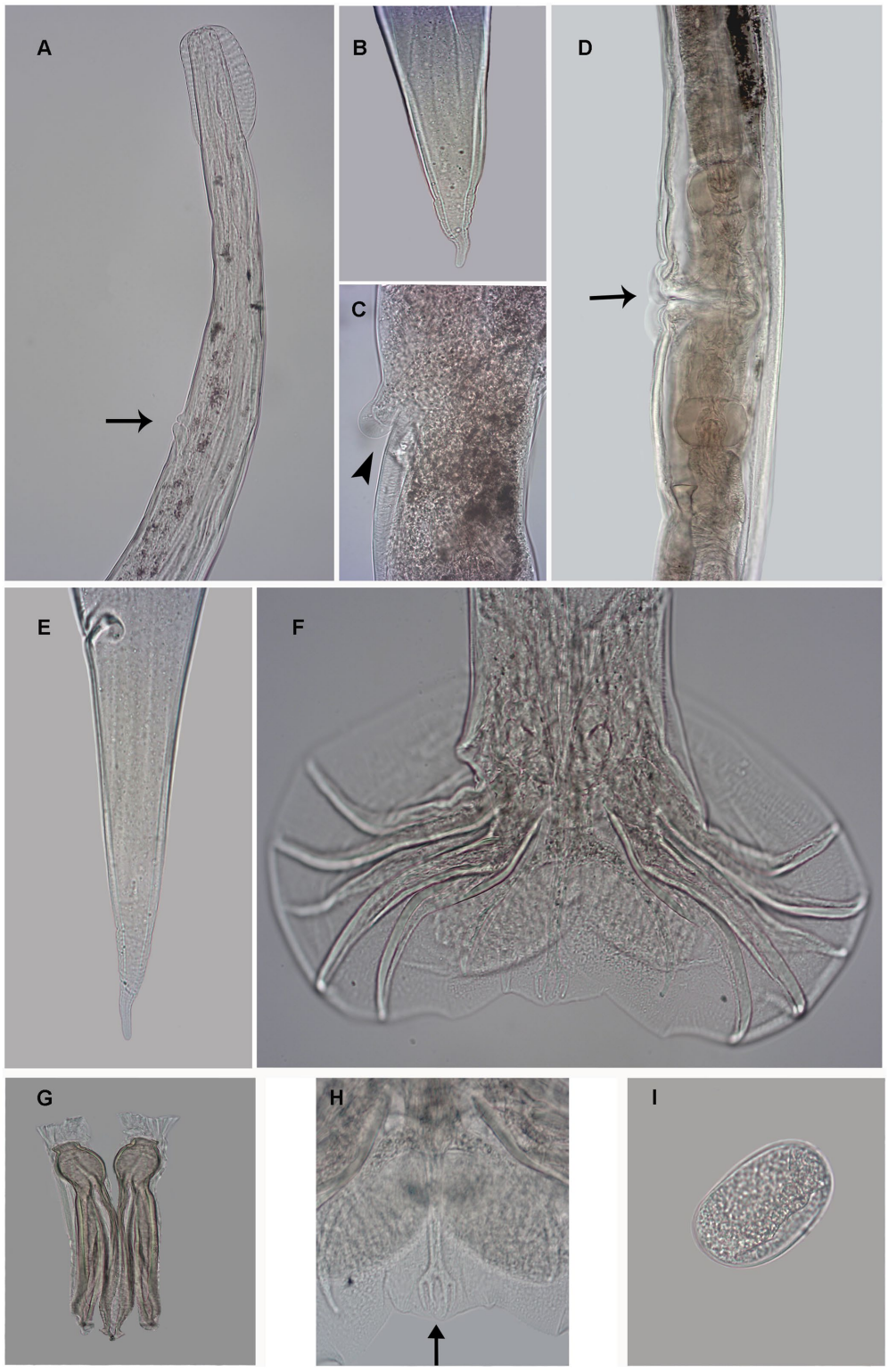

FIGURE 1
Photomicrography of female *Delicata tatouay* n. sp. **(A)** Anterior extremity, excretory pore (arrow). **(B)** Detail tail tip female. **(C)** Detail vulva (arrowhead). **(D)** Vulva (arrow). **(E)** Posterior extremity, ventro-lateral view of the anus. **(F)** Male, caudal bursa, ventral view. **(G)** Spicules. **(H)** Detail Dorsal rays (arrow) **(I)** Egg. Scale bars: **(A, D, E, F)** = 100 µm; **(B, C, G, H)** = 50 µm; **(F)** = 10 µm.

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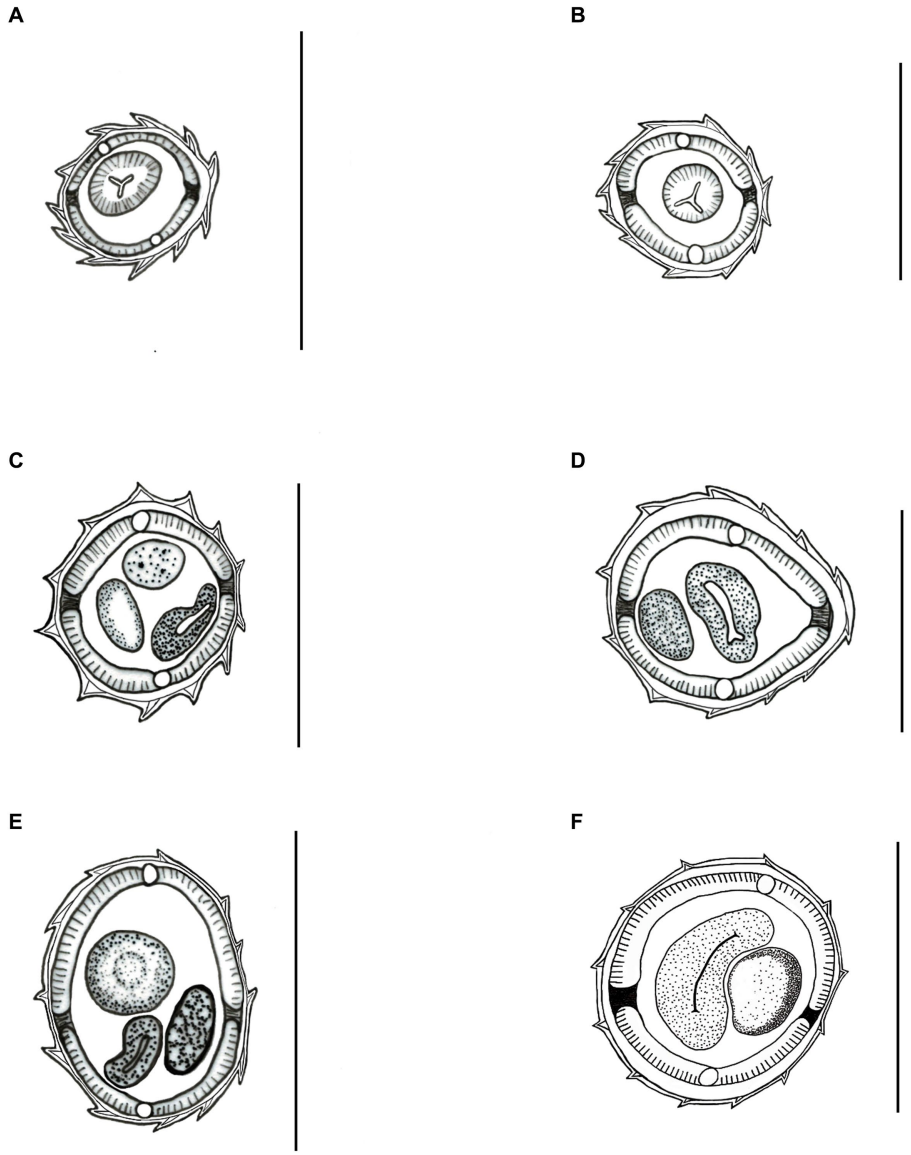

FIGURE 2
Light microscopy drawing of synlophe in transverse sections of the body from *Delicata tatouay* n. sp. **(A–C)** Female. **(A)** At the oesophago-intestinal junction; **(B)** at mid-body; **(C)** at level of the anus. **(D–F)** Male. **(D)** At the oesophago-intestinal junction; **(E)** at the mid-body; **(F)** at the level of the near caudal bursa. Scale bars: **(A–F)** = 50 µm.

Taxonomic summary

Delicata tatouay n. sp.

Type host: *Cabassous tatouay*.

Site of infection: small intestine.

Type locality: Highway BR-040, Areal municipality (22°13′55.35”S, 43°7′3.93”W), State of Rio de Janeiro, Brazil.

Deposition of type specimens: Helminthological collection of the Oswaldo Cruz Institute in Rio de Janeiro state (CHIOC). Holotype accession number: CHIOC 39647 a; allotype accession number: CHIOC 39647 b; paratype accession numbers: CHIOC 39647 c (one male and seven females).

Etymology: The species epithet is due to the specific name of the host.

Discussion

The new species belongs to the genus *Delicata,* presenting a cephalic end without a cuticular ring and a lack of cuticular plates, a female amphidelphic vulva far from the anus, a tail rounded with a caudal spine, a male with ray 5 at the same length or longer than ray 6, the presence of small post-cloacal papillae 7 at the caudal bursa, and parasites of the intestine of Xenarthra (20). This is the first species belonging to the genus *Delicata* described by *Cabassous tatouay.*

The present species is distinguished from almost all other species of *Delicata* by the longest length of the body, except for *D. khalili* and *D. appendiculata*, which show similar lengths. In addition, *Delicata tatouay* n. sp. differs from *D. soyerae*, *D. cameroni*, *D. abbai*, *D. delicata*,

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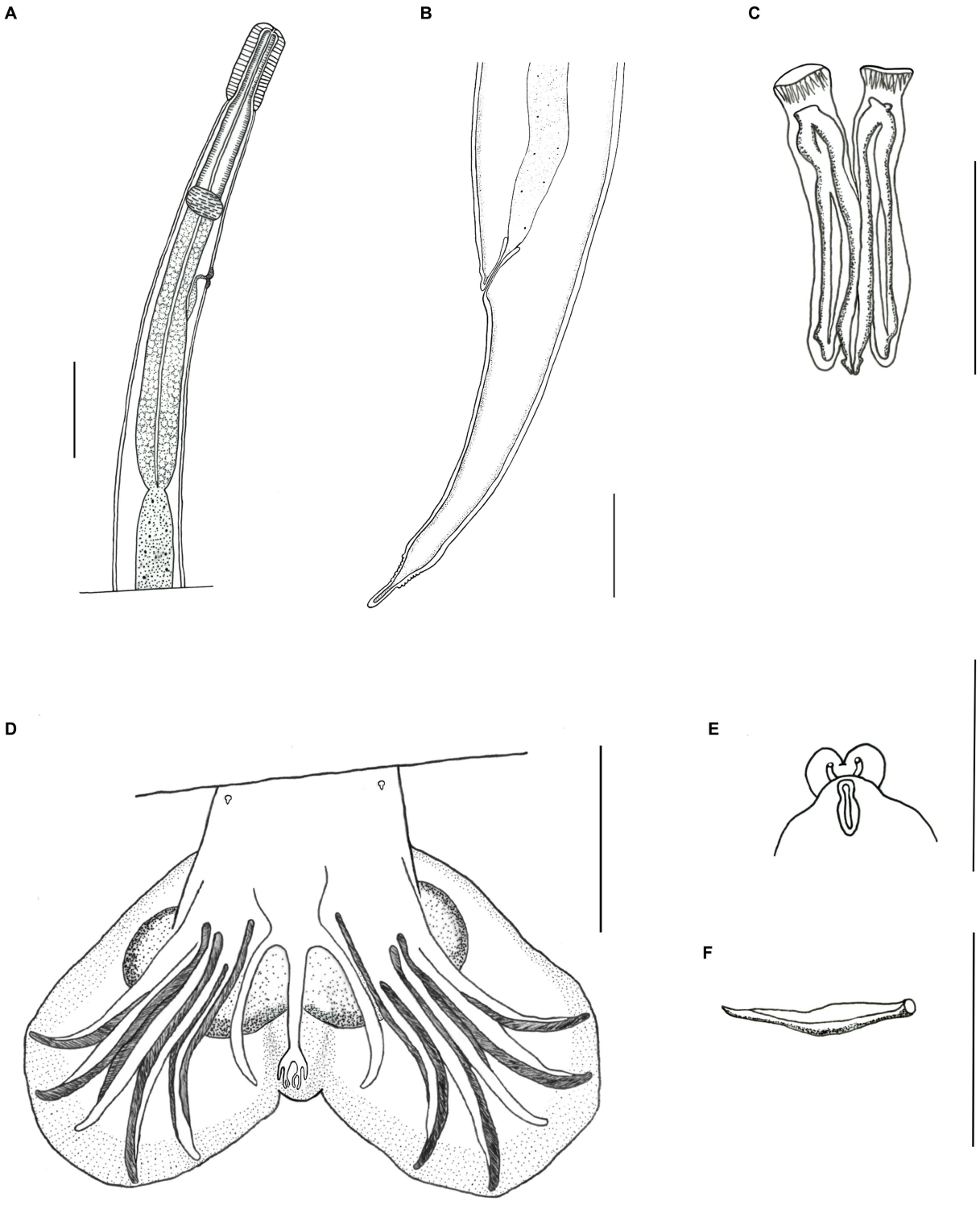

FIGURE 3
Light microscopy drawing of *Delicata tatouay* n. sp. **(A)** Female, anterior extremity, lateral view. **(B)** Female, lateral view, posterior extremity. **(C)** Male, spicules. **(D)** Male, caudal bursa, ventral view. **(E)** Male, genital cone. **(F)** Male, gubernaculum. Scale bars: **(A, B, D)** = 100 µm; **(C, E, F)** = 50 µm.

D. appendiculata, *D. uncinata*, and *D. similis* by the longest spicules (Tables 1, 2). In contrast, the species *D. ransoni*, *D. khalili*, *D.* var*iabilis*, and *D. perronae* have larger spicules than the new species.

The most similar species is *D. speciosa*, but it can be differentiated because it presents a robust branch from rays 2 and 3, rays 4 larger, and rays 8 longer from those of *Delicata tatouay* n. sp. Moreover, the synlophe at the middle body of *D. speciosa* is markedly distinguished from *D. tatouay* n. sp. The first presents only four ridges situated at the ventral side and two small lateral cuticular dilatation, and the second presents 12 ridges (six ventral and six dorsal) in both sexes. Finally,

TABLE 1 Morphometric data on male species of the genus *Delicata* in America.

Species	*Delicata khalili*	*Delicata appendiculata*	*Delicata perronae*	*Delicata soyerae*	*Delicata pseudoappendiculata*	*Delicata delicata*	*Delicata ransomi*	*Delicata uncinata*	*Delicata similis*	*Delicata variabilis*	*Delicata cameroni*	*Delicata speciosa*	*Delicata abbai*	*Delicata tatouay*
Host	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua longicaudata*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Dasypus novemcinctus*	*Dasypus hybridus*	*Dasypus novemcinctus*	*Dasypus hybridus*	*Cabassous tatouay*
Length	6.50	3.00	3.80	2.50	2.80	3.50	4.90	3.30	2.00	2.80	4.20	4.923	3.05	5.97
Width	170	77	45	41	–	78	130	70	51	62	90	65	80	80.5
Cephalic Vesicle L	50	46	62	70	–	49	56	54	40	35	32	75	38	80.5
Cephalic Vesicle W	–	–	25	21	–	–	–	–	–	–	–	–	31	33.5
Nerve ring	–	–	105	137	–	–	–	–	120	120	150	180	140	212
Deirids	–	–	124	175	–	–	–	–	–	–	–	–	155	235
Excretory Pore	–	–	140	165	–	–	–	–	160	240	200	400	190	276
Esophagus	–	380	220	200	230	360	350	320	300	290	290	315	–	500
Type	–	–	–	–	–	–	–	–	–	–	–	2-1-2	2-1-2	2-1-2
Spicule	250	115	520	105	100	99	163	81	81	180	72	222/179	56	134/143
Ratio of spicule/ body total length	3.84%	3.83%	13.68%	4.20%	3.57%	2.82%	3.32%	2.45%	4.05%	6.42%	1.71%	4.50%	1.83%	2.34%
Gubernaculum L	115	69	80	58	–	63	127	–	48	29	48	139	34	35.6
Gubernaculum W	–	–	–	–	–	–	–	–	–	–	–	–	6	16
Locality	Brazil	Brazil	Brazil	Brazil	Trinidad	Brazil	Brazil	Brazil	Brazil	Brazil	Brazil	Brazil	Argentina	Brazil
Author	Travassos (21)	Travassos (21)	Durette-Desset et al. (22)	Durette-Desset et al. (22)	Cameron (23)	Travassos (24)	Travassos (24)	Travassos (25)	Travassos (25)	Travassos (25)	Travassos (25)	Lux Hoppe et al. (26)	Ezquiaga et al. (1)	Present study

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TABLE 2 Morphometric data on female species of the genus *Delicata* in the Americas.

Species	*Delicata khalili*	*Delicata appendiculata*	*Delicata perronae*	*Delicata soyerae*	*Delicata pseudoappendiculata*	*Delicata delicata*	*Delicata ransomi*	*Delicata uncinata*	*Delicata similis*	*Delicata variabilis*	*Delicata cameroni*	*Delicata speciosa*	*Delicata abbai*	*Delicata tatouay*
Host	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua tetradactyla*	*Tamandua longicaudata*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Cabassous unicinctus*	*Dasypus novemcinctus*	*Dasypus hybridus*	*Dasypus novemcinctus*	*Dasypus hybridus*	*Cabassous tatouay*
Length	7.50	6.30	4.10	3.60	3.40	5.00	5.30	5.50	–	3.00	4.60	5.59	4.00	7.73
Width	170	110	43	45	–	87	150	90	–	67	110	95	70	90.7
Cephalic Vesicle L	90	77	62	80	–	56	78	60	–	37	43	70	45	79
Cephalic Vesicle W	–	–	21	23	–	–	–	–	–	–	–	–	30	36.5
Nerve ring	120	–	93	150	–	–	–	–	–	130	150	155	97	215
Deirids	–	–	112	185	–	–	–	–	–	–	–	–	110	225
Excretory Pore	–	–	120	172	–	–	–	140	–	210	270	337	130	286.4
Esophagus	–	500	–	230	230	370	460	340	–	290	290	–	262	533.6
Vulva	1.15	730	680	590	500	1.00	1.30	1.30	–	710	1.10	1.065	1.025	1.480
Vagina Vera	–	–	–	15	–	–	–	–	–	–	–	116	21	44
Vestibulo Ant	–	–	80	50	–	–	35	–	–	–	–	–	47	61.7
Sphincter Ant.	–	–	26	25	–	–	–	–	–	–	–	–	20	39.8
Sphincter Ant.	–	–	–	–	–	–	–	–	–	–	–	–	25	47.6
Infundibulum	–	–	35	25	–	–	–	–	–	–	–	–	40	135.8
Uterine branch	630	–	310	320	–	–	–	–	–	–	–	–	552	1.253
Vestibulo Post.	–	–	60	30	–	–	–	–	–	–	–	–	50	63.7
Sphincter Post. L	–	–	30	20	–	–	–	–	–	–	–	–	20	40
Sphincter Post. W	–	–	–	80	–	–	–	–	–	–	–	–	25	47.3
Infundibulum	–	–	32	25	–	–	–	–	–	–	–	–	40	123.3
Uterine branch	460	–	280	–	–	–	–	–	–	–	–	–	425	820
Eggs	77	69	68	63	–	63	63	70	–	–	67	58	62.6	56.5
Eggs	38	38	25	26	–	38	35	37	–	–	40	32	38.5	32.7
Tail	200	100	77	90	100	85	140	130	–	90	150	96	155	112.4
Phasmids	–	–	–	–	–	–	–	–	–	–	–	–	34	28
Caudal spine	15	–	13.5	22	–	–	–	13	–	16	18	9.6	12	20
Locality	Brazil	Brazil	Brazil	Brazil	Trinidad	Brazil	Brazil	Brazil	Brazil	Brazil	Brazil	Brazil	Argentina	Brazil
Author	Travassos (21)	Travassos (21)	Durette-Desset et al. (22)	Durette-Desset et al. (22)	Cameron (23)	Travassos (24)	Travassos (24)	Travassos (25)	Travassos (25)	Travassos (25)	Travassos (25)	Lux Hoppe et al. (26)	Ezquiaga et al. (1)	Present study

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Delicata tatouay n. sp. is the only species presenting a terminal spine in the tail with rattlesnake tail-like transversal striations.

Durette-Desset (20) characterized the synlophe of genus *Delicata* as having two lateral alae. However, Ezquiaga et al. (1) questioned this feature, suggesting not to use this character to propose a new genus once there is no synlophe of all known species, mainly *D. delicata*, that represents the type species of the genus. In addition, the known synlophe of *D. soyerae*, *D. perronae*, *D. abbai*, *D. ransomi*, and *Delicata tatouay* n. sp. have demonstrated great variability in the number of ridges. In fact, a review of the genus is required to elucidate these generic diagnostic features.

Records of the greater naked-tailed armadillo are scarce in some regions (4, 27), although it is considered globally of "least concern" (12, 28). In addition, there is almost no information about the helminth fauna of this host (18). Indeed, there is still an important lack of knowledge about parasites infecting wildlife (29), especially in highly diverse countries such as Brazil. Using road-killed vertebrates to identify new species of helminths is important not only for helminthology, but it is also essential to develop ecological research on host–parasite interaction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://zoobank.org/, B2C78C9B-035C-4DF6-A0B4-8D7822313471.

Ethics statement

The animal study was approved by SISBIO License Number: 30727-9. License: No. 1187/2013 Abio: 514/2014. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RO: Conceptualization, Methodology, Resources, Writing – original draft. BA: Methodology, Resources, Writing – review & editing. NO: Methodology, Resources, Writing – review & editing. CB: Methodology, Resources, Writing – review & editing. AM: Conceptualization, Methodology, Resources, Writing – original draft.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1325263/full#supplementary-material

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First record of *Alectorobius coniceps* (Ixodoidea: Argasidae) and *Dermacentor* sp. (Ixodoidea: Ixodidae) in Pakistan

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Alectorobius species are soft ticks primarily infesting birds, such as swallows, while *Dermacentor* species are hard ticks mainly infesting mammals, such as small ruminants. This study for the first time reported on the morphological and molecular bases of two tick species, namely *A. coniceps* and a *Dermacentor* sp. in Pakistan. The former species was examined in swallows' nests in Khyber Pakhtunkhwa province, while the latter species was examined in small ruminants in Balochistan province. In total, 25 ticks were collected, with 14 ticks morphologically identified as *A. coniceps* (males = 9 and females = 5) and 11 ticks identified as *Dermacentor* sp. (males = 7 and females = 4). Following morphological identification, molecular identification was gained by obtaining 16S rDNA and *cox1* sequences for these ticks. The BLAST results for the 16S rDNA and *cox1* sequences from *A. coniceps* shared a maximum identity of 97.46% and 96.49% with the same species from Malta. The BLAST analysis of the 16S rDNA and *cox1* sequences from *Dermacentor* sp. showed maximum identities of 98.42% and 97.45% with *Dermacentor pavlovskyi* from China. The phylogenetic analysis based on 16S rDNA and *cox1* of *A. coniceps* showed a close evolutionary relationship with the same species. The case of *Dermacentor* sp., based on 16S DNA and *cox1*, indicated a close evolutionary relationship with *Dermacentor pavlovskyi* from China.

KEYWORDS

***Alectorobius coniceps*, *Dermacentor* sp., 16S rDNA, *cox1*, Pakistan**

Introduction

Ticks are arthropods that fall under Arachnida and are further categorized into three families: hard ticks (Ixodidae), soft ticks (Argasidae), and Nuttalliellidae (1, 2). With medical significance, they are obligate ectoparasites of semi-terrestrial and terrestrial vertebrates (3–5). Usually, hard ticks take a single extended blood meal during each of their life stages, while soft ticks consume multiple brief blood meals during the nymphal and adult stages (6–8).

The genus *Ornithodoros* is recognized as the most diverse among soft ticks, comprising approximately 130 species (9–11). They have been documented on a wide variety of hosts, including amphibians, birds, mammals, and reptiles (12–16). Previously, this genus was believed to have seven subgenera, including *Alectorobius*, which encompassed ticks such as *Ornithodoros* (*Alectorobius*) *coniceps* (17, 18). However, *Alectorobius* was later reclassified as a distinct genus (19), and this reclassification is adopted in this study. These ticks are distributed worldwide primarily infesting birds and occasionally parasitize humans (6, 20). These parasites have been observed infesting pigeons, ruddy shelducks, swallows, swifts, sparrows, and chickens (6, 13, 20, 21). Throughout their lifespan, they may parasitize a single host or multiple hosts (6, 20). However, they exhibit nidicolous behavior, briefly attaching to their hosts and generally residing in their nests (6, 13, 22). *Alectorobius coniceps* is an ornithophilic species belonging to the mentioned genus, and there are limited morphologically based records of this species from the Oriental region (23).

With approximately 40 species, the *Dermacentor* is the fourth most diverse genus among hard ticks (24). Approximately half of its species are found in the Palearctic region, with the remaining species distributed across Afrotropical, Nearctic, and Oriental regions (24–26). They are primarily three-host ticks, with a prominent preference for mammals, including wild and domestic, and occasionally humans (24, 26, 27). The adult and nymph stages of these parasites have been observed on a range of larger mammals including pigs, deer, antelope, bison, elk, goats, sheep, cattle, camels, horses, and dogs, whereas their larval stages have been found infesting smaller hosts such as rodents and lagomorphs (24, 27). The subgenus *Asiacentor* is mainly found in Asia, and *Dermacentor pavlovskyi* is regarded as the type species for this subgenus (28–31). These ticks have primarily been found infesting small ruminants, such as goats and sheep (29, 31, 32). The known distribution of *D. pavlovskyi* includes Central Asia and China, which are parts of the Palearctic region (26, 29, 31, 32), and there have been no recorded data of this species in the Oriental region.

Currently, there is a lack of consensus on the systematic classification and taxonomy of argasid ticks, including the genus *Alectorobius* (18, 19). Similarly, although the current understanding recognizes seven subgenera within the genus *Dermacentor* (24, 33, 34), the systematics and taxonomy of this genus pose significant challenges (27, 35). In such circumstances, combining morphological and molecular studies on ticks, especially those that are poorly understood, could be crucial for clarifying their phylogenetic position (36–38). Moreover, understanding tick species from different geographic locations is important for shedding light on the evolutionary history of ticks. Although demonstrating characteristics of both the Palearctic and Oriental regions, the knowledge regarding the genera *Alectorobius* and *Dermacentor* is limited within Pakistan. No tick species from the *Alectorobius* genus has been reported, while only *Dermacentor raskemensis* and *Dermacentor marginatus* have been identified morphologically or molecularly within the *Dermacentor* genus in this country (39–43). To address this knowledge gap, in this study, we reported the occurrence and genetic characterization of *Dermacentor* and *Alectorobius* species in Pakistan using morphological and molecular approaches.

Materials and methods

Study area

This study was carried out in the districts of Quetta (30°08′55.3″N, 66°57′42.1″E) and Charsadda (34°10′17.5″N, 71°45′31.7″E) in the provinces of Balochistan and Khyber Pakhtunkhwa, Pakistan, respectively. The geocoordinates of the collection areas were determined using the Global Positioning System (GPS), and the study map was designed using ArcGIS v. 10.3.1 (Figure 1).

Tick collection and preservation

Tick specimens were collected from sheep and nests of swift birds in 2023 from the districts of Quetta and Charsadda, respectively. In order to avoid any external damage to the specimens, the ticks were carefully detached from the host body and nests using tweezers. The specimens were rinsed in distilled water followed by 70% ethanol and subsequently preserved in 100% ethanol in 1.5 mL Eppendorf tubes.

Morphological identification of ticks

The collected specimens were morphologically identified using a stereo-zoom microscope (StereoBlue-euromex SB.1302-1, Arnhem, Netherlands) using standard morphological identification keys for *Dermacentor* spp. (28–30) and *Alectorobius* spp. (21, 44, 45).

DNA extraction and PCR

A total of 14 ticks including five *Dermacentor* spp. and nine *Alectorobius* spp. specimens selected for DNA extraction and were dried in an incubator for 30 min. With the use of sterilized scissors and a micro pestle, the specimens were homogenized in 200 µL of phosphate-buffered saline (PBS). The phenol-chloroform method was used to extract the genomic DNA (46), and 30 µL of "nuclease-free" PCR water was utilized to dilute the extracted DNA pellet. The genomic DNA was measured through NanoDrop (Nano-Q, Optizen, Daejeon, South Korea) and kept at −20°C for further experiments.

Conventional PCR (GE-96G, BIOER, Hangzhou, China) was used to amplify partial fragments of mitochondrial 16S rRNA and *cox1* from the extracted genomic DNA of ticks (Table 1). Each PCR reaction mixture was performed in a total 25 µL volume—containing 1 µL each primer—forward and reverse (10 µM), 2 µL of genomic DNA template (100 ng/µL), 8.5 µL of "nuclease-free" PCR water, and 12.5 µL of DreamTaq green MasterMix (2x; Thermo Scientific, Waltham, MA, United States). A positive control (DNA of *Argas persicus* or *Hyalomma anatolicum*) and a negative control (PCR water that had been "nuclease-free" instead of DNA) were included in each PCR reaction. Lists of the primers used in the present study along with the thermocycler conditions are shown in Table 1.

The products of the PCR were electrophoresed on a 2% agarose gel and observed under ultraviolet light in a Gel Documentation System (RB Flash Digi, Robus Technologies, United Kingdom). The DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA,

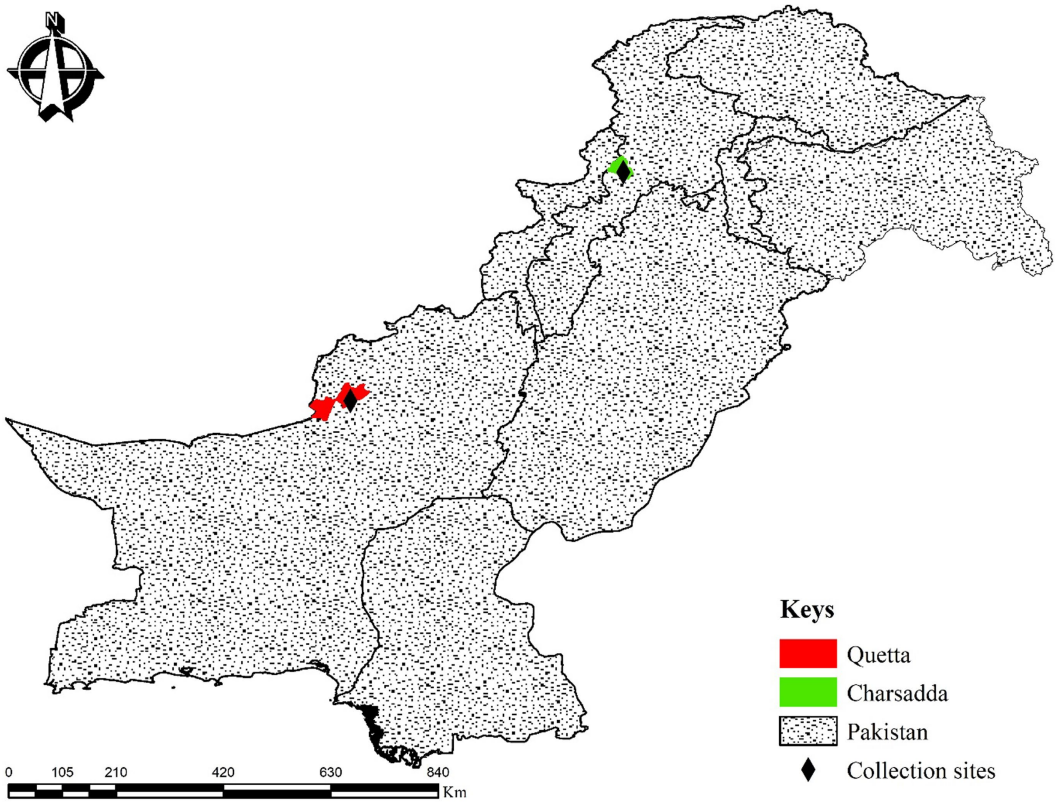

FIGURE 1
Map showing the collection sites of ticks during this survey.

TABLE 1 List of primers that were used to amplify the ticks' targeted DNA.

Gene	Primer sequences 5′-3′	Amplicon size	Annealing temperature	References
cox1	HC02198: TAAACTTCAGGGTGACCAAAAAATCA	649 bp	95°C 5 min, 40× (95°C 30 s, 48°C 60 s, 72°C 1 min), 72°C 5 min	Folmer et al. (47)
	LCO1490: GGTCAACAAATCATAAAGATATTGG			
16S rDNA	16S + 1: CCGGTCTGAACTCAGATCAAGT	460 bp	95°C 3 min, 40× (95°C 30 s, 56°C 60 s, 72°C 1 min), 72°C 7 min	Mangold et al. (48)
	16S − 1: GCTCAATGATTTTTTAAATTGCTG			

United States) was used to purify the PCR-positive samples in accordance with the instructions provided by the manufacturer.

DNA sequencing and phylogenetic analysis

All amplified amplicons of *cox1* and 16S rDNA partial fragments were sequenced bidirectionally (Macrogen Inc., Seoul, South Korea) using the Sanger sequencing method. The obtained sequences were cropped through SeqMan v. 5 (DNASTAR, Inc., Madison/WI, United States) to remove poor reading sequences and subjected to Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) at the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). After BLAST, high identity sequences were downloaded in FASTA format from the NCBI. The obtained sequences were aligned with the downloaded sequences using ClustalW multiple alignments in BioEdit Sequence Alignment Editor v. 7.0.5 (49). The phylogenetic trees were constructed individually for each gene sequence of the tick, using the maximum likelihood method with the Tamura-Nei model in Molecular Evolutionary Genetics Analysis (MEGA-X), with a bootstrapping value of 1,000 (50). The coding sequences were aligned using MUSCLE alignments (51).

Results

Ticks and their geographic origin

A total of 25 tick specimens were collected and morphologically identified into two distinct species, *A. coniceps* and *Dermacentor* sp., both of which were found in different areas. These comprised 14 out

of 25 (56%; 9 males and 5 females) *A. coniceps* from three different nests of swift birds in the district of Charsadda, Khyber Pakhtunkhwa and 11 out of 25 (44%; 7 males and 4 females) *Dermacentor* sp. from sheep in the district of Quetta, Balochistan.

Morphology of *Alectorobius coniceps*

Female

They are broadly rounded posteriorly, obtusely angled anteriorly with a small, bluntly subtriangular hood, and the color is light to dark brownish to black. Approximately 10 setae and large central pores are found on the anterior labium, and irregularly divided longitudinal striations are found on the posterior labium. The coxal and supracoxal folds are conspicuous. The transverse part of the pre-anal groove is small. With comparatively larger disks adjacent to it, the posteromedian groove extends from the anus to the paired organ. The posterior paired organ can be found at approximately posterior 1 out of 7 to 1 out of 9 of the body. The anterior valve is small and finely striated in shape, whereas the posterior valve is wider and less finely striated in shape with coxa IV disks at each apex. The spiracular plates are large and positioned laterally to coxa IV. The capitulum in the camerostome is between the coxa I and the hood. Basis capituli ventral surface is pebbled and approximately two times as broad as long. Legs are narrow and long, and surfaces are pebbled. Tarsi are narrow, elongate, and abruptly tapering distally (Figure 2).

Male

Except for sexual characteristics and size, male ticks are similar to female ticks. The integument around the genital operculum is finely and densely pebbled spiculate, and the posterior integument is transversely rugose. Nymphs resembled adults, except for the lack of external genitalia. Base capituli posthypostomal setae extending to the level of midpalpal segment 2 length. Legs have moderate length and humps (Figure 2).

Morphology of *Dermacentor* sp.

Female

The female tick's body is elongated, oval, and brownish-red in color. Legs and capitulum are lighter. Except for punctations and grooves, the scutum is oval and whitish in color. Eyes are in front of the middle lateral border. The genital opening is level on coxae II and III. The spiracles are well developed. The capitulum is long and hairy with slightly developed whitish patterns dorsally. Cornua formed barely tubercles, and porose regions are subcircular. Palps are twice as long as they are wide; article I is small; article II and III are both well developed; and article II has a slight posterodorsal spine, while article IV is small and cylindrical. Coxa I has long, triangular, close-spaced, and parallel spurs with tapering or narrowly rounded tips; the external spur is usually nearly equal to or slightly shorter than the internal; and both the spurs are directed slightly posterolateral. Each part of coxae II and III has a sharp external spur and a smaller, broadly oval internal spur; coxa IV is enlarged and rounded with a narrow triangular external spur with a tapering apex. Legs are ornamented, hairy, and lack spines. The tarsi are slightly raised (Figure 3).

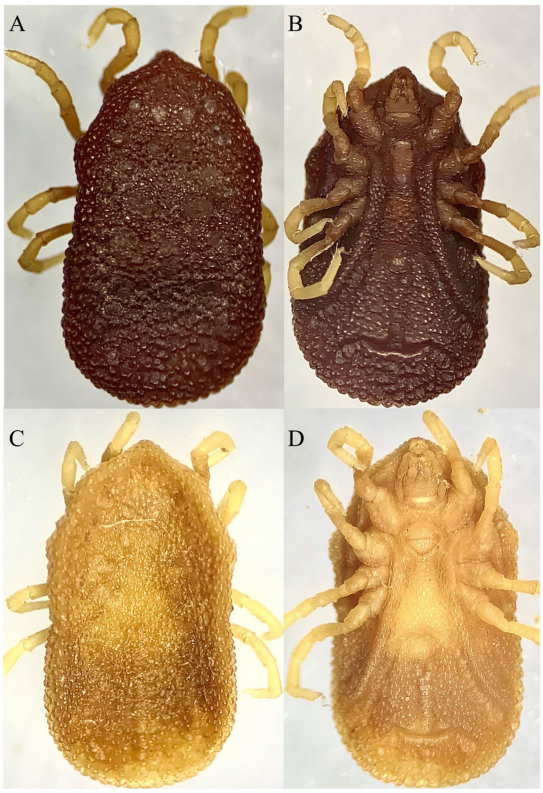

FIGURE 2
Male (**A**: dorsal and **B**: ventral) and female (**C**: dorsal and **D**: ventral) views of *Alectorobius coniceps* collected in this study from nests of swift birds in district Charsadda, KP.

Male

They are oval and brownish-red in color. The scutum is partially curved, and the whole scutum is white except for the grooves and punctations. The cervical grooves are twisted outward, while the marginal grooves are deep. The outside festoons are wider than the inner ones. The punctations can be seen in some short hairs, some of which are dispersed. Eyes are on a level on coxa II. The genital aperture is parallel to coxa II, and the genital grooves extend almost parallel to coxa IV. They have thin and well-developed spiracles. The cornua is tapered to large spines. The palps are twice as long as the hypostome. Articles II and III are well developed, article II has a tiny spine postero-dorsally, article III is triangular dorsally, and article IV is small and cylindrical. Legs are similar to or resemble those of the female (Figure 3).

Molecular analysis

The BLAST analysis of the 16S rDNA sequence belonging to morphologically identified *A. coniceps* showed 97.46% maximum identity with the same species. In the phylogenetic tree, the 16S rDNA sequence for *A. coniceps* was clustered with the same species reported from Malta (MK946450) and grouped in a sister clade with *Alectorobius capensis* (KU946450) and *Alectorobius sawaii* (MK606017). The BLAST analysis of the 16S rDNA sequence for *Dermacentor* sp. showed 98.42% maximum identity with *Dermacentor*

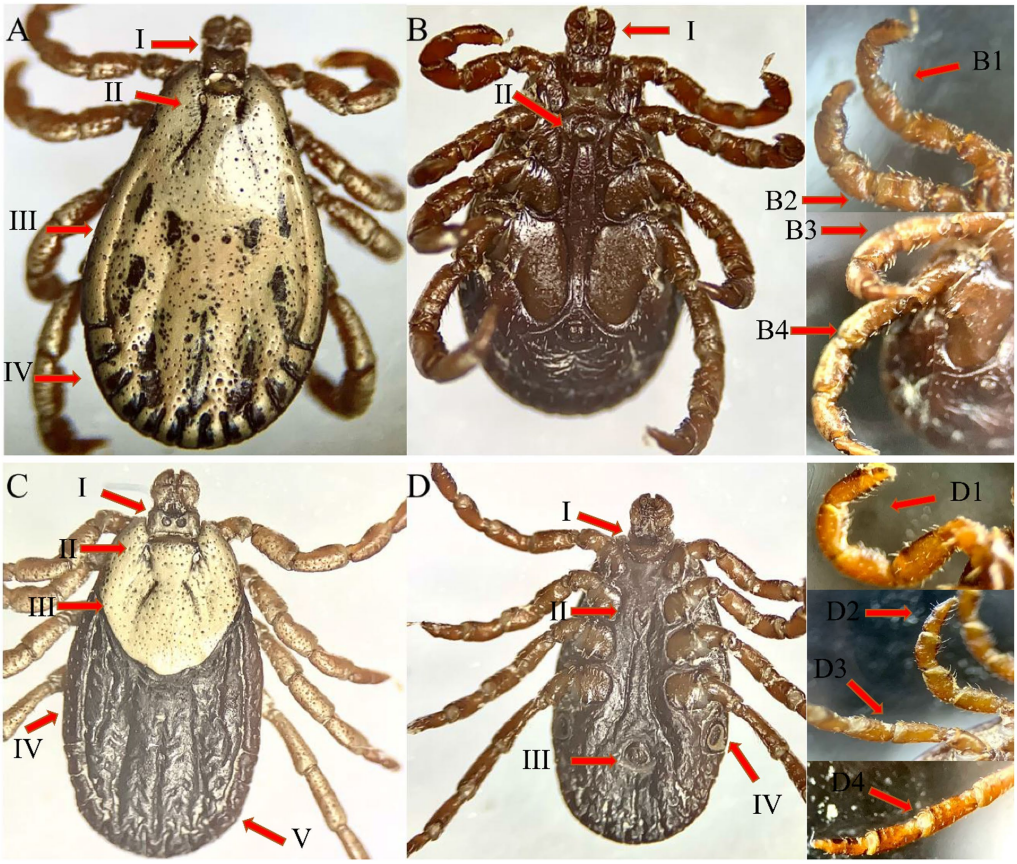

FIGURE 3
The male [**A**: dorsal—dorsally capitulum (I), cervical groove (II), Lateral groove (III), and festoon (IV), **B**: ventral—ventrally capitulum (I), and genital aperture, leg 1 (B1), leg 2 (B2), leg 3 (B3), leg 4 (B4)] and female [**C**: dorsal—dorsally capitulum (I), cervical groove (II), scutum (III), lateral groove (IV) and festoon (V), **D**: ventral—ventrally capitulum (I), genital aperture (II), anal groove (III) and spiracles (IV), leg 1 (D1), leg 2 (D2), leg 3 (D3), leg 4 (D4)] views of *Dermacentor* sp. collected in this study from district Quetta, Balochistan.

pavlovskyi, followed by 96.35% with *Dermacentor marginatus*, 95.79% with *Dermacentor raskemensis*, 95.60% with *Dermacentor niveus*, 94.23% with *Dermacentor nuttalli*, 94.20% with *Dermacentor silvarum*, and 93.70% with *Dermacentor sinicus*. Phylogenetically, the 16S rDNA sequence for *Dermacentor* sp. clustered with the *D. pavlovskyi* reported from China (OK493293-OK493294) and grouped in a sister clade with *D. raskemensis, D. nuttalli, D. marginatus, D. silvarum*, and *D. sinicus* (Figure 4). The obtained 16S rDNA sequence for *A. coniceps* and *Dermacentor* sp. were deposited to the GenBank under accession numbers: OR643824 and OR643821, respectively.

The BLAST analysis of the mitochondrial *cox1* partial sequence obtained for *A. coniceps* showed 96.49% maximum identity with the same species. In the phylogenetic tree, the *cox1* sequence for *A. coniceps* clustered with the corresponding sequence was reported from Malta (MK946447). While the BLAST analysis of the obtained *cox1* sequence for *Dermacentor* sp. showed 97.45% maximum identity with *D. pavlovskyi* followed by 93.85% with *D. raskemensis*, 92.09% with *D. nuttalli*, 91.92% with *D. sinicus*, and 91.39% with *D. marginatus*, *D. silvarum*, and *D. niveus*. In the phylogeny, the *cox1* sequence for *Dermacentor* sp. clustered with the *D. pavlovskyi* species was reported from China (OK489456) and grouped in a sister clade with *D. raskemensis*, *D. nuttalli*, *D. sinicus*, *D. marginatus*, and *D. silvarum* (Figure 5). The obtained *cox1* sequences for *A. coniceps* and *Dermacentor* sp. were deposited to the GenBank under accession numbers OR660126 and OR643819, respectively.

The sequences were used in the phylogenetic analysis for *Alectorobius* spp. (Tables 2, 3) and *Dermacentor* spp. (Tables 4, 5), and their identities with the species of the corresponding genus are shown in Table 2.

Discussion

Ticks of the genus *Alectorobius* and *Dermacentor* have medical and economic importance worldwide (20, 34, 52). However, they are among the most neglected tick genera in the Oriental region, including Pakistan. Importantly, research in Pakistan has considerably focused on the exploration of ticks and tick-borne pathogens in the last half-decade (4, 5, 9, 39, 53–65). This study provides the first morphological and molecular record of *A. coniceps* and *Dermacentor* sp. from the Oriental region, including Pakistan.

Alectorobius spp. are distributed globally possibly due to their association with birds, facilitating an efficient distribution (6, 13, 20). Among *Alectorobius* ticks, *A. coniceps* has been collected from various locations such as caves, crevices, cliffs, ravines, nests, stables, wells, and lofts in both the Palearctic and Oriental regions (20, 21, 66). In

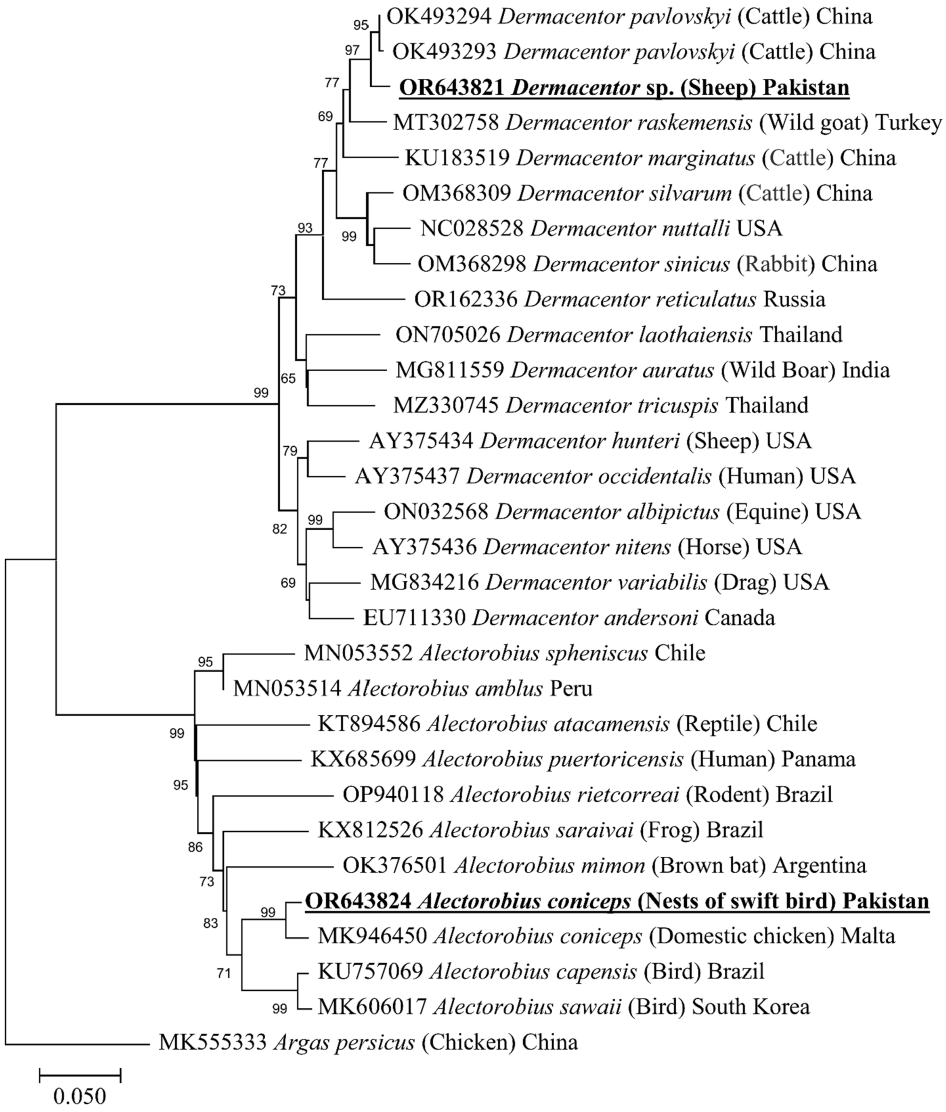

FIGURE 4
Maximum likelihood phylogenetic tree based on partial mitochondrial 16S ribosomal DNA sequences for *Alectorobius* spp. and *Dermacentor* spp. The 16S rDNA sequence of *Argas persicus* was used as an outgroup. The levels of bootstrap support (>65%) for phylogenetic groupings are given at each node; the accession numbers are followed by the species names, hosts, and locations (if applicable). The obtained sequences are shown in bold-underlined font.

this study, *A. coniceps* ticks were collected from swallows' nests in Khyber Pakhtunkhwa, which is located at the junction of the Palearctic and Oriental regions. Moreover, the larval stages of *Alectorobius* ticks may feed on the same bird species, and therefore, they are relatively well understood from the Palearctic region (13, 21, 67). With this tendency, the adults and nymphs of *A. coniceps* were collected in this study. Future studies should also prioritize the investigation of larval stages from the Oriental region, as, to the best of our knowledge, this stage has not been described in the Oriental region.

Dermacentor spp. are believed to have evolved in central Asia, and this region exhibits the highest diversity of *Dermacentor* spp. (26, 68, 69). Among *Dermacentor* ticks, *D. pavlovskyi* has been documented infesting goats and sheep in mountainous regions, as reported in earlier studies (29, 31, 32). Similarly, the area (Balochistan), where *Dermacentor* sp., a closely related tick to *D. pavlovskyi*, was collected in the present study, is situated adjacent to the Palearctic region and has a mountainous terrain with an approximate elevation of 5,500 feet. Other than open areas, *D. pavlovskyi* ticks have been found on wild animals in nature reserves and national parks. For instance, these ticks were collected by the Republican Tropical Station in 1941–1955 in the Aksu-Dzhabagly nature reserve in Kazakhstan (31). Interestingly, the current study area is located near the renowned national park, "Hazarganji-Chiltan." It could be assumed that *D. pavlovskyi* or closely related ticks have an affinity for wild goats and sheep in such protected areas from where they could invade domestic animals. The tendency of larval *Dermacentor* ticks to infest small animals has resulted in a limited understanding of this stage. Consequently, this study collected adult and nymphal stages of *Dermacentor* sp. ticks, and no larval stage of this species was collected.

Molecular-based analysis is pivotal for comprehending the debated systematics and taxonomy of tick species, including the genus *Alectorobius* and the genus *Dermacentor* (19, 37, 70, 71). Consequently,

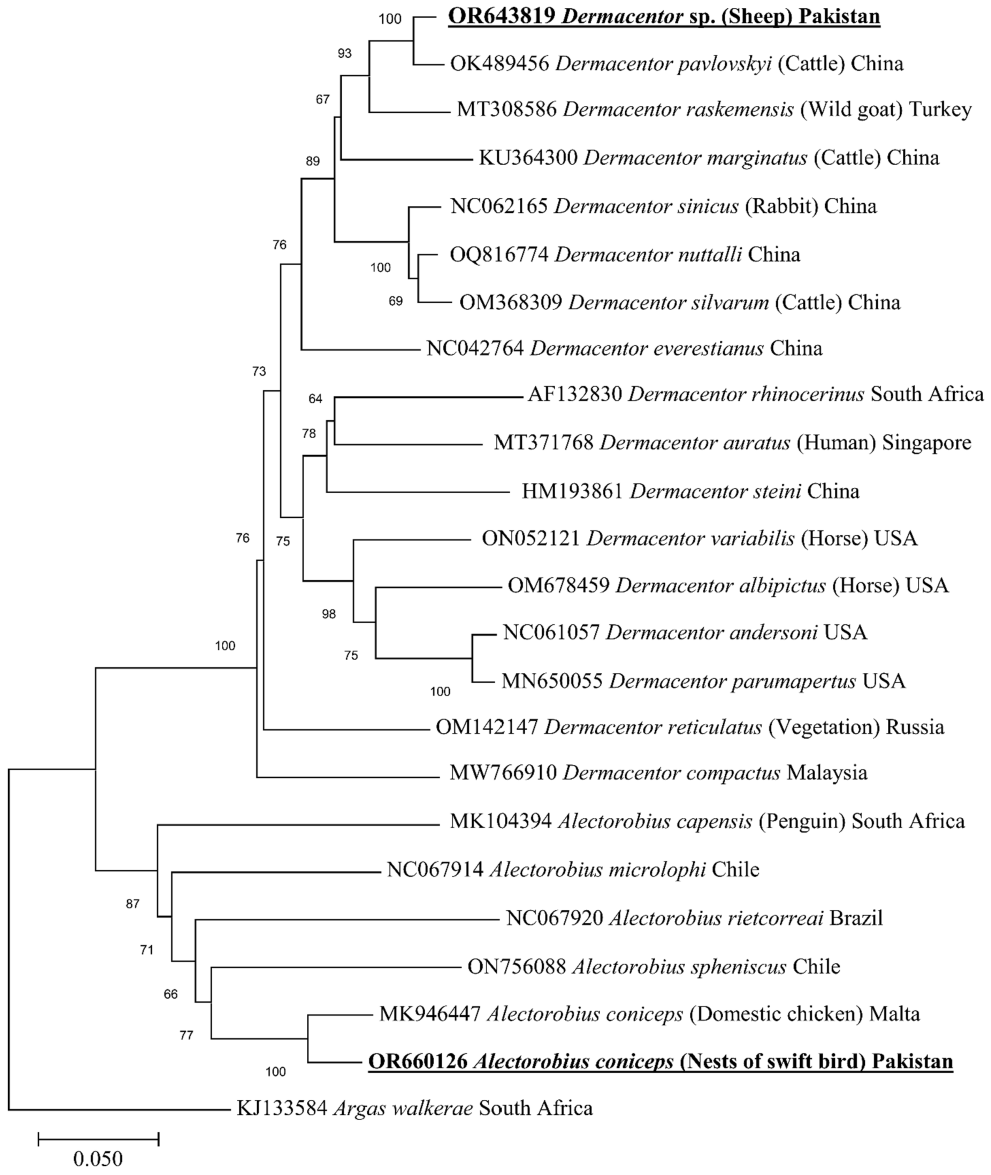

FIGURE 5
Maximum likelihood phylogenetic tree based on partial mitochondrial *cox1* sequence for *Alectorobius* spp. and *Dermacentor* spp. The *cox1* sequence of *Argas walkerae* was used as an outgroup. The levels of bootstrap support (>65%) for phylogenetic groupings are given at each node; the accession numbers are followed by the species names, hosts, and locations (if applicable). The obtained sequences are shown in bold-underlined font.

A. coniceps and *Dermacentor* sp. ticks of the current study were subjected to molecular-based analysis involving 16S rDNA and *cox1* sequences. The analysis revealed that the *A. coniceps* ticks from this study displayed variations of 2.54 and 3.51% with their respective species from Malta, based on 16S rDNA and *cox1* sequences, respectively. Similarly, *Dermacentor* sp. ticks exhibited variations of 1.68% and 2.58% with *D. pavlovskyi* from China as determined by 16S rDNA and *cox1* sequences, respectively. At present, although ticks having this range of variations are considered a single species (72, 73, 4), *Dermacentor* sp. could not be validated as *D. pavlovskyi* due to some morphological variations. In contrast to *D. pavlovskyi* and *Dermacentor montanus* (28, 29), *Dermacentor* sp. examined in this study lacks prominent spines on their legs. In comparison to female ticks of *D. montanus* and *D. pavlovskyi*, *Dermacentor* sp. exhibited a genital aperture with a more wing-like shape. Furthermore, other morphological differences among these species were observed in punctuations, cervical grooves, and lateral grooves. However, further comprehensive studies such as mitochondrial genome sequencing of these ticks are essential to gain an accurate understanding of their genetic structure. Furthermore, these intraspecific variations can be attributed to various factors, including tick population size, ecology, and geographic isolation (37, 74). In addition to its closest evolutionary relationship with the same species, *A. coniceps* displayed proximity to *A. capensis*, confirming their classification into the same species complex (6, 13). Although the closest evolutionary relationship of *Dermacentor* sp. with *D. pavlovskyi* was observed, however, its

TABLE 2 Obtained 16S rDNA (OR643824; <460 bp) sequence identities with the diversity of *Alectorobius* species.

Accession numbers	OR643824	MK946450	KU757069	MK606017	KX812526	MN053552	KX685699	KT894586	OK376501	OP940118	MN053514
OR643824	100										
MK946450	97.46	100									
KU757069	92.39	92.39	100								
MK606017	91.83	91.83	98.83	100							
KX812526	89.83	89.83	90.61	90.4	100						
MN053552	88.17	88.17	89.53	89.1	86.35	100					
KX685699	88.03	88.03	87.15	87.34	87.35	85.98	100				
KT894586	87.96	87.96	88.16	88.86	89.37	85.96	87.68	100			
OK376501	87.82	87.82	89.44	89.69	88.32	85.95	85.75	86.92	100		
OP940118	87.43	87.43	88.84	89.04	87.88	85.48	87.38	87.66	86.68	100	
MN053514	87.32	87.32	89.2	89.44	86.35	92.38	86.59	85.71	84.78	87.85	100

TABLE 3 Obtained *cox1* (OR660126; <649 bp) sequence identities with the diversity of *Alectorobius* species.

Accession numbers	OR660126	MK946447	NC067914	MK104394	ON756088	NC067920
OR660126	100					
MK946447	96.49	100				
NC067914	86.48	86.23	100			
MK104394	85.66	83.62	81.34	100		
ON756088	84.98	84.2	77.91	81.4	100	
NC067920	84.04	84.6	78.08	76.07	76.18	100

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TABLE 4 Obtained 16S rDNA (OR643821; <460 bp) sequence identities with the diversity of *Dermacentor* species.

Accession numbers	OR643821	OK493294	OK493293	MT302758	KU183519	OM368309	NC028528	OM368298	OR162336	ON705026	MG811559	MZ330745	AY375434	AY375437	ON032568	AY375436	MG834216	EU711330
OR643821	100																	
OK493294	98.42	100																
OK493293	98.16	99.78	100															
MT302758	95.79	96.33	96.11	100														
KU183519	95.31	95.62	95.38	96.59	100													
OM368309	94.2	93.98	93.76	93.35	94.39	100												
NC028528	94.23	94.1	93.79	92.95	93.67	98.82	100											
OM368298	93.7	93.58	93.36	92.95	93.93	97.83	97.63	100										
OR162336	90.98	91.34	91.13	91.4	91.48	85.42	85.29	85.47	100									
ON705026	88.62	88.7	88.46	89.71	89.59	84.3	85.2	85.6	87.3	100								
MG811559	88.39	88.97	88.73	88.52	88.81	85.1	85.29	85.47	86.53	91.77	100							
MZ330745	87.57	89.7	89.47	90.39	89.29	85.2	84.3	85.3	86.6	91.71	91.07	100						
AY375434	86.54	88.94	88.72	88.12	87.02	84.9	83.4	84.55	84.44	89.32	90.31	87.99	100					
AY375437	86.54	88.94	88.72	88.55	87.47	85.42	85.29	85.47	83.2	88.19	89.37	88.66	93.44	100				
ON032568	88.16	88.17	87.96	88.63	88.14	84	81.69	82.08	82.05	88.46	88.73	88.79	92.76	93.67	100			
AY375436	87.89	89.61	89.39	89.44	88.62	84.1	82.5	86.1	85.4	89.35	89.61	89.27	93.89	94.75	95.12	100		
MG834216	88.62	89.83	89.61	89.51	87.98	86.4	85.29	85.47	84.3	89.4	89.88	88.81	94.1	94.53	93.67	94.54	100	
EU711330	87.83	89.08	88.87	89.6	88.41	82.98	83.44	83.4	83.06	90.02	90.19	90.28	93.9	93.7	88.11	94.35	94.99	100

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TABLE 5 Obtained *cox1* (OR643819; <649 bp) sequence identities with the diversity of *Dermacentor* species.

Accession numbers	OR643819	OK489456	MT308586	KU364300	OM368309	OQ816774	NC062165	OM142147	ON052121	MT371768	OM678459	NC061057	NC042764	MW766910	HM193861	AF132830	MN650055
OR643819	100																
OK489456	97.45	100															
MT308586	93.85	94.48	100														
KU364300	91.39	92.12	92.14	100													
OM368309	91.39	91.98	92.04	90.49	100												
OQ816774	92.09	92.53	92.71	91.44	86.56	100											
NC062165	91.92	92.14	91.8	91.34	92.3	97.17	100										
OM142147	88.58	88.47	88.66	88.27	88.09	88.04	88.37	100									
ON052121	86.49	87.41	87.59	86.16	83.2	86.69	83.45	86.01	100								
MT371768	86.24	86.62	86.28	85.71	86.39	86.71	86.68	85.17	86.86	100							
OM678459	87.59	87.28	87.01	86.52	82.58	86.35	82.68	86.15	88.36	86.32	100						
NC061057	87.39	87.14	87	85.56	83.52	87.53	83.51	86.01	89.71	86.3	88.49	100					
NC042764	90.16	91.31	90.68	89.53	88.6	90.69	87.07	89.07	83.2	87.22	82.93	83.21	100				
MW766910	85.89	87.03	88.56	85.53	86.95	86.64	86.79	87.3	84.55	86.5	84.91	83.45	84.5	100			
HM193861	86.75	86.79	85.28	84.89	85.58	85.83	86.2	86.1	85.45	87.69	84.31	85.5	85.31	85.19	100		
AF132830	84.34	84.74	84.74	85.13	85.88	84.15	84.72	88.23	85.88	85.29	88.6	85.08	85.55	86.12	85.32	100	
MN650055	87.25	87.09	87.6	84.48	87.6	87.77	87.44	87.7	89.22	85.46	88.7	84.4	85.3	86.02	85.57	85.48	100

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closeness with other species of the same subgenus, such as *D. montanus*, could not be verified due to the lack of authentic genetic data in GenBank.

Conclusion

This study for the first time presented both morphological and molecular data on poorly known ticks, *A. coniceps*, and *Dermacentor* sp., closely related to *D. pavlovskyi*, from the junction of the Palearctic and Oriental regions in Pakistan. The geographic, morphological, and genetic data of these tick species may aid future studies on tick systematic and taxonomy. Furthermore, the study suggests that the study area, showcasing a combination of traits from two different zoogeographic regions, could harbor a notable diversity of ticks.

Data availability statement

The data presented in the study have been deposited in the GenBank repository with the following accession numbers: OR643824, OR643821, OR660126, and OR643819.

Ethics statement

The animal studies were approved by the Advance Studies Research Board (ASRB: Dir/A&R/AWKUM/2022/9396) Committee members of Abdul Wali Khan University, Mardan, KP, Pakistan, gave their approval for the proposed study. The owners of the animals gave their verbal consent for the observation and tick collections. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AAli: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. MK: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. ZU: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. MN: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. K-HT: Data curation, Formal analysis, Validation, Writing – review & editing. AAlo: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. MA: Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. TT: Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing.

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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.

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Paraparesis due to angio-neurotropic *Gurltia paralysans* in a domestic cat (*Felis catus*) and retrospective study on feline gurltiosis cases in South America

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Introduction: The nematode *Gurltia paralysans* is a neglected angio-neurotropic parasite causing chronic meningomyelitis in domestic cats (*Felis catus*) as well as wild felids of the genus *Leopardus* in South America. Adult *G. paralysans* nematodes parasitize the leptomeningeal veins of the subarachnoid space and/or meningeal veins of the spinal cord parenchyma. The geographic range of *G. paralysans* encompasses rural and peri-urban regions of Chile, Argentina, Uruguay, Colombia and Brazil.

Methods: This case report presents clinical and pathological findings of a *G. paralysans*-infected cat suffering from severe thrombophlebitis and meningomyelitis resulting in ambulatory paraparesis. Neurological examination of affected cat localized the lesions at the thoracolumbar (T3–L3) and lumbosacral (L4–Cd4) segments. Molecular and morphological characteristics of extracted nematodes from parasitized spinal cord veins confirmed *G. paralysans*. Additionally, data obtained from a questionnaire answered by cat owners of 12 past feline gurltiosis cases (2014–2015) were here analyzed. Questionnaire collected data on age, gender, geographic location, type of food, hunting behavior, type of prey, and other epidemiological features of *G. paralysans*-infected cats.

Results and Discussion: Data revealed that the majority of cats originated from rural settlements thereby showing outdoor life styles with hunting/predatory behaviors, being in close contact to wild life [i.e. gastropods, amphibians, reptiles, rodents, birds, and wild felids (*Leopardus guinia*)] and with minimal veterinary assistance. Overall, this neglected angio-neurotropic *G. paralysans* nematode still represents an important etiology of severe thrombophlebitis and meningomyelitis of domestic cats living in endemic rural areas with high biodiversity of definitive hosts (DH), intermediary (IH), and paratenic hosts (PH). The intention of this study is to generate awareness among veterinary surgeons as well as biologists on this neglected feline neuroparasitosis not only affecting domestic cats but also endangered wild felid species of the genus *Leopardus* within the South American continent.

KEYWORDS

Gurltia paralysans, feline gurltiosis, neuroparasitosis, domestic cats, paraparesis

1 Introduction

Gurltia paralysans is a neglected and re-emerging nematode placed in the family Angiostrongylidae (superfamily Metastrongyloidea) and the only member so far reported for the genus *Gurltia* (1, 2). *G. paralysans* causes severe meningomyelitis, known as feline gurltiosis, affecting mainly domestic cats (*Felis catus*) and wild felid species of *Leopardus* (2). The parasite has been recorded in various South American countries, including Chile, Argentina, Colombia, Uruguay and Brazil (3–13). Recently, there have been reports of the first case outside of South America in Tenerife (Canary Islands, Spain), and a sporadic anecdotal case in USA previously (14). Adult *G. paralysans* nematodes (female 20–30 mm × 0.1 mm; males 12–15 mm × 0.1 mm) reside in the subarachnoid space, specifically in the thoracic, lumbar and sacral spinal cord segments of affected wild or domestic cats which represent the definitive hosts (DH). *G. paralysans*-induced meningomyelitis causes symptoms of progressive hindlimb weakness, pelvic limb ataxia, tail paralysis, urinary, and fecal incontinence (2–5). The life cycle of *G. paralysans* is still not fully understood, but hypothetically either an infected-mollusk intermediate host (IH) or an infected-bird, –amphibian and/or –reptile, acting as paratenic host (PH), is ingested by a domestic- or a wild cat (2, 6). The infective third-stage larvae (L3) migrate through the mucosal layer of the digestive system to the venous or lymphatic system of the abdominal viscera, and then via veins connections or anastomosis of the azygos or caval venous system with thoracic, lumbar or sacral intervertebral veins to reach the vertebral venous plexus. These vascular connections could also explain the presence of *G. paralysans* eggs and adults in remote anatomic sites, such as the cerebrum, cerebellum and, in more recent reports, in the anterior chamber of the eye (14–19).

Infectious etiologies of feline meningomyelitis include viruses (e.g., PIF, FelV, and FIV), and bacteria (e. g. *Pasteurella multocida*), fungal, protozoal, and nematode agents (e. g. *Toxoplasma gondii*, *Sarcocystis* spp., *Baylisascaris procyonis*, *Dirofilaria immitis*, and *Aelurosrongylus abstrusus*) (2, 20, 21). Consistently, cases of aberrant or ectopic larval migration to central nervous system (CNS) of *B. procyonis*, *D. immitis*, and *A. abstrusus* have been reported and thereby producing spinal cord lesions in domestic cats (22, 23). Nonetheless, *G. paralysans* is an emerging metastrongyloid nematode with a marked angio-neurotropic character of adult nematodes migrating to the veins of spinal subarchnoid space for mating purposes (2, 6).

The objectives of this article were to report on a recent case of chronic paraparesis associated to a domestic cat in Southern Chile and retrospectively analyze epidemiological aspects of 12 previous cases of this uncommon and neglected feline neuroparasitosis.

2 Case presentation

A 3 year old, 3.5 kg body weight (BW), female domestic cat was referred in January 2023 to the Veterinary Hospital at the University Austral of Chile (UACH), for chronic and progressive pelvic limb ataxia that had progressed to non-ambulatory paraparesis over a period of 3 months. The cat was kept indoor/outdoor in a rural settlement close to the city Puerto Varas in Southern Chile. There were external abrasions on dorsal metatarsal regions indicating chronic weakness of pelvic limbs. Neurologic examination revealed physiological mental status and cranial nerve activities, non-ambulatory paraparesis, decreased muscle tone of pelvic limbs, decreased flexor reflexes in pelvic limbs, normal patellar reflexes with no alteration in postural and spinal reflexes of forelimbs and tail paralysis. The bladder was full and difficult to express but there was no defecation disorder. Spinal hyperesthesia was detected in response to palpation of lumbar and lumbosacral regions. The neurological examination indicated that neuroanatomical lesion localization was at the T3–L3 and L4–Cd4 spinal cord segments. Complete blood count, biochemistry panel, fecal examination, spinal radiographs were unremarkable. Differential diagnosis included spinal trauma, infectious agents (i.e. viral, bacterial, fungal, protozoa, and nematodes), non-infectious inflammatory disorders (i.e. meningomyelitis of unknown origin [MUO]) and neoplastic etiologies (i.e. lymphoma and meningioma). Due to owner request, the cat was euthanized and *post mortem* examination was performed.

In order to detect *Gurltia* DNA, polymerase chain reaction (PCR) targeting the 28S rDNA was performed. A serum sample was obtained for a semi-nested PCR analysis for *G. paralysans* DNA (U2 universal oligonucleotide and *G. paralysans*-specific oligonucleotide E1: Gp28Sa3) and *Aelurostrongylus abstrusus* (U1 universal oligonucleotide and *A. abstrusus*-specific E1: Aa28Ss2) based on previously reported studies (1) (Figure 1). DNA extraction from the serum sample was performed according to the manufacturer instructions, using an E.Z.N.A.® Tissue DNA Kit D3396-02 (Omega Bio-tek, Inc., Norcross, GA, United States). The first PCR amplified DNA from both parasite species using universal oligonucleotides (forward: AaGp28Ss1 5′-CGAGTRATATGTATGCCATT-3′, reverse: AaGp28Sa1 5′- AGGCATAGTTCACCATCT-3′) based on identical conserved sequences. The second (semi-nested) specific PCR differentiated *G. paralysans* (universal forward primer AaGp28Ss1, *Gurltia* reverse primer Gp28Sa3 5′-TCTTGCCGCCATTATAGTAG-3′) from *Aelurostrongylus abstrusus* DNA (*Aelurostrongylus*-specific forward primer Aa28Ss2 5′-CGTTGATGTTGATGAGTATC-3′, universal reverse primer AaGp28Sa1). Reaction conditions for the first PCR were as follows: 5 min initial denaturation at 94°C 35 to 40 cycles of 30 s at 94°C, 30 s at 54°C and 30 s 72°C extension, followed by a 5 min 72°C elongation. From first PCR, 1 µL of the reaction was used as template for the second semi-nested PCRs under the following conditions: 94°C for 1 min, 35 cycles of 30 s at 94°C, 30 s at 56°C, 30 s at 72°C and a final elongation for 5 min 72°C. PCR products were analyzed by agarose gel electrophoresis (2%). Sequencing of the amplicon was performed in an automated DNA analyzer (Applied Biosystems). Amplification products were run on 2.5% ethidium bromide agarose gels and visualized under ultraviolet light.

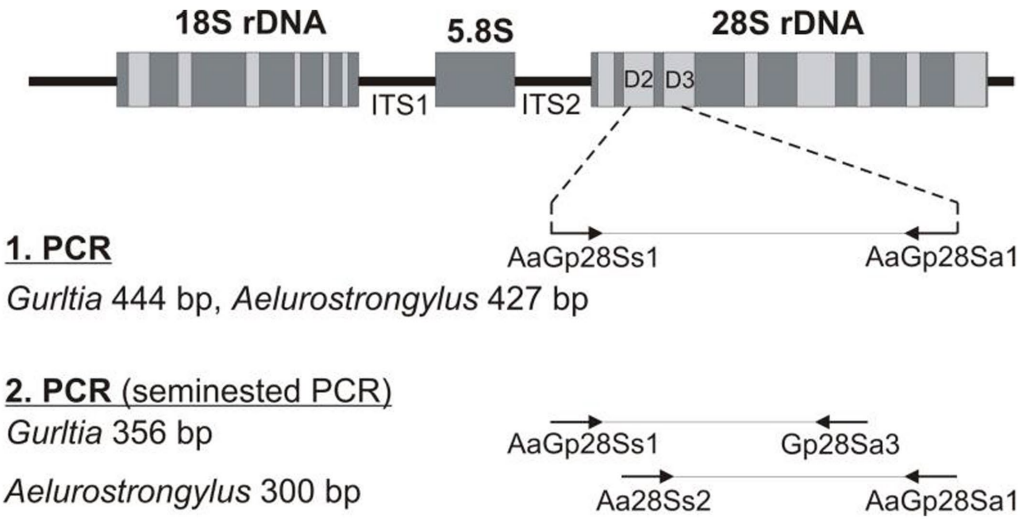

FIGURE 1
Schematic representation of the seminested PCR technique used for the *in vivo* diagnosis of *G. paralysans*- and *Aelurostrongylus abstrusus* infections.

2.1 Data collection

Additionally, data obtained from a questionnaire answered by cat owners of 12 past feline gurltiosis cases (2014–2015) were analyzed (Tables 1, 2 and 3). Questionnaire data included demographic- and risk factors using face-to-face interviews with cat owners. Data collected included age, gender, types of food, hunting behavior, type of prey, lifestyle, presence of contacting wildlife animals and duration of clinical signs. The food type included the categories of canned food, dry food and homemade food. The lifestyle categories were "indoor housing", "outdoor housing", "access to the outdoors" and "outdoor hunting".

3 Results

Results obtained from the molecular analysis confirmed identification of *G. paralysans* (GenBank: J975484) but were negative for *A. abstrusus*. On macroscopic *post mortem* evaluation, no significant lesions were observed in the brain up to C8 spinal cord segment. Submeningeal congestive vessels, i.e. indicative of progressive thrombophlebitis, were observed at the cervicothoracic, mid thoracic and lumbar spinal cord segments (Figure 2). Moving adult nematodes were observed in the leptomeningeal vasculature near the dorsal nerve rootlets of the lumbar segment by examination under a dissecting miscroscope and then carefully extracted "... (Figure 3A). One of the extracted specimens was approximately 13 mm in length and morphological characteristics were consistent with a male nematode of *G. paralysans* (Figure 3B). Brain and spinal cord samples from affected domestic cat were fixed in neutral buffered 10% formaldehyde. Samples were embedded in paraffin wax, and sections (4 µm) were stained with hematoxylin and eosin (HE). Histopathologic evaluation of the formalin-fixed section of spinal cord revealed meningomyelitis extending the cervicothoracic to the sacral regions. Lesions included congestion of spinal cord and leptomeningeal vasculature and presence of thrombi in the subarachnoid veins and sections of adult specimens of *G. paralysans* in the spinal cord parenchyma (Figure 4).

In the descriptive analysis of the 12 previous cases of feline gurltiosis, variables such as "age > 3 years" and/or "not castrated" were more frequent observed in affected cats (Table 1). The absence of veterinary care and infrequently or never given anthelmintic treatments were more likely observed in affected than regularly treated cats (Table 2). Additionally, cats living "rural" and with "outdoor activities" were more frequently affected by this neuroparasitosis (Table 3).

4 Discussion

The feline patient described in this report presented chronic progressive ambulatory paraparesis. The most frequent clinical manifestation of neurological feline gurltiosis is chronic and progressive ambulatory pelvic limb weakness that may end in paraplegia (3–5, 9, 11). Regarding the length of clinical signs, our retrospective study found that the majority of cases (5/12) had a chronic duration (> 1 month) in agreement with other reports. In line, previous studies have shown that the duration of clinical signs can range from 2 weeks to 48 months (5, 9–11, 24). Other signs observed and that have been reported include pelvic limb ataxia, pelvic limb proprioceptive deficit, pelvic limb tremor, pelvic limb muscle atrophy, tail tremors, tail atony, and fecal/urinary incontinence (3–5, 8, 11, 16, 17). Neurological signs had correlative neuroanatomical lesions observed at necropsy and in histopathological samples (2, 5, 24). Haematological abnormalities associated with feline gurltiosis included non-regenerative anaemia and low mean corpuscular haemoglobin concentrations (hypochromia) (2, 5) revealing chronic inflammatory disease or chronic blood loss (2, 5). The eosinophilia associated with parasitic infections has commonly been reported in domestic animals, but is not a frequently observed feature in domestic cats with feline gurltiosis (5), which has also been reported in dogs with neural angiostrongylosis (25, 26). No clinical signs of

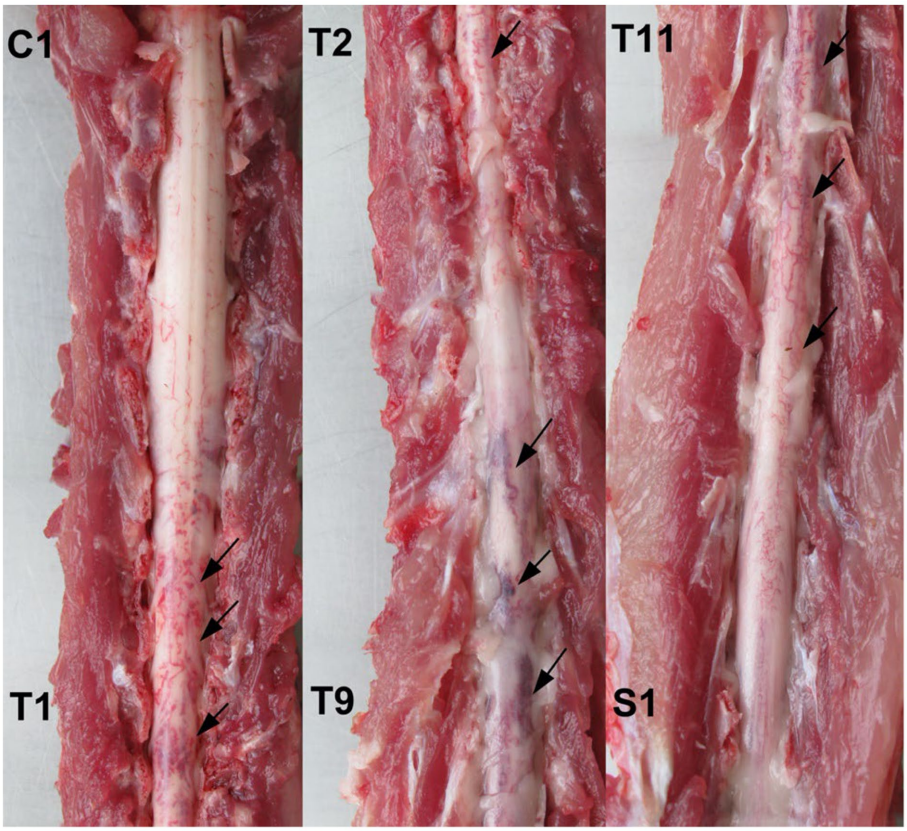

FIGURE 2
Post mortem spinal cord specimen from an affected domestic cat with *G. paralysans* infection. Multiple areas of submeningeal congestion (arrows) are observed in the cervicothoracic-, thoracic-, and lumbar spinal cord segments.

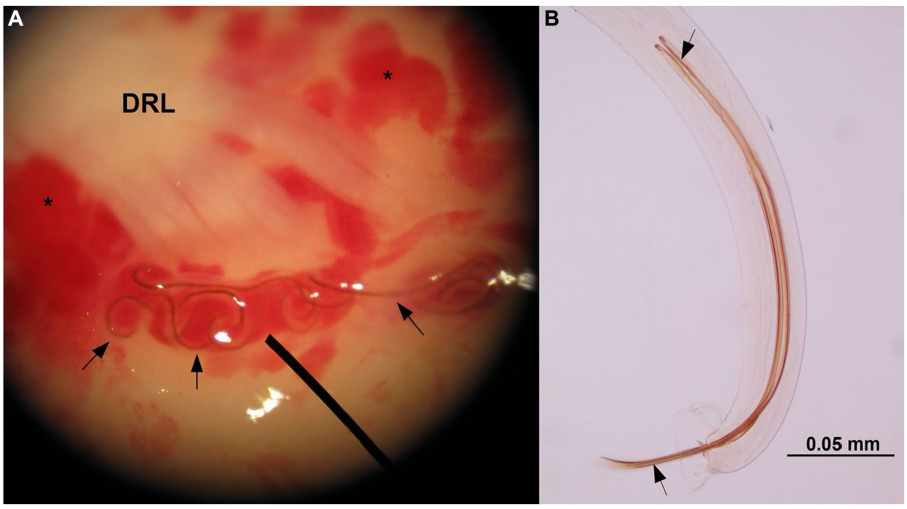

FIGURE 3
Stereoscopic microscope illustration indicating the presence of adult nematodes of *G. paralysans* (arrows) in a vein at the base of the dorsal rootlets (DRL) of a lumbar spinal nerve. Multiple areas of congestive leptomeningeal vessels are observed (asterisks). Video is available in the Supplementary material **(A)**. Caudal end of an adult male specimen of *G. paralysans* indicating the spicules (arrows).

coagulopathy have been observed in naturally *G. paralysans*-infected cats. However, high levels of urea in the blood (uremia) have been reported probably arising from neurogenic urinary dysfunction (5). A bronchial lavage analysis of five naturally *G. paralysans*-infected cats showed absence of larval stages and eggs (27). Ocular lesions including uveitis, chorioretinitis, posterior synechiae, and corneal oedema, have

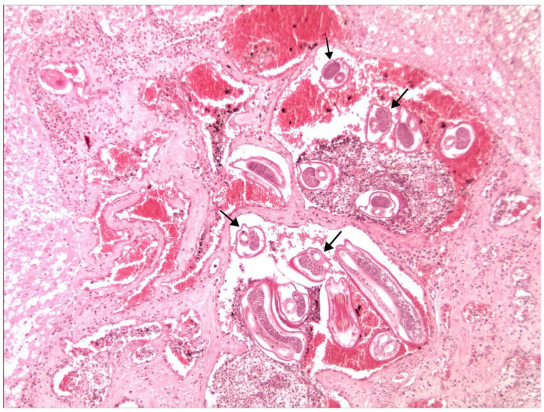

FIGURE 4
Histopathological sections of the lumbar spinal cord parenchyma of the affected cat. Several adult *G. paralysans* specimen cross section (arrows) are observed within the spinal cord vasculature and surrounded by blood and inflammatory cells. H&E. Original magnification 100X.

TABLE 1 Variable demographic description collected from cat owners of 12 past feline gurltiosis cases in Southern Chile.

Variable	*n*°	%
Age		
<1 y	1	8.3
>3 y	7	58.3
ND	4	33.3
Total	12	100.0
Breed		
DSH	5	41.7
DLH	3	25.0
ND	4	33.3
Total	12	100.0
Sex		
Female	6	50.0
Male	6	50.0
Total	12	100.0
Spayed/castrated		
No	10	83.3
Yes	2	16.7
Total	12	100.0

DSH, domestic short hair cat; DLH, domestic long hair cat; ND, non data available.

TABLE 2 Variable demographic information collected from cat owners of 12 past feline gurltiosis cases in Southern Chile.

Veterinary care	*n*°	%
No	9	75.0
Yes	3	25.0
Total	12	100.0
Vaccination/anthelmintic status		
Updated	1	8.3
Sometimes	3	25.0
Never	8	66.7
Total	12	100

recently been reported to be associated with the presence of a motile adult specimen of *G. paralysans* in the anterior chamber of the eye of a domestic cat (14).

In line, necropsy findings of *G. paralysans*-infected domestic cat reported in this study, included presence of adult females and males in meningeal veins thereby inducing diffuse leptomeningeal congestion of the lumbar, sacral, and caudal spinal cord segments (Figure 5). These findings are in line with pathological lesions observed in other previous studies (3, 4). Numerous intravascular eggs, nematode larvae and pre-adult stages can be identified histologically in the meningeal veins of the spinal cord, associated with vascular congestion, thrombosis, and thickening of the subarachnoidal vessels (3–5, 11). Presence of mild smooth-muscle hypertrophy, moderate adventitial fibroplasia, and marked subintimal fibrosis of the spinal cord venules (phlebosclerosis) have been reported as well (11). In some specimens, concentric thickening of the venule wall may produce stenosis of the vessel lumen (11). Sections of normal or dilated and tortuous varicose venules may contain thrombi with various levels of organization (11). The spinal cord parenchyma may show areas of malacia, microcavitation, multiple haemorrhages, extensive areas of malacia, with gitter cells and adjacent reactive gliosis and foci of mineralization (4, 11, 24). Lymphocytic infiltrate, intermingled with fewer macrophages, primarily infiltrate the subarachnoid space, forming a perivascular pattern. Mature eosinophils distributed randomly within the leptomeninges have also been observed, which are consistent with broad spinal leptomeningitis and thrombophlebitis (4, 11). Some animals may also show granulomatous leptomeningitis or suppurative leptomeningitis (11). Expression of GFAP, CNPase, factor VIII, CD3 and CD45 in affected spinal cord segments are suggestive of reactive gliosis and chronic inflammatory spinal lesions consequent to the ischemia caused by *G. paralysans*-mediated vascular injury (28). However, no cases of feline gurltiosis have been observed with clinical encephalic syndromes.

The morphological features of the specimen recovered from the affected spinal cord were consistent with an adult male of *G. paralysans* (Figure 3). This identification was further confirmed by a semi-nested PCR showing the expected amplification products and matching with *G. paralysans* sequence.

Historically, the first cases of feline gurltiosis were reported in 1933 in domestic cats from temperate rainforest areas around Valdivia in Southern Chile (29, 30). Subsequently, cases have been reported in other regions of Chile including the IX (i.e. La Araucanía)- and in the X (i.e. Los Lagos) region (2–4, 31) (Figure 4). Since then, other cases have been diagnosed in Argentina (8, 10), Uruguay (9), Colombia (7), and Brazil (11, 24, 25) (Table 4). In Chile, cases of feline gurltiosis have been predominantly reported in rural areas, but understanding of the spatial distribution in this environment is limited (3–5, 19, 29, 30). As above stated, feline gurltiosis has been diagnosed in three different rural regions of Southern Chile: (i) the Araucanía region (e.g., Lastarria), (ii) the Los Rios region (e.g., Punucapa, Niebla, Paillaco, and Futrono), and (iii) the Los Lagos region (e.g., Futrono, Pichirropulli, and Ancud) (4) and

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TABLE 3 Environmental and feeding variables reported from cat owners of 12 past feline gurltiosis cases in Southern Chile.

Variable	Cases											
	1	2	3	4	5	6	7	8	9	10	11	12
Habitat												
Cat goes outside	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cat lives outdoor	no	✓	✓	no	✓	✓	✓	✓	✓	✓	✓	✓
Cat interacts with other outdoor cats	✓	no	✓	no	✓	no	✓	✓	✓	✓	✓	✓
Environment												
Proximity to native forest	✓	✓	✓	no	✓	✓	✓	✓	✓	✓	✓	✓
Food												
Processed cat food	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Home-made food	✓	–	–	–	✓	✓	✓	✓	–	–	–	–
Milk	–	–	–	–	–	–	–	–	–	–	✓	✓
Hunting	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Type of prey												
Birds	✓	nd	✓	nd	✓	✓	✓	✓	✓	✓	✓	✓
Insects	nd	nd	nd	✓	✓	✓	nd	nd	nd	nd	✓	✓
Rodents	✓	✓	✓	nd	✓	✓	✓	✓	✓	✓	✓	✓
Small lizards	✓	nd	✓	nd	✓	✓	nd	nd	nd	✓	✓	✓
Presence of wild animals												
Wild felids (*Leopardus guigna*)	✓	–	–	–	✓	–	✓	✓	✓	✓	✓	✓
Frogs	–	–	✓	–	✓	–	–	–	–	–	✓	✓
Foxes (*Pseudalopex griseus, Pseudolopex culpaeus*)	–	✓	–	–	–	–	–	–	–	–	–	–
Puma (*Puma concolor*)	–	✓	–	–	–	–	–	–	–	–	–	–
Others (small lizards, birds, and rodents)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

nd, non data available.

more recently in the cities of Puerto Montt, Valdivia, and Temuco (31). In Colombia, six cases of feline gurltiosis in domestic cats were diagnosed in Antioquia (municipalities Tarso and Amagá), with signs of spinal hyperesthesia and paraparesis (7). In Argentina, *G. paralysans* was reported in one cat in the Baradero area of Buenos Aires province, and in three cases in rural areas of the Santa Fé province, specifically in the districts of Las Colonias, San Cristóbal, and Castellanos (8). In Uruguay, between 2008 and 2009, two cases of feline parasitic meningomyelitis caused by *G. paralysans* were documented in the rural region of Fray Bentos (9). Gurltiosis has been identified in Brazil since the mid-1990s, and is known locally as "bambeira," "derrengado," or "renga," which mean wobbly or lame. In 2013, there were four reported cases of cats infected with *G. paralysans* in the state of Rio Grande do Sul (11), and eleven cases were later found in Pernambuco (municipalities of Caetés and Capoeiras), Northeastern Brazil (24). More recently, a 2-year-old cat in São Paulo state in Southeastern Brazil was diagnosed with feline gurltiosis, indicating a wide distribution of the parasite throughout Brazilian territory (25). Additionally, two wild cats, specifically *Leopardus wieddii* and *Leopardus triginus*, were discovered in Parana (Chapecó), showing typical severe spinal cord lesions associated with feline gurltiosis (32, 33). The first case outside of America was documented in Tenerife, Canary Islands, Spain involving a parasitic ocular infection by *G. paralysans* (14). In 1993, a cat in the United States of America (USA) exhibited neurological signs and necropsy findings in the lumbar spine consistent with feline gurltiosis (34). The occurrence of feline gurltiosis, in both Spain and in USA, may have been caused by the introduction of *G. paralysans*-infected domestic cats from South American endemic areas, or by the importation of infected IH or PH, similar to the closely related *Angiostrongylus cantonensis* to the Canary Islands of Spain (35). However, further research is needed for defenitive conclusions.

The epizootiology of the disease has been linked with rural and rainforest areas, in both temperate and tropical humid ecosystems with abundant vegetation (2, 3, 6, 9, 11, 24, 29, 30). In our retrospective study, all 12 cases of feline gurltiosis were from rural areas. The prevalence of *G. paralysans* in South America is unknown, but is likely to be underestimated and the disease under-diagnosed as recently demonstrated (1, 31). Modeling studies indicate that certain regions in Southern Chile and Argentina, as well as areas in Brazil, Uruguay, and Colombia, are at high risk of the spread of metastrongyloid nematodes based on their climatic suitability (2, 36). Feline gurltiosis has no seasonal occurrence pattern and can be detected throughout the year (3, 4, 9). Regarding the presence of wildlife near the habitat of *G. paralysans*-infected domestic cats, owners indicated the presence of terrestrial gastropods (IH), wild felid species guiña (*Leopardus guigna,* DH), small lizards, frogs, snails/slugs and birds (PH). In terms of predation, owners declared that birds, rodents, lizards and insects were the main prey of hunting. These prey species have previously been mentioned as possible PH in the epizootiology of feline gurltiosis

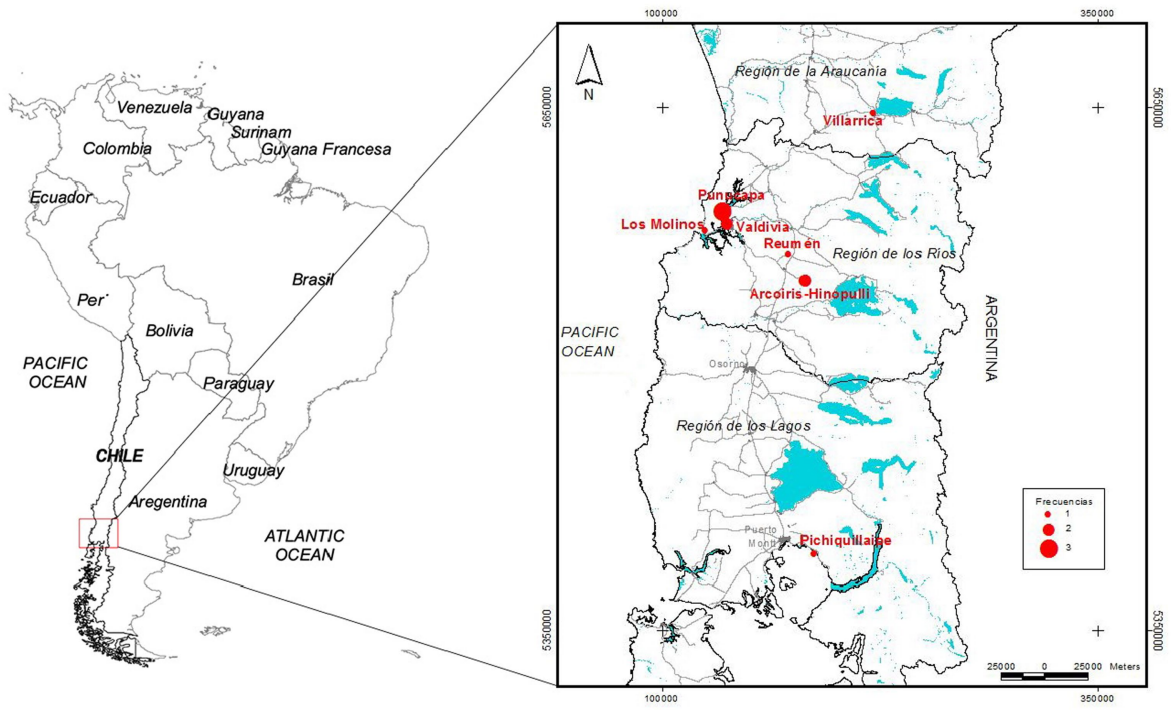

FIGURE 5
Geographic distribution of the 12 cases of feline gurltiosis, frequencies and wetland locations in Southern Chile.

(2, 6). Contact with synanthropic animals, such as birds and rodents, might increase the susceptibility to new parasitic infections as reported elsewhere (37, 38). Additionally, in our study high frequency of infected cats showed an outdoor behavior mentioned by the owners, indicating increase exposure or opportunity to ingest *G. paralysans*-infected IH or PH. These results support the fact that outdoor lifestyle by cats increases the risk of infection. The high rainfall that occurs in Southern Chile and the abundant vegetation and large hydrographic areas (rivers, lakes) support the presence of possible IH or PH that could facilitate transmission from wild guiñas (*L. guigna*) to domestic cats mainly in rural areas.

In one report, domestic cats with feline gurltiosis showed co-infections with *A. abstrusus*, although none of them showing respiratory signs (5). This might suggest that *G. paralysans* and *A. abstrusus* may share the same IH and/or PH (2). Factors affecting the distribution of gastropod species are important in determining whether the life cycle of *G. paralysans* can be completed and whether there is a potential contact with suitable DH and/or PH. Previous epizootiological studies have indicated that the endemic range of closely related *Angiostrongylus vasorum* has expanded into new countries and regions (39–41) and might be extrapolated for *G. paralysans*. Models have been used to predict the distribution of *A. vasorum* and the risk of infection based on climatic variables and their effects on the survival rates of infected IH (40). Similar modeling information is required for *G. paralysans* to predict the real distribution range in South American countries with similar climate conditions, high biodiversity of IH, PH and DH. As such, the causes of apparent re-emergence of metastrongyloid parasitoses in domestic animals are still unknown, but several factors may explain the recent increases in reports of feline gurltiosis in several countries (24, 25). Factors such as global warming, changes in the population dynamics of IH and PH, and movement of animals (DH) may explain the increase in reports of feline gurltiosis (39, 40). A recent large scale serological/molecular investigation in Southern Chile, including 171 examined animals, revealed a 54.4% occurrence of feline gurltiosis in domestic cats (31). Nonetheless, specific local and/or global geographic criteria studies, or prevalence studies on wild felids are necessary to better understand their role in transmission. Finally, epidemiological investigations based on Geographic Information System (GIS) considering climatic factors, vegetation indices, humidity, temperature, altitude and biodiversity of DH, IH, and PH are urgently needed to comprehend not only the establishment but also the spread of feline gurltiosis into previously non-endemic regions.

Although questionnaires have obvious limitations, they may be useful for further investigations of re-emerging diseases and for raising awareness among clinicians and owners on neglected feline gurltiosis. Critical risk factors for cats, such as access to wildlife environments with high biodiversity (DH, IH, and PH) and hunting habits, may increase the exposure to *G. paralysans* and therefore be considered in future questionnaire-related studies.

Treatment of feline gurltiosis relays on several anthelmintic drugs including macrocyclic lactones such as ivermectin, moxidectin, selamectin and/or milbemycin (2). The usual dose of ivermectin is 300–400 mg/kg/BW, three times weekly associated with corticosteroids (e.g., prednisolone). Additionally, benzimidazoles such as fenbendazole or ricobendazole have been mentioned as effective anthelmintic drugs in literature (2, 8). However, anthelmintic treatments have been beneficial only in mild or moderate cases of suggestive feline gurltiosis but not in animals with severe non-ambulatory paraparesis, paraplegia or in cases with substantial neurological deficit.

TABLE 4 Profile of feline *G. paralysans*-induced meningomyelitis and ocular cases including age, geographic area, spinal/ophthalmic location, imaging systems and diagnosis published since 2010.

Author	Location	Cases	Age	Clinical signs	Imaging	Final Diagnosis	PCR confirmation	Extracted nematodes
Gómez et al. 2010	Chile	3	1–4 y	T3–L3 L4–S3	Myelo CT	Histopathology	NP	2F; 2 M 12F; 2 M 12F; 2 M
Moroni et al. 2011	Chile	3	1–3 y	T3–L3 L4–Cd4	NR	Histopathology	NP	–
Alzate et al. 2011	Colombia	6	6–8 m	T3–L3 L4–Cd4	Rx	Histopathology	NP	–
Guerrero et al. 2011	Argentina	1	2y	T3–L3	NR	Histopathology	NP	–
Rivero et al. 2011	Uruguay	2	ND	T3–L3	NR	Histopathology	NP	–
Mieres et al. 2013	Chile	9	8 m–10y	T3–L3 L4–Cd4	Myelo CT, MRI	Histopathology	NP	–
Togni et al. 2013	Brazil	4	ND	T3–L3 L4–S3	NR	Histopathology	NP	–
Bono et al. 2016	Argentina	3	ND	T3–L3	NR	Histopathology	NP	–
Moroni et al. 2017	Chile	1	8 m	T3–L3	Myelo CT	Histopathology	NP	11F; 1 M
Udiz-Rodriguez et al. 2018	Spain	1	2y	Anterior chamber of the eye	NR	Microsurgical extraction of parasites	Yes	1 M
Melo-Neto et al. 2018	Brazil	11	ND	T3–L3	NR	Histopathology	Yes (9/11)	–
Gómez et al. 2020	Chile	10	4 m–36 m	T3–L3 L4–S3	NP	Histopathology Angio Detect TM IDEXX	NP	10F, 2 M
Gutiérrez et al. 2020	Chile	1	4y	L4–S3	NR	NP	Yes	–
Mello Emboaba et al. 2023	Brazil	1	2y	T3–L3	CT	Histopathology	No	–

Myelo CT, computed tomography-myelography; MRI, magnetic resonance imaging; Rx, conventional radiography; ND, non data available; NP, not performed; F, female; M, male.

5 Conclusion

We reported a case of parasitic meningomyelitis due to *G. paralysans* infection in a domestic cat based on clinical, molecular, morphological and *post mortem* results. Additionally, results of the descriptive epidemiological analysis of 12 previous cases of feline gurltiosis in this study suggest that infection with *G. paralysans* should be suspected based on clinical signs of chronic paraparesis, epidemiological characteristic of wildlife environment proximity and hunting habits, regional endemicity, typical pathomorphological lesions of spinal cord veins, and isolation and characterization of specimens. This report underlines the clinical and epizootiological aspects of this neglected parasitosis in domestic and wild felids. Further studies are needed to better understand the relationships among possible demographic and environmental factors and infection of *G. paralysans* in domestic cats. Taking into account that *G. paralysans* also infect various endangered wild felids, clinicians, biologists, and ecologists involved in feline conservation programs in South, Central and North America should be aware of this neglected parasite.

Data availability statement

Information for existing publicly accessible datasets is contained within the article.

Ethics statement

The animal studies were approved by Comité de Bioética "Uso de Animales en Investigación" Universidad Austral de Chile. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MG: Conceptualization, Investigation, Validation, Writing – original draft, Writing – review & editing. PM: Conceptualization, Investigation, Methodology, Writing – review & editing. ManM: Writing – review & editing, Investigation, Methodology, Validation. MarM: Formal analysis, Investigation, Methodology, Writing – review & editing. VB: Investigation, Methodology, Validation, Writing – review & editing. CR: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. AT: Investigation, Methodology, Supervision, Validation, Writing – review & editing, Writing – original draft. CH: Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1322819/full#supplementary-material

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Case report: First documented case of cerebral angiostrongyliasis caused by *Angiostrongylus costaricensis* in a free-ranging opossum

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Angiostrongylus costaricensis is a metastrongyloid nematode that primarily infects the mesenteric arteries of wild rodents. This parasite is endemic in several regions of the American continent, and in humans, causes a disease known as abdominal angiostrongyliasis. Despite the important health implications of this nematode, there are limited studies investigating the involvement of wild animals in its life cycle. In this study, we present the clinical manifestations, pathologic findings, and molecular diagnosis, to the best of our current knowledge, of the first documented onset of cerebral angiostrongyliasis because of *A. costaricensis* infection in a juvenile free-ranging opossum (*Didelphis marsupialis*). Histopathological findings stress the presence of eosinophilic meningoencephalitis with nematodes present within the lesions, and PCR was positive for *cox*1 and ITS1 reactions. The obtained sequences for a 279 bp fragment of ITS1 were 100% identical to *A. costaricensis* from Costa Rica. This case highlights the substantial difficulties in diagnosing neuroangiostrongyliasis, yet underscores the importance of considering *A. costaricensis* as a potential culprit behind neurological conditions in wild marsupials. It acts as an urgent call to action to improve surveillance programs tracking infectious and parasitic diseases causing mortality in wildlife populations.

KEYWORDS

case report, parasitic diseases, angiostrongyliasis, wildlife reservoir, zoonosis

1 Introduction

Angiostrongylus costaricensis is a metastrongyloid nematode that primarily infects the mesenteric arteries of wild rodents. The life cycle of this parasite involves the infection of eight different taxonomic families of terrestrial gastropods as competent intermediate hosts, while definitive hosts include the cotton rat (*Sigmodon hispidus*) and other rodent species (1). During its development into the adult phase within vertebrates, *A. costaricensis*

can take two distinct migratory routes. The primary route, referred to as the lymphatic-venous-arterial pathway, involves the migration of the worms through the lymphatic system and systemic arterial circulation until they establish their final niche within the mesenteric arteries. The hepatic-venous pathway, as a secondary route, solely documented in the context of experimental infections of *S. hispidus* (2).

Human beings typically serve as unintentional hosts due to the absence of egg deposition or L1 larval release into the intestinal lumen, a process that is characteristic of natural definitive hosts (3). Instead, the presence of parasite triggers a robust inflammatory response mediated by eosinophils, resulting in a disease known as abdominal angiostrongyliasis (4, 5). This condition is characterized by notable pathological features including marked infiltration of eosinophils into the intestinal wall, granulomatous formations, and eosinophilic inflammation of the blood vessels (vasculitis) most prominently affecting the ileocecal region of the intestine (1).

This parasite, first identified in Costa Rica in 1971, has attracted increasing attention due to reported cases in humans spanning a substantial geographic range throughout the American continent, with a notable concentration in Central and South America (1). Additionally, few cases reported in Africa and Europe imported from the Americas (6–9). In Costa Rica, a considerable number of children have been diagnosed with the disease, attributed to the consumption of mollusks hidden within vegetables (10).

A considerable proportion of wild species are prone to become infected through the ingestion of intermediate hosts (11). Nevertheless, despite the significant health implication of this nematode, there are limited studies investigating the involvement of wild animals in its life cycle, leaving this aspect of the community-epidemiology continuum unknown (1, 12, 13). Multiple cases of abdominal angiostrongyliasis due to *A. costaricensis* in wildlife species have been documented in the literature, including members of Procyonidae (raccoons), non-human primates and marsupials of the Didelphimorphia order (opossums) (14–16). Nevertheless, these reports have employed microscopic and histopathological techniques to identify parasites within lesions. Consequently, the verification of the diagnosis has proven unattainable. Subsequent research has revealed that certain cases were caused by Angiostrongylus species distinct from *A. costaricensis* (17).

Opossums belonging to the genus *Didelphis*, fulfill significant ecological functions, encompassing vital roles in seed dispersal as well as in the regulation of insect and gastropod populations (18). The common opossum, *Didelphis marsupialis* (Didelphidae), exhibits a broad geographic range across the Americas, spanning from Mexico to South America. Within Costa Rica, this species has been documented at elevations ranging from sea level to 2,000 meters above sea level, mainly occupying sites of high contact with human populations given their synanthropic nature (19).

Despite substantial evidence for *D. marsupialis* serving as a natural host for various zoonotic parasites, a survey of the published literature reveals no prior documented cases of neuroangiostrongyliasis attributable to *A. costaricensis* infection in this marsupial species (13). Hence, this study elucidates a noteworthy instance of neurological disease linked to infection caused by the parasite in a juvenile free ranging opossum (*Didelphis marsupialis*). This investigation entails a comprehensive analysis of clinical manifestations, pathological findings, and molecular diagnostics, revealing the potential epidemiological significance of the case.

2 Methods

This research, which focused on an animal that had already died naturally without any therapeutic intervention, in accordance with local legislation and institutional requirements, did not require ethical review or approval. This research was approved by the local wildlife authority, through permit R-SINAC-ACG-PI-026-2019.

A juvenile opossum (*Didelphis marsupialis*) exhibiting neurological symptomatology was discovered by agricultural laborers in a rural locality of the Guanacaste region, Costa Rica (10.501099° N, 84.9241900° W) (Figure 1A). Veterinary personnel transported the affected animal to a nearby wildlife rehabilitation facility for clinical evaluation. Upon examination, observed neurological signs included vestibular syndrome, disorientation, lethargy, and seizures. Regrettably, the opossum succumbed swiftly and naturally without any therapeutic intervention. Postmortem, the carcass was preserved via frozen storage at −20°C prior to being transported to the pathology laboratory for analysis.

This case was examined within the context of an initial pilot program designed to establish passive disease surveillance protocols within wildlife populations in Costa Rica. Macroscopic examination of all organs was performed following previously described protocols, with specimens preserved for histopathological assessment and relevant complementary tests conducted (20). These procedures considered both the clinical history and postmortem findings associated with the case. Brain samples were procured during the necropsy procedure and frozen at −20°C. Subsequently, sections of fresh brain tissue were meticulously affixed onto glass slides and subjected to microscopic scrutiny. The primary objective of this examination was to ascertain the potential presence of nematode parasites. Segments of the nematodes were subsequently isolated from the brain tissue and examined under a light microscope for further morphological and taxonomic analysis.

DNA was isolated from the retrieved portions of nematodes found within the brain parenchyma by using the DNeasy Blood & Tissue kit (Qiagen, Germany) according to the instructions of the manufacturer but eluting the purified DNA in 30 µl of elution buffer. Three different PCRs were run to amplify a fragment of the cytochrome oxidase subunit 1 (*cox*1) of the Phylum Nematoda and two independent reactions for amplifying the ITS1 loci of *Angiostrongylus* spp. and the phylum Nematoda. Accordingly, primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') were used to amplify the 390 bp fragment of the *cox*1 with an initial denaturation step at 95°C, 35 cycles of amplification at 95°C for 1 min, 57°C for 1 min and 72°C for 45 s, and a final elongation step for 7 min (21).

A 400 bp fragment of the ITS-1 was amplified with rDNA2 (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA158S (5'-ACGAGCCGAGTGATCCACCG-3') (22) primers, following the previously described conditions (23). Finally, a PCR amplifying a 290 bp fragment of the ITS1 of

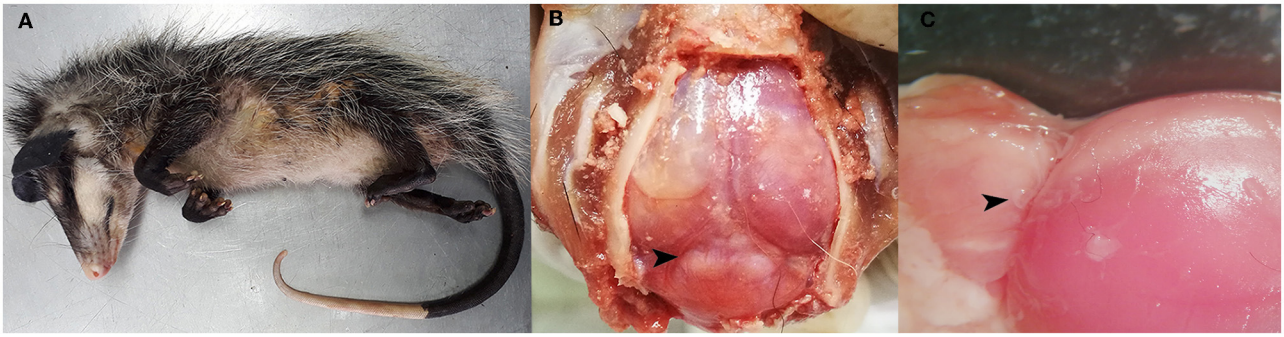

FIGURE 1
Post-mortem analysis findings. **(A)** Juvenile opossum (*Didelphis marsupialis*). **(B)** Multifocal areas of variable sizes, slightly raised and whitish in color, observed in the parietal lobes and occipital lobe of the brain, as well as in the cerebellar vermis (arrowhead). **(C)** Nematodes located in the leptomeninges above the cerebellar vermis and the occipital lobe (arrowhead).

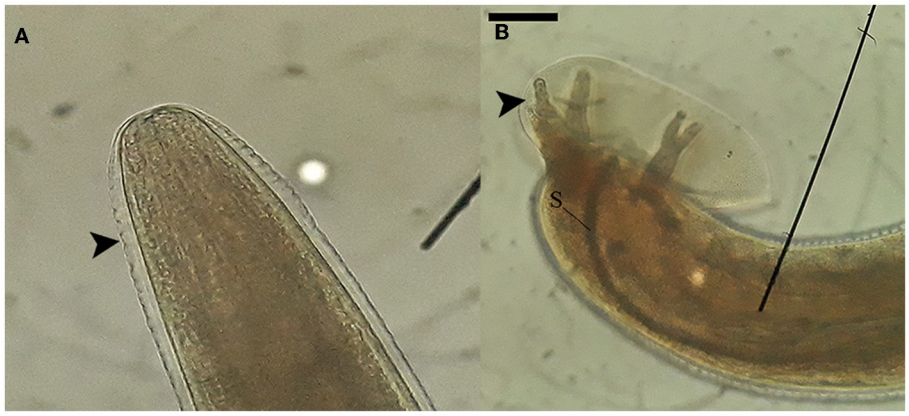

FIGURE 2
Morphological characteristics of nematodes collected from the neuroparenchyma. **(A)** The anterior end of the nematode possesses a rounded shape with a circular mouth opening. The cuticle of the nematode exhibits' transverse striations, as indicated by the arrowhead. **(B)** The posterior segment of a male nematode reveals the presence of the copulatory bursa, bursal rays (arrowhead), and the spicule (S). These images were captured using at a magnification of 100x, and the scale bar represents a length of 200 μm.

Angiostrongylus spp. was run using primers AngioF1674 (5'-GTCGTAACAAGGTATCTGTAGGTG-3') and designed reverse primer AcosR1 (5'-GTCTATACGAGCGAACGCATAC-3') with an initial denaturation at 95°C, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final denaturation step at 72°C (24). All amplicons were run in 1.5% agarose gels stained with SYBR-Safe. Positive reactions were purified using Exo-SAP and Sanger sequenced using the BigDye terminator cycle sequencing chemistry (Macrogen, South Korea). The acquired sequences underwent purification and were subsequently subjected to comparative analysis against the GenBank database.

In accordance with the established rabies protocols by the National Animal Health Service concerning animals displaying neurological symptoms, an analysis was conducted on brain tissue to detect the presence of rabies virus. Total RNA was extracted utilizing the commercially available DNeasy Blood and Tissue kit (Qiagen, Germany) following the manufacturer's standard protocol. The amplification of the nucleoprotein gene was conducted using the RT-PCR technique, employing the primers RAB504 (5′-TATACTCGAATCATGAATGGA GGTCGACT-3′) and RAB304 (5′-ACGCTTAACAACAACAARATCARAG-3′). The diagnostic procedure adhered to the established protocol (25, 26).

3 Results

Post-mortem analysis revealed poor overall body condition and ∼5 mL of sanguineous abdominal effusion, further showed evidence of a mild catarrhal enteritis within the gastrointestinal tract. Nonetheless, the central nervous system exhibited the most remarkable lesions, characterized by multiple areas of diverse sizes that exhibited slight elevation and a whitish coloration. Lesions were evident upon examination of the parietal and occipital cerebral lobes, as well as within the cerebellar vermis (Figure 1B). After the removal of the dura mater, we observed a small and pale pink nematode associated with these lesions (Figure 1C). A subset of the postmortem analysis findings is depicted in Figure 1. Further examination of the remaining organ systems did not reveal any gross lesions.

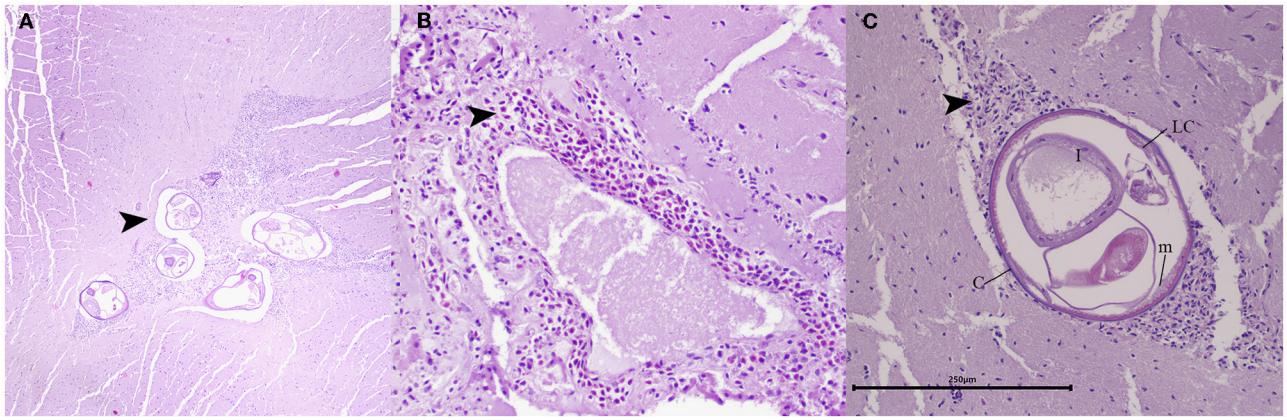

FIGURE 3
Histopathology of the cerebellum in juvenile opossum (*D. marsupialis*). **(A)** Lesions in the cerebellum: showing transverse sections of fully developed nematodes in the neuroparenchyma, affecting both gray matter and white matter, and surrounded by a mild mixed inflammatory infiltrate (arrowhead). H&E, magnification: 400x. **(B)** Lesions in pia mater: presenting a moderate inflammatory infiltrate, primarily composed of eosinophils, with a mild number of macrophages and lymphocytes (arrowhead). H&E, magnification: 200x. **(C)** The mature nematode: measuring approximately 250 µm in size, was characterized by a smooth cuticle (c), coelomyarian musculature (m), lateral cords (LC) and an intestine containing multinucleated cells (I). Encircling the nematode, a mixed inflammatory infiltrate comprising eosinophils, macrophages, and lymphocytes was discerned (arrowhead). H&E, magnification: 200x, scale bar: 250 µm.

Nematode segments obtained from the brain tissue contained preserved sections of both the anterior and posterior regions of the worm. Specifically, the posterior segment exhibited distinctive features such as the copulatory bursa accompanied by bursal rays and the spicule, as visualized in Figure 2. These morphologic features enabled the distinction of these nematodes from other species that erratically migrate in mammalian brains (such as *Baylisascaris spp., Parelaphostrongylus spp., Elaphostrongylus spp.*) and placed them into the taxonomic order of Strongylida.

During histopathological examination, cross sections of fully developed nematodes measuring ~250 µm (Figure 3A). These nematodes exhibited a smooth cuticle, polymyarian and coelomyarian musculature with lateral cords and an intestine containing multinucleated cells (Figure 3C). The nematodes were in the neuroparenchyma, affecting both gray matter and white matter of the cerebellum (Figure 3A), surrounded by a mild mixed inflammatory infiltrate consisting of eosinophils, macrophages, lymphocytes, and plasma cells. An analogous inflammatory infiltrate exhibited a perivascular pattern of distribution within the pia mater upon histological examination (Figure 3B). Despite the presence of various artifacts associated with specimen freezing, which hindered a comprehensive analysis of tissue damage, the findings suggested the presence of eosinophilic meningoencephalitis with nematodes present within the lesions.

PCR was positive for the *cox*1, and the ITS1 reactions. Despite this, distinct sequences were procured only for the 290-base pair ITS1 fragment. A 279 bp fragment of the ITS1 was obtained which showed 100% of identity to *A. costaricensis* (accession number GU58774) from Costa Rica with 99% of coverage. This sequence was 88.93% like *Angiostrongylus chabaudi* (accession number KM979214) and 89.44% similar to *Angiostrongylus vasorum* (accession number GU045374).

4 Discussion

Eosinophilic meningitis, caused by *Angiostrongylus cantonensis*, has well documented in wild animals (27). However, as far as our current knowledge is concerned, this represents the initial documented occurrence of cerebral angiostrongyliasis resulting from *A. costaricensis* infection. Previous studies have associated *A. costaricensis* infection in wild species with the development of granulomatous lesions in mesenteric arteries, as it normally occurs in humans (14, 15, 27). Prior case reports have documented an aberrant migration of this parasitic nematode within human testicular and hepatic tissues, eliciting accompanying inflammatory responses (8, 28). Despite the considerable diagnostic challenges posed by neuroangiostrongyliasis, previously reported cases in both humans and wild species, have consistently exhibited presumptive associations with *A. cantonensis* (27, 29).

ITS1 regions show high intra and interspecies variability in nematodes and other helminth species, making it a suitable marker for molecular identification (30). In the current study, A. *costaricensis* was identified with 100% identity and coverage and the next match was to A. *cantonensis* with 87.7% of identity, thus confirming our identification. Nevertheless, it should be highlighted that few *Angiostrongylus* spp. sequences are available in gene databases to allow comparisons between species, even though some research groups have made the efforts to deposit large datasets in Genbank (31). Therefore, molecular typing of collected *Angiostrongylus* spp. and other less-prevalent helminths is encouraged to increase the robustness of databases.

In experimental infections of *S. hispidus* with eggs of *A. costaricensis*, the normal migratory pathway in the rodent showed the presence of L3 and L4 larvae within blood vessels without associated inflammation in organs such as the kidney, lung, and

brain after 9 days post-infection (2). Nevertheless, in this case, the identification of dead L4 larvae outside the blood vessels within the neuroparenchyma, accompanied by inflammation, suggests an aberrant migration, presenting a migratory pathway like to *A. cantonensis* (32, 33).

Even though an aberrant migration can occur due to proximate factors like a weakened host immune system rather than reflecting evolutionary adaptation. In this case, the inability to properly navigate the vascular system and susceptibility to immune attack in the CNS suggests the parasite lacks proximate adaptations to optimize infection in this host (34). Mechanisms to avoid immunogenic contact like surface molecular disguise, immunomodulation, or sensory and navigational changes have likely not yet evolved. While a weakened immune system could contribute, the overall evidence implies this parasite is still in early stages of adaptation to this host species (35). The aberrant migration and immune-mediated death of larvae point to inadequate proximate adaptations rather than a sole issue of host immunity (34).

Abdominal angiostrongyliasis has emerged as a noteworthy public health issue in Costa Rica, and its prevalence is often underestimated in other regions of the Americas. Nevertheless, the epidemiological significance of human infections is considered limited, as humans represent dead-end accidental hosts within the parasite's life cycle (7, 10). Despite the observation of inflammation in association with the presence of the parasite in the present case, without detectable eggs or L1 larvae in tissue or blood vessels, like humans, the migratory pathway of the parasite and the immune response of the opossum remain unclear. Thus, further extensive inquiries are justified to elucidate the role of opossums within the life cycle of *A. costaricensis* and establish its eco-epidemiological significance, especially considering its prevalence in both urban and rural localities and proximate associations with human and domestic animal population.

The mechanism of *A. costaricensis* exposure in this exceptional case of neuroangiostrongyliasis in a wild opossum remains unidentified. It is conceivable that the opossum might have consumed infected gastropods, given the frequent reports of human infections in the specific region where it discovered (10). Furthermore, the occurrence of the giant African snail (*Achatina fulica*) has been officially recorded within the vicinity of the area where the specimen was located (36). Although efforts make to control and eliminate this invasive species within the country, it well known that giant African snails are efficient intermediate hosts for various *Angiostrongylus* spp. and, consequently, should be consider a potential reservoir for *A. costaricensis*.

A differential diagnosis for neurological diseases observed in free-ranging marsupials should encompass the consideration of *A. costaricensis* as a potential etiological agent. Pharmacological and surgical management have proven effective in cases of aberrant migrations of *Angiostrongylus* spp. reported in domestic animals (37–39). The diverse migratory patterns of larvae within the host's body give rise to a wide spectrum of clinical signs, making resolution challenging in wild animals. This presents an extraordinary challenge in cases of cerebral angiostrongyliasis, where severe clinical symptoms manifest with limited therapeutic success despite extraordinary efforts (40, 41).

The diagnosis of neuroangiostrongyliasis presents challenges, particularly in wild animals, due to the lack of standardized diagnostic protocols. While serological tests are available for humans, diagnostic tests for veterinary species remain limited or untested (4). Consequently, it is probable that the identification of this disease will primarily rely on passive surveillance of severe cases and postmortem examinations, as exemplified in our case (42, 43). This leaves the establishment of preventive measures against exposure of animals in captivity and the control of intermediate hosts as the only viable options (44).

This case highlights the pressing need for a comprehensive One Health approach, emphasizing the initiative-taking measures required to bolster surveillance for infectious and parasitic causes of wildlife mortality. It underscores the critical connection between wildlife health and public health, emphasizing the importance of preventive strategies to mitigate potential implications on both fronts. Only through enhanced wildlife surveillance can we deepen our comprehension of the role played by these animals in the eco-epidemiology of pathogens. Such understanding is vital for assessing the risk of pathogen infections to humans and domestic animals, as well as the risks associated with the introduction of exotic parasites for the conservation of wildlife populations. Armed with this knowledge, we can implement preventive measures more effectively to mitigate these risks (45).

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.

Ethics statement

The requirement of ethical approval was waived by Ministeriode Ambiente y Energia, Costa Rica (MINAE), Sistema de Areas de Conservacion (SINAC) for the studies involving animals because we did work with wildlife carcass. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the participants for the publication of this case report.

Author contributions

TS-S: Conceptualization, Investigation, Methodology, Resources, Validation, Visualization, Writing—original draft, Writing—review & editing. FA-V: Conceptualization, Investigation, Methodology, Resources, Validation, Visualization, Writing—original draft, Writing—review & editing. MC-S: Investigation, Resources, Writing—review & editing. AC: Investigation, Writing—review & editing. AR: Investigation, Methodology, Resources, Validation, Writing—original draft, Writing—review & editing. MB: Conceptualization, Investigation, Resources, Validation, Writing—original draft, Writing—review & editing.

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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 reviewer GD is currently organizing a Research Topic with the author AR.

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Chewing lice of wild birds in Iran: new data and a checklist of avian louse species reported in Iran

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Between September 2019 and December 2023, a total of 612 wild birds representing 16 orders, 33 families, 60 genera, and 78 species from nine provinces of Iran with different climates namely Hamedan (*n* = 54), Sistan-va-Baluchestan (*n* = 372), Kerman (*n* = 73), South Khorasan (*n* = 52), Mazandaran (*n* = 7), Chaharmahal-va-Bakhtiari (*n* = 2), Gilan (*n* = 2), Golestan (*n* = 18), North Khorasan (*n* = 9), and Razavi Khorasan (*n* = 23) were examined for chewing lice infestation. Naked eye examination revealed that 58 birds (9.5%) were infested with at least one chewing louse species. Collected lice specimens belonged to 28 species from the families Philopteridae, Menoponidae and Laemobothriidae including *Strigiphilus strigis* (*n* = 55, 15.6%), *Falcolipeurus quadripustulatus* (*n* = 41, 11.6%), *Craspedorrhynchus platystomus* (*n* = 40, 11.3%), *Colpocephalum turbinatum* (*n* = 36, 10.2%), *Laemobothrion maximum* (*n* = 25, 7.1%), *Nosopon lucidum* (*n* = 20, 5.6%), *Degeeriella fulva* (*n* = 18, 5.1%), *Colpocephalum eucarenum* (*n* = 16, 4.5%), *Laemobothrion vulturis* (*n* = 15, 4.2%), *Anaticola crassicornis* (*n* = 13, 3.7%), *Craspedorrhynchus aquilinus* (*n* = 9, 2.5%), *Degeeriella fusca* (*n* = 7, 2.0%), *Aegypoecus trigonoceps* (*n* = 7, 2.0%), *Quadraceps obscurus* (*n* = 6, 1.7%), *Colpocephalum impressum* (*n* = 6, 1.7%), *Trinoton querquedulae* (*n* = 6, 1.7%), *Colpocephalum heterosoma* (*n* = 5, 1.4%), *Colpocephalum nanum* (*n* = 5, 1.4%), *Lunaceps holophaeus* (*n* = 4, 1.1%), *Quadraceps* spp. (*n* = 4, 1.1%), *Actornithophilus uniseriatus* (*n* = 2, 0.6%), *Nosopon chanabense* (*n* = 2, 0.6%), *Actornithophilus cornutus* (*n* = 1, 0.3%), *Cuclotogaster heterographus* (*n* = 1, 0.3%), *Falcolipeurus suturalis* (*n* = 1, 0.3%), *Laemobothrion atrum* (*n* = 1, 0.3%), *Colpocephalum gypsi* (*n* = 1, 0.3%), and *Rallicola cuspidatus* (*n* = 1, 0.3%). All of these species except six, i.e., *Trinoton* spp., *C. aquilinus*, *L. vulturis*, *L. maximum*, *C. impressum*, *C. turbinatum*, and *C. heterographus* are recorded for the first time from Iran. This study is the largest epidemiological study to date performed in the country. Data reported herein contribute to our knowledge about diversity of avian chewing lice from wild birds in Iran. In this paper, an updated checklist of louse species reported from Iran according to their avian hosts is presented.

KEYWORDS

birds of prey, chewing louse species, fauna, host–parasite associations, Iran, Middle-east, new record, Phthiraptera

Introduction

Lice are small (0.35–11 mm long as adults), wingless, dorsoventrally flattened insects. They are obligatory, permanent ectoparasites of birds and mammals throughout the world which typically, parasitize individuals in small numbers and cause no apparent discomfort however, some of the lice can cause skin lesions and act as vectors or intermediate hosts of several bacteria, viruses and filarial parasites (1, 2). In addition, it has been shown that *Piagetiella titan* infesting white pelicans may invade the oral cavity causing erosions and petechial hemorrhages (3–5).

Lice (Insecta: Psocoptera: Phthiraptera) with about 5,000 known species, present on roughly 4,000 species of birds and 800 mammals, are categorized in four suborders (6). Species of the suborder Anoplura have adopted to suck blood from capillaries of mammals and ingest it, while Amblycera, Ischnocera, and Rhynchophthirina (formerly known as Mallophaga) have chewing mouth pieces, adapted to eat hairs and feathers, and sometimes also the skin and blood of birds and mammals (7). Avian chewing lice belong to one of two sub-orders: Amblycera, which occur on feathers and skin, or Ischnocera, which are more restricted to feathers (1). Most of the lice species are strongly associated with hosts, their phylogeny parallels that of hosts, sometimes with different speeds however, "host specificity" cannot be assumed (7, 8). Among different fields of wildlife parasitology, studying avian chewing lice is important as their epizootiology is largely associated with geographical distribution of their hosts.

Iran is a country in western Asia with a territory of 1,648,195 km^2. It is the second largest country in the Middle East and the 17th largest in the world. In the country, 550 avian species are distributed which is almost equal to the richness of birds in Europe (9, 10). However, there is limited and scanty information about their parasites fauna specially the chewing lice (11–14) with several published in Persian language and presented in local congresses (15–19). Considering the scarcity of published records of lice in Iran, we aimed to gather new data and present an updated checklist of birds' Phthiraptera occurring in the country.

Materials and methods

Between September 2019 and December 2023, totally 612 wild birds belonging 16 orders, 33 families, 60 genera, and 78 species from Hamedan (n = 54), nine different regions of Sistan-va-Baluchestan (n = 372), Kerman (n = 73), South Khorasan (n = 52), Mazandaran (n = 7), Chaharmahal-va-Bakhtiari (n = 2), Gilan (n = 2), Golestan (n = 18), North Khorasan (n = 9), and Razavi Khorasan (n = 23) were collected (Figure 1). The birds were euthanized by the Provincial Department of Environment because of general health failure or were found dead in the environment. The time lapse from death to examination of birds for lice infestation could not be estimated however, only fresh carcasses were examined. Individual birds were sent to Laboratory of Parasitology, Faculty of Veterinary Medicine, Bu-Ali Sina University in sealed plastic bags for examination or were examined in the field. The bird identifications were made using the reference book *Atlas of Birds of Iran* (9), and a standard examination for searching chewing lice was performed (20). The collected lice were placed in tubes containing 70% ethanol, cleared in 10% KOH for at least 1 day, mounted in Canada balsam on glass slides (21), and identified according to the original descriptions or keys (7, 22–35) using a Leica DM750 camera mounted trinocular microscope with Leica DFC295 application unit.

We also collected all the available information about chewing lice infesting birds in Iran. The databases and search engines employed for the literature review were Phthiraptera.info,[1] PubMed,[2] Google,[3] Scientific Information Database of Iran,[4] the collection of defended theses at all Iranian Universities,[5] and the collection of proceedings of Iranian congresses.[6] Valid names of the louse and bird species were obtained from Global Biodiversity Information Facility resources (36).

Results

In total, 352 lice specimens including *Strigiphilus strigis* n = 55; 15.6% (Pontoppidan, 1763), *Falcolipeurus quadripustulatus* n = 41; 11.6% (Burmeister, 1838), *Craspedorrhynchus platystomus* n = 40; 11.3% (Burmeister, 1838), *Colpocephalum turbinatum* n = 36; 10.2% (Denny, 1842), *Laemobothrion maximum* n = 25; 7.1% (Scopoli, 1763), *Nosopon lucidum* n = 20; 5.6% (Rudow, 1869), *Degeeriella fulva* n = 18; 5.1% (Giebel, 1874), *Colpocephalum eucarenum* n = 16; 4.5% (Burmeister, 1838), *Laemobothrion vulturis* n = 15; 4.2% (Fabricius, 1775), *Anaticola crassicornis* n = 13; 3.7% (Scopoli, 1763), *Craspedorrhynchus aquilinus* n = 9; 2.5% (Denny, 1842), *Degeeriella fusca* n = 7; 2.0% (Denny, 1842), *Aegypoecus trigonoceps* n = 7; 2.0% (Giebel, 1874), *Quadraceps obscurus* n = 6; 1.7% (Burmeister, 1838), *Colpocephalum impressum* n = 6; 1.7% (Rudow, 1866), *Trinoton querquedulae* n = 6; 1.7% (Linnaeus, 1758), *Colpocephalum heterosoma* n = 5; 1.4% (Clay, 1951), *Colpocephalum nanum* n = 5; 1.4% (Piaget, 1890), *Lunaceps holophaeus* n = 4; 1.1% Burmeister, 1838, *Quadraceps* spp. (new species) n = 4; 1.1% (Clay and Meinertzhagen, 1939), *Actornithophilus uniseriatus* n = 2; 0.6% (Piaget, 1880), *Nosopon chanabense* n = 2; 0.6% (Ansari, 1951), *Actornithophilus cornutus* n = 1; 0.3% (Giebel, 1866), *Cuclotogaster heterographus* n = 1; 0.28% (Nitzsch, 1866), *Falcolipeurus suturalis* n = 1; 0.3% (Rudow, 1869), *Laemobothrion atrum* n = 1; 0.3% (Nitzsch, 1818), *Colpocephalum gypsi* n = 1; 0.3% (Eichler & Zlotorzycka, 1971), and *Rallicola cuspidatus* n = 1; 0.3% (Scopoli, 1763) were collected from 58/612 birds (9.5%). Collected lice specimens belonged to 31 species from the families Philopteridae, Menoponidae, and Laemobothriidae. All of the identified lice species except *C. aquilinus*, *L. vulturis*, *L. maximum*, *C. impressum*, *C. turbinatum*, and *C. heterographus* are recorded for the first time from Iran (Table 1).

Number of lice specimens collected from examined birds ranged from 1 to 55, the latter was a *Bubo bubo* Linnaeus, 1758. Mixed lice infestation was found in 11 birds, i.e., in one *Philomachus pugnax* (Linnaeus, 1758), two *Himantopus himantopus* Linnaeus, 1758, two *Anas crecca* Linnaeus, 1758, one *Aquila nipalensis* Hodgson, 1833, one *Aquila rapax* Temminck, 1828, two *Gyps fulvus* Hablitz, 1783, and two

1 http://phthiraptera.myspecies.info/
2 www.pubmed.gov
3 www.google.com
4 www.sid.ir
5 https://irandoc.ac.ir/
6 https://www.civilica.com/

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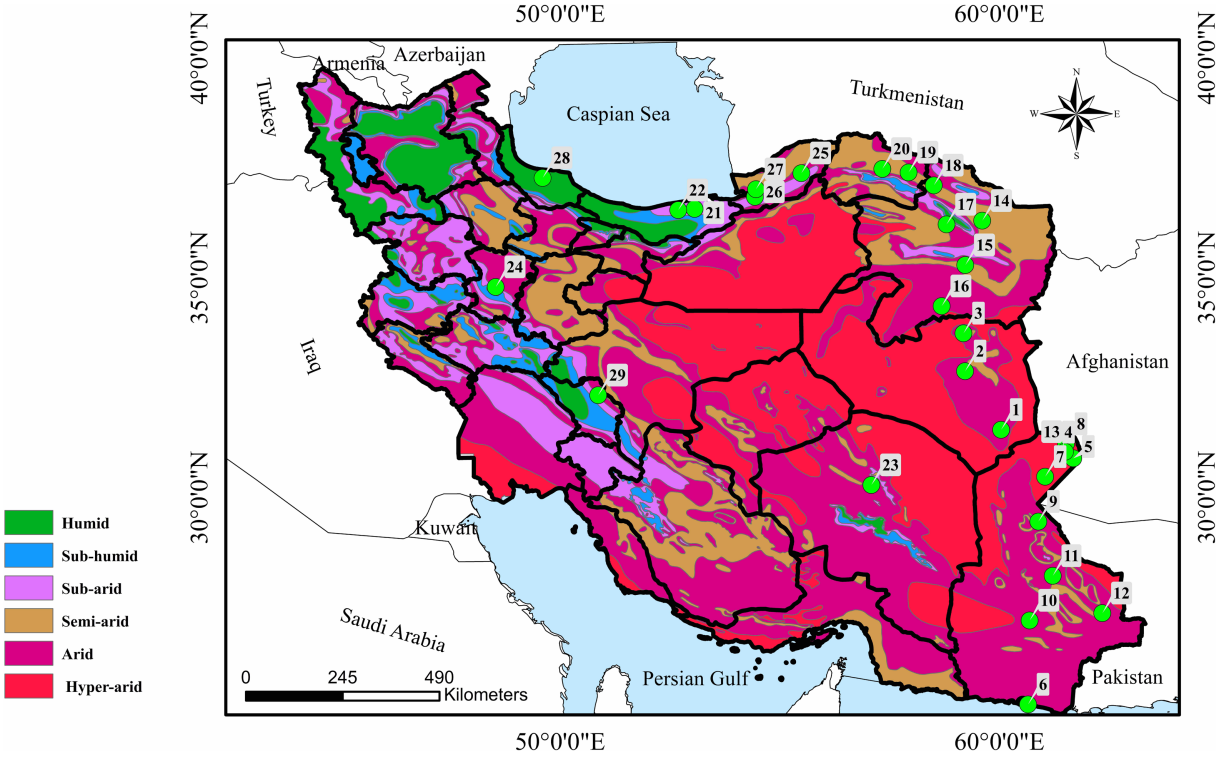

FIGURE 1
Map of Iran showing 29 sampling localities in nine provinces with number of birds examined in each city. (1) Nehbandan (n = 9), (2) Birjand (n = 10), (3) Qaen (n = 6), (4) Zabol (n = 93), (5) Zehak (n = 136), (6) Chabahar (n = 15), (7) Hamun (n = 59), (8) Hirmand (n = 29), (9) Zahedan (n = 26), (10) Iranshahr (n = 4), (11) Khash (n = 5), (12) Saravan (n = 5), (13) Nimruz (n = 27), (14) Mashhad (n = 12), (15) Torbat-e Heydariyeh (n = 4), (16) Gonabad (n = 2), (17) Neyshabur (n = 2), (18) Quchan (n = 3), (19) Shirvan (n = 5), (20) Bojnoord (n = 4), (21) Sari (n = 4), (22) Babol (n = 3), (23) Kerman (n = 73), (24) Hamedan (n = 54), (25) Kalaleh (n = 7), (26) Gorgan (n = 7), (27) Aqqala (n = 4), (28) Rasht (n = 2), and (29) Shahrekord (n = 2).

Buteo buteo Linnaeus, 1758. Photomicrographs of examined lice specimens are presented in Figures 2–14.

Identification of few specimens could not be performed to species level including one damaged female *Strigiphilus* sp. collected from *Asio otus* Linnaeus, 1758, one female *Pectinopygus* spp. collected from *Anas clypeata* Linnaeus, 1758 which is an unusual host and possibly was a contamination, one *Laemobothrion* spp. nymph, and four *Quadraceps* spp. (Figures 10A–C, 13B, 14D). In addition, nits collected from one *Falco tinnunculus* Linnaeus, 1758 could not be identified.

In Supplementary Table 1, the information in Table 1 in addition to world conservation status according to International Union for Conservation of Nature (IUCN) and names of birds in Persian language are presented.

Discussion

This study is the largest epidemiological study to date performed in Iran. However, low number of collected lice from birds could be due to the fact that most of the ectoparasites including lice leave dead hosts rather quickly. Data reported herein contribute to our knowledge about diversity of avian chewing lice from wild birds in Iran and in a broader context in western Asia. Lice species in this study belonged to both Ischnocera (15 species), Amblycera (14 species). We compiled our data and previous information about avian lice species in Iran in Table 2. So far, lice infestation of birds belonging to 16 orders, 33 families, 60 genera, and 78 species and subspecies has been recorded from Iran. In Supplementary Table 2, the information in Table 2 in addition to world conservation status and names of birds in Persian language are presented.

Review of all relevant publications indicated that in some reports from Iran, researchers identified the lice specimens only to genus level, i.e., *Brueelia* (nine documents), *Philopterus*, *Menacanthus* (six documents), *Ricinus*, *Lipeurus* (three documents), *Sturnidoecus*, *Trinoton*, *Menopon*, and *Goniodes* (two documents), *Ardeicola*, *Colpocephalum*, *Craspedorrhynchus*, *Laemobothrion*, *Strigiphilus*, and *Myrsidea* (one document) (14, 16, 17, 19, 38, 40, 47, 50, 51, 62). The reason could be damage of the specimens, observation of a louse with morphological differences from identification keys or difficulty in identification of the species. It is necessary that researchers will try their best to identify the lice to species level correctly and provide the drawings, measurements, or photos.

Observation of one male poultry head louse specimen, *Cuclotogaster heterographus* (Nitzsch, 1866) which was collected from the buzzard *Buteo buteo* (Linnaeus, 1758) in this study was probably because the buzzard preyed with a galliform bird and the

TABLE 1 Distribution of louse species of wild birds in some regions of Iran (September 2019 and December 2023) according to their host bird species.

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
	ACCIPITRIFORMES												
	Accipitridae												
1		*Accipiter badius* (Gmelin, 1788)	Shikra	-	-	-	Zahedan	0/1					
8		*Accipiter nisus* (Linnaeus, 1758)	Eurasian sparrowhawk	-	-	-	Hamun	0/2					
							Hamedan	0/5					
							Zabol	0/1					
1		*Aegypius monachus* (Linnaeus, 1758)	Cinereous vulture	-	-	-	Zahedan	0/1					
4		*Aquila chrysaetos* (Linnaeus, 1758)	Golden eagle	*Craspedorrhynchus aquilinus* (Denny, 1842)	Ischnocera	Philopteridae	Hamedan	3/3	5	4	0	0	9
							Kerman	0/1					
1		*Aquila heliaca* (Savigny, 1809)	Asian imperial eagle	*Laemobothrion maximum* (Scopoli, 1763)	Amblycera	Laemobothriidae	Hamedan	1/1	0	0	3	0	3
3		*Aquila nipalensis* (Hodgson, 1833)	Steppe eagle	*Laemobothrion maximum* (Scopoli, 1763)	Amblycera	Laemobothriidae	Hamedan	2/3	2	4	0	0	6
				Laemobothrion vulturis (Fabricius, 1775)				1/3	0	2	2	0	4
				Colpocephalum impressum Rudow, 1866		Menoponidae		1/3	0	1	0	0	1
				Craspedorrhynchus aquilinus (Denny, 1842)	Ischnocera	Philopteridae		1/3	0	1	0	0	1
				Falcolipeurus suturalis (Rudow, 1869)				1/3	0	1	0	0	1

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
1		*Aquila rapax* (Temminck, 1828)	Tawny eagle	*Laemobothrion vulturis* (Fabricius, 1775)	Amblycera	Laemobothriidae	Hamedan	1/1	1	0	0	0	1
				Colpocephalum impressum (Rudow, 1866)		Menoponidae		1/1	4	0	1	0	5
				Nosopon chanabense (Ansari, 1951)				1/1	0	2	0	0	2
14		*Buteo buteo* (Linnaeus, 1758)	Buzzard	*Degeeriella fulva* (Giebel, 1874)	Ischnocera	Philopteridae	Hamedan	1/14	10	8	0	0	18
				Degeeriella fusca (Denny, 1842)				1/14	2	4	1	0	7
				Cuclotogaster heterographus (Nitzsch, 1866)				1/14	1	0	0	0	1
				Craspedorrhynchus platystomus (Burmeister, 1838)				2/14	18	19	3	0	40
				Colpocephalum nanum (Piaget, 1890)	Amblycera	Menoponidae		1/14	0	2	2	1	5
				Colpocephalum turbinatum (Denny, 1842)				1/14	24	12	0	0	36
				Laemobothrion maximum (Scopoli, 1763)		Laemobothriidae	Kerman	1/12	3	3	6	0	12
3		*Buteo rufinus* (Cretzschmar, 1829)	The long-legged buzzard	*Laemobothrion maximum* (Scopoli, 1763)	Amblycera	Laemobothriidae	Kerman	1/1	1	1	1	0	3
							Zabol	0/1					
							Zahedan	0/1					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
3		*Circus aeruginosus* (Linnaeus, 1758)	Eurasian marsh-harrier	*Nosopon lucidum* (Rudow, 1869)	Amblycera	Menoponidae	Hamedan	1/3	6	12	2	0	20
2		*Circaetus gallicus* (Gmelin, 1788)	Short-toed snake eagle	-	-	-	Zahedan	0/2					
4		*Gyps fulvus* (Hablizl, 1783)	Griffon vulture	*Laemobothrion vulturis* (Fabricius, 1775)	Amblycera	Laemobothriidae	Hamedan	2/4	1	5	4	0	10
				Colpocephalum gypsi (Eichler & Zlotorzycka, 1971)		Menoponidae	Zabol	1/4	1	0	0	0	1
				Colpocephalum spp.				1/4	0	0	0	1	1
				Falcolipeurus quadripustulatus (Burmeister, 1838)	Ischnocera	Philopteridae		2/4	23	18	0	0	41
				Aegypoecus trigonoceps (Giebel, 1874)			Kerman	1/4	3	3	1	0	7
	ANSERIFORMES												
	Anatidae												
6		*Aythya ferina* (Linnaeus, 1758)	Common pochard	-	-	-	Zehak	0/3					
							Zabol	0/1					
							Chabahar	0/2					
10		*Anas crecca* (Linnaeus, 1758)	Common Teal	*Trinoton querquedulae* (Linnaeus, 1758)	Amblycera	Menoponidae	Zehak	5/8	0	3	0	0	3
				Anaticola crassicornis (Scopoli, 1763)	Ischnocera	Philopteridae	Chabahar	0/2	3	5	1	0	9

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
7		*Anas platyrhynchos* (Linnaeus, 1758)	Mallard	*Anaticola crassicornis* (Scopoli, 1763)	Ischnocera	Philopteridae	Zehak	0/1	3	1	0	0	4
							Zabol	2/2					
				Trinoton querquedulae (Linnaeus, 1758)	Amblycera	Menoponidae	Hamedan	1/1	0	1	0	0	1
							Chabahar	0/3					
1		*Anas penelope* (Linnaeus, 1758)	Eurasian wigeon	*Laemobothrion* spp.	Amblycera	Laemobothriidae	Zabol	1/1	0	0	1	0	1
9		*Spatula clypeata* (Linnaeus, 1758)	Northern shoveler	*Pectinopygus* spp.	Ischnocera	Philopteridae	Zabol	1/3	0	1	0	0	1
							Zehak						
							Chabahar						
1		*Mergus merganser* (Linnaeus, 1758)	Common merganser	-	-	-	Zabol	0/1					
2		*Spatula querquedula* (Linnaeus, 1758)	Garganey	*Trinoton querquedulae* (Linnaeus, 1758)	Amblycera	Menoponidae	Zehak	1/2	1	0	0	0	1
2		*Tadorna tadorna* (Linnaeus, 1758)	Common shelduck	-	-	-	Zehak	0/2					
	BUCEROTIFORMES												
3	**Upupidae**	*Upupa epops* (Linnaeus, 1758)	Eurasian hoopoe	-	-	-	Nehbandan	0/1					
							Birjand	0/1					
							Zabol	0/1					
	CHARADRIIFORMES												
	Recurvirostridae												
4		*Himantopus himantopus* (Linnaeus, 1758)	Black-winged stilt	*Actornithophilus uniseriatus* (Piaget, 1880)	Amblycera	Menoponidae	Zehak	4/4	1	0	1	0	2
				Quadraceps spp.	Ischnocera	Philopteridae			3	0	1	0	4
	Scolopacidae												

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
3		*Philomachus pugnax* (Linnaeus, 1758)	Ruff	*Lunaceps holophaeus* (Burmeister, 1838)	Mallophaga	Philopteridae	Zehak	3/3	2	2	0	0	4
				Actornithophilus cornutus (Giebel, 1866)	Amblycera	Menoponidae			1	0	0	0	1
2		*Tringa stagnatilis* (Bechstein, 1803)	Marsh sandpiper	*Quadraceps obscurus* (Burmeister, 1838)	Ischnocera	Philopteridae	Zehak	2/2	3	3	0	0	6
14		*Phalaropus lobatus* (Linnaeus, 1758)	Red-necked phalarope	-	-	-	Zehak	0/14					
	Laridae												
17		*Sterna repressa* (Hartert, 1916)	White-cheeked tern	-	-	-	Zehak	0/7					
							Zabol	0/4					
							Hamun	0/6					
	CAPRIMULGIFORMES												
	Caprimulgidae												
8		*Caprimulgus aegyptius* (Lichtenstein, 1823)	Egyptian nightjar	-	-	-	Hamun	0/3					
							Zabol	0/1					
							Nimruz	0/2					
							Zehak	0/2					
	COLUMBIFORMES												
	Columbidae												
22		*Streptopelia decaocto* (Frivaldszky, 1838)	Eurasian collared dove	-	-	-	Hirmand	0/4					
							Zabol	0/4					
							Nimruz	0/3					
							Zehak	0/6					
							Hamun	0/5					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
36		*Spilopelia senegalensis* (Linnaeus, 1766)	Laughing dove	-	-	-	Hirmand	0/1					
							Zabol	0/8					
							Nimruz	0/2					
							Zehak	0/5					
							Sari	0/1					
							Kerman	0/2					
							Zahedan	0/2					
							Mashhad	0/3					
							Kalaleh	0/2					
							Gorgan	0/2					
							Nehbandan	0/1					
							Birjand	0/1					
							Hamun	0/6					
	FALCONIFORMES												
	Falconidae												
46		*Falco tinnunculus* (Linnaeus, 1758)	Kestrel	*Laemobothrion maximum* (Scopoli, 1763)	Amblycera	Laemobothriidae	Hamedan	1/12	0	0	1	0	1
							Kerman	0/26					
							Zabol	0/4					
							Zahedan	0/4					
4		*Falco cherrug* (Gray, 1834)	Saker falcon	-	-	-	Kerman	0/4					
1		*Falco naumanni* (Fleischer, 1818)	Lesser kestrel	-	-	-	Zabol	0/1					
2		*Falco peregrinus* subsp. *pelegrinoides* (Temminck, 1829)	Barbary falcon	-	-	-	Kerman	0/2					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
	GALLIFORMES												
	Phasianidae												
22		*Ammoperdix griseogularis* (Brandt, 1843)	See-see partridge	-	-	-	Zahedan	0/8					
							Nehbandan	0/3					
							Iranshahr	0/1					
							Khash	0/3					
							Chabahar	0/1					
							Birjand	0/1					
							Qaen	0/1					
							Torbat-Heidarie	0/2					
							Gonaabaad	0/1					
							Saravan	0/1					
11		*Alectoris chukar* (Gray, 1830)	Chukar	-	-	-	Iranshahr	0/3					
							Saravan	0/4					
							Chabahar	0/1					
							Zahedan	0/3					
15		*Coturnix coturnix* (Linnaeus, 1758)	Common quail	-	-	-	Kerman	0/14					
							Zabol	0/1					
5		*Francolinus francolinus* (Linnaeus, 1766)	Black francolin	-	-	-	Hirmand	0/1					
							Zehak	0/1					
							Hamun	0/3					
	GRUIFORMES												
	Rallidae												
1		*Rallus aquaticus* (Linnaeus, 1758)	Water rail	*Rallicola cuspidatus* (Scopoli, 1763)	Ischnocera	Philopteridae	Hamedan	1/1	1	0	0	0	1

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
18		*Fulica atra* (Linnaeus, 1758)	Coot	*Laemobothrion* (*Eulaemobothrion*) *atrum* (Nitzsch, 1818)	Amblycera	Laemobothriidae	Zehak	0/12					
							Zabol	1/3	0	1	0	0	1
							Hamun	0/2					
							Nimruz	0/1					
	OTIDIFORMES												
	Otididae												
1		*Chlamydotis macqueenii* (Gray, 1832)	MacQueen's bustard	-	-	-	Kerman	0/1					
	PASSERIFORMES												
4	**Acrocephalidae**	*Acrocephalus scirpaceus* (Hermann, 1804)	Eurasian reed warbler	-	-	-	Zehak	0/2					
							Zabol	0/2					
	Alaudidae												
5		*Alaemon alaudipes* (Desfontaines, 1789)	Greater hoopoe-lark				Hirmand	0/2					
							Zabol	0/2					
							Zehak	0/1					
11		*Alauda arvensis* (Linnaeus, 1758)	Eurasian skylark	-	-	-	Hamun	0/2					
							Zehak	0/1					
							Hirmand	0/2					
							Zabol	0/4					
							Nimruz	0/2					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
35		*Galerida cristata* (Linnaeus, 1758)	Crested lark	-	-	-	Hamun	0/4					
							Zabol	0/4					
							Hirmand	0/4					
							Birjand	0/2					
							Neishaboor	0/1					
							Quchan	0/1					
							Torbat-Heidarie	0/1					
							Gonaabaad	0/1					
							Gorgan	0/2					
							Shirvan	0/2					
							Bojnurd	0/2					
							Sari	0/2					
							Qaen	0/1					
							Nimruz	0/4					
							Zehak	0/4					
4		*Melanocorypha calandra* (Linnaeus, 1766)	Calandra lark	-	-	-	Mashhad	0/4					
	Cisticolidae												
7		*Prinia gracilis* (Lichtenstein, 1823)	Graceful prinia	-	-	-	Zehak	0/3					
							Hamun	0/2					
							Zabol	0/2					
	Fringillidae												
5		*Serinus pusillus* (Pallas, 1811)	Red-fronted serin	-	-	-	Kerman	0/5					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
5		*Carduelis carduelis* (Linnaeus, 1758)	European goldfinch	-	-	-	Mashhad	0/1					
							Birjand	0/1					
							Gorgan	0/1					
							Kalaleh	0/1					
							Kerman	0/1					
11		*Rhodospiza obsoleta* (Lichtenstein, 1823)	Desert finch	-	-	-	Nehbandan	0/2					
							Qaen	0/3					
							Mashhad	0/3					
							Bojnurd	0/1					
							Birjand	0/1					
							Torbat-Heidarieh	0/1					
	Hirundinidae												
7		*Hirundo rustica* (Linnaeus, 1758)	Barn swallow				Hirmand	0/2					
							Hamun	0/1					
							Zehak	0/4					
	Passeridae												
19		*Passer hispaniolensis* (Temminck, 1820)	Spanish sparrow	-	-	-	Nimruz	0/3					
							Zabol	0/5					
							Zehak	0/6					
							Hamun	0/2					
							Hirmand	0/3					
23		*Passer domesticus* (Linnaeus, 1758)	House sparrow	-	-	-	Shirvan	0/3					
							Gorgan	0/1					
							Kalaleh	0/4					
							Aqqala	0/4					
							Rasht	0/2					
							Sari	0/1					
							Babol	0/3					
							Birjand	0/3					
							Nehbandan	0/1					
							Quchan	0/1					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
30		*Passer montanus* (Linnaeus, 1758)	Eurasian tree sparrow	-	-	-	Hamun	0/7					
							Zabol	0/9					
							Hirmand	0/3					
							Zehak	0/5					
							Nimruz	0/6					
	Pycnonotidae												
21		*Pycnonotus leucotis* (Gould, 1836)	White-eared bulbul	-	-	-	Hamun	0/4					
							Zabol	0/6					
							Hirmand	0/4					
							Zehak	0/5					
							Nimruz	0/2					
	Laniidae												
4		*Lanius phoenicuroides* (Schalow, 1875)	Red-tailed shrike	-	-	-	Hirmand	0/1					
							Hamun	0/1					
							Zehak	0/1					
							Zabol	0/1					
	Leiothrichidae												
14		*Turdoides caudata* (Dumont, 1823)	Common babbler	-	-	-	Hamun	0/4					
							Zabol	0/3					
							Hirmand	0/2					
							Zehak	0/3					
							Nimruz	0/2					
	Motacillidae												

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
11		*Motacilla alba* (Linnaeus, 1758)	White wagtail	-	-	-	Gorgan	0/1					
							Quchan	0/1					
							Bojnurd	0/1					
							Neishaboor	0/1					
							Mashhad	0/1					
							Zabol	0/1					
							Qaen	0/1					
							Nehbandan	0/1					
							Zehak	0/2					
							Hamun	0/1					
	Muscicapidae												
7		*Oenanthe albonigra* (Hume, 1872)	Hume's wheatear	-	-	-	Zehak	0/2					
							Hamun	0/1					
							Zabol	0/4					
1		*Cercotrichas galactotes* (Temminck, 1820)	Rufous-tailed scrub robin	-	-	-	Zabol	0/1					
	Scotocercidae												
5		*Scotocerca inquieta* (Cretzschmar, 1830)	Streaked scrub warbler	-	-	-	Zehak	0/2					
							Hamun	0/1					
							Zabol	0/2					
	PELECANIFORMES												
	Ardeidae												

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
6		*Ardea cinerea* (Linnaeus, 1758)	Gray heron	-	-	-	Hamedan	0/1					
							Zehak	0/5					
5		*Ardea alba* (Linnaeus, 1758)	Great egret				Zehak	0/3					
							Hamun	0/2					
1		*Botaurus stellaris* (Linnaeus, 1758)	Great Bittern (Eurasian bittern)	-	-	-	Hamedan	0/1					
2		*Ixobrychus minutus* (Linnaeus, 1766)	Little bittern	-	-	-	Zahedan	0/2					
	Pelecanidae												
4		*Pelecanus crispus* (Bruch, 1832)	Dalmatian pelican	*Colpocephalum eucarenum* Burmeister, 1838	Amblycera	Menoponidae	Zehak	0/2	6	5	5	0	16
							Zabol	1/2					
	PHOENICOPTERIFORMES												
	Phoenicopteridae												
3		*Phoenicopterus ruber* (Linnaeus, 1758)	American flamingo	*Colpocephalum heterosoma* Piaget, 1880, small specimen (Clay, 1951)	Amblycera	Menoponidae	Zabol	1/1	1	3	0	0	4
				Colpocephalum heterosoma Piaget, 1880, large specimen			Zehak	0/2	1	0	0	0	1
	PODICIPEDIFORMES												
	Podicipedidae												
9		*Podiceps cristatus* (Linnaeus, 1758)	Great crested grebe	-	-	-	Zehak	0/6					
							Zabol	0/3					

(Continued)

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TABLE 1 (Continued)

Host information				Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
	PTEROCLIDIFORMES												
	Pteroclidae												
1		*Pterocles orientalis* (Linnaeus, 1758)	Black-bellied Sandgrouse	-	-	-	Hamedan	0/1					
	PICIFORMES												
	Picidae												
1		*Dendrocopos syriacus* (Hemprich & Ehrenberg, 1833)	Syrian woodpecker	-	-	-	Hamedan	0/1					
	SULIFORMES												
	Phalacrocoracidae												
7		*Phalacrocorax carbo* (Linnaeus, 1758)	Great cormorant	-	-	-	Zabol	0/2					
							Zehak	0/2					
							Chabahar	0/3					
	STRIGIFORMES												
	Strigidae												
2		*Asio otus* (Linnaeus, 1758)	Long-eared owl	*Strigiphilus* sp.	Ischnocera	Philopteridae	Hamedan	1/2	0	1	0	0	1
9		*Athene noctua* (Scopoli, 1769)	Little owl	-	-	-	Hamedan	0/1					
							Kerman	0/4					
							Shahrekord	0/2					
							Khash	0/2					
8		*Bubo bubo* (Linnaeus, 1758)	Eagle owl	*Strigiphilus strigis* (Pontoppidan, 1763)	Ischnocera	Philopteridae	Hamedan	2/2	26	29	0	0	55
							Zahedan	0/1					
							Zabol	0/1					
							Kerman	0/4					

(Continued)

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TABLE 1 (Continued)

	Host information			Parasite information									
n birds examined	Host bird taxonomy	Host scientific name	Host vernacular name	Louse species	Suborder	Family	City	*n* total/ infested	Louse prevalence (*n*)				
									Male	Female	Nymph	Damaged	Total
1		*Otus scops* (Linnaeus, 1758)	European scops owl	-	-	-	Hamedan	0/1					
2		*Otus brucei (Hume, 1873)*	Pallid scops owl	-	-	-	Kerman	0/2					
	Tytonidae												
4		*Tyto alba* (Scopoli, 1769)	Barn owl	-	-	-	Hamedan	0/1					
							Kerman	0/2					
							Zahedan	0/1					
Total 612								58	157	157	35	2	352

[a]according to International Union for Conservation of Nature (IUCN) Red List of Threatened Species (www.iucnredlist.org). Names of orders are capitalized, and names of families are showed in bold.

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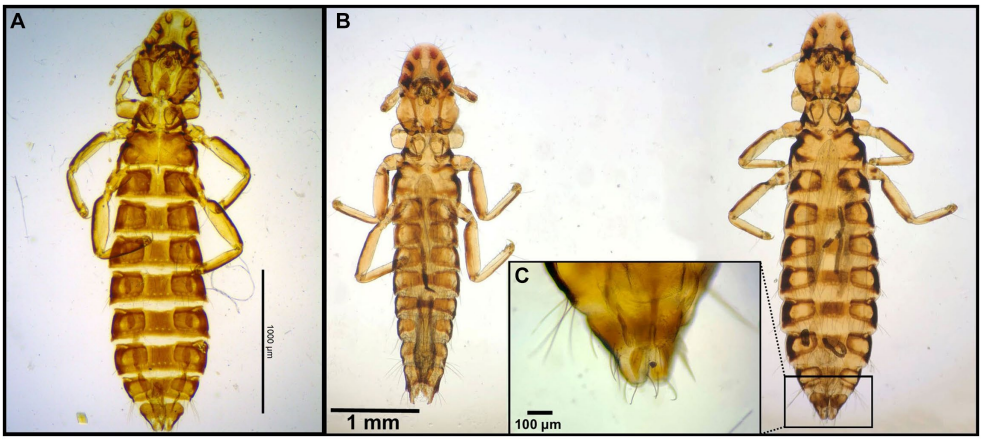

FIGURE 2
Chewing lice identified in this study part I: **(A)** *Falcolipeurus suturalis* ♀; **(B)** *Falcolipeurus quadripustulatus* left ♂, right ♀; and **(C)** ♀ posterior end. The map was drawn by using ArcGIS software version 10.3 (https://enterprise.arcgis.com/en/portal/).

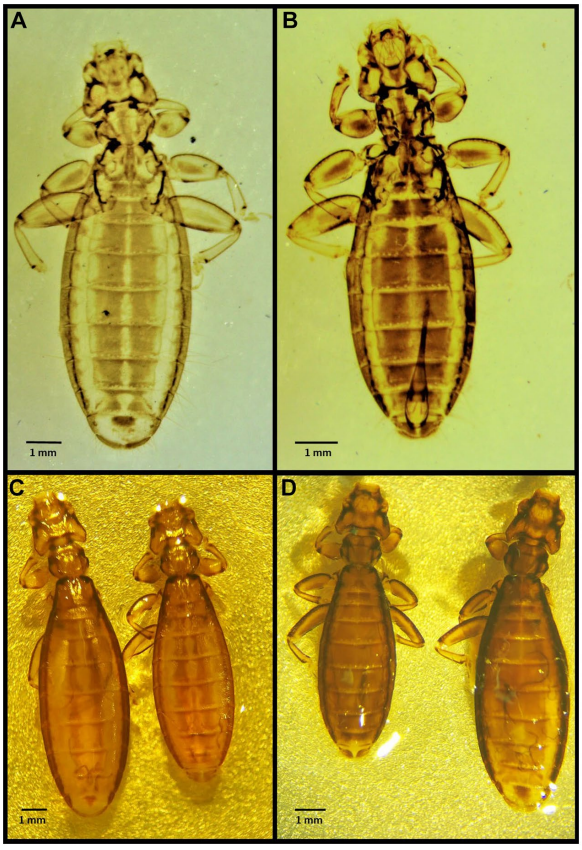

FIGURE 3
Chewing lice identified in this study part II: **(A)** *Laemobothrion maximum* ♀; (**B**) *Laemobothrion maximum* ♂; **(C)** Stereomicroscope picture of *Laemobothrion vulturis* left ♀, right ♂; and **(D)** Stereomicroscope picture of *Laemobothrion maximum* left ♂, right ♀.

louse was mechanically transferred to the predator. In some reports from Iran, lice species that normally infest other bird orders were documented on abnormal bird species. For instance, *Trinoton* sp. that infest Anseriform birds were collected from raptors *Buteo rufinus* and *Gyps fulvus* (16, 17). It can be assumed that lice infestation occurred during feeding the raptors from their preys. In addition, in some reports, *Menacanthus stramineus*, *Menopon gallinae*, and *Cuclotogaster heterographus* that live on Galliformes

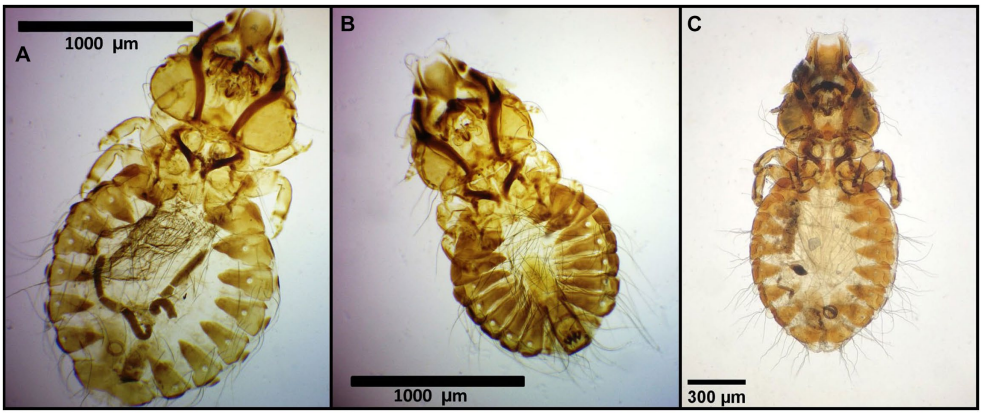

FIGURE 4
Chewing lice identified in this study part III: **(A)** *Craspedorrhynchus aquilinus* ♀, **(B)** *Craspedorrhynchus aquilinus* ♂; and **(C)** *Craspedorrhynchus platystomus* ♀.

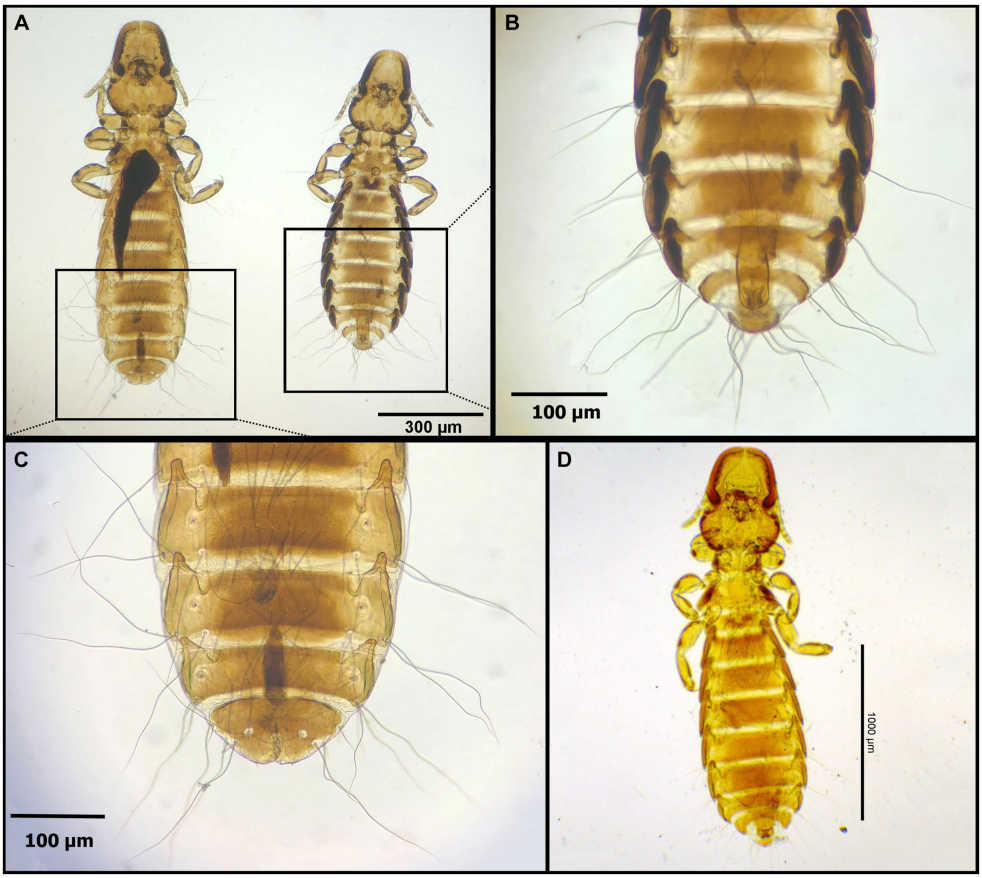

FIGURE 5
Chewing lice identified in this study part IV: **(A–C)** *Degeeriella fusca*, **(A)** left ♀, right ♂; **(B)** ♂, posterior part of the abdomen; **(C)** ♀, posterior part of the abdomen; and **(D)** *Degeeriella fulva* ♂.

were collected from mallards and geese (42, 43) as well as pigeons (47). These findings could be due to keeping mixed species together by nomads which is a normal practice in Iran although misidentification cannot be ruled out. Special caution should be taken for interpretation of such findings.

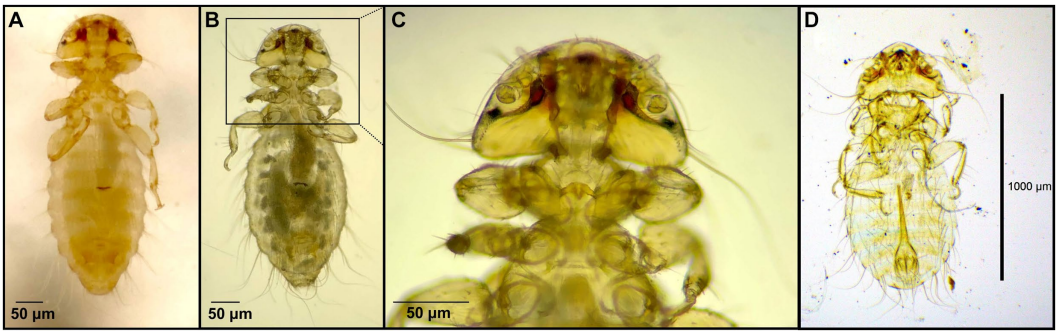

FIGURE 6
Chewing lice identified in this study part V: **(A–C)** *Nosopon chanabense* ♀; **(A)** Stereomicroscope picture; **(B)** Light microscope picture; **(C)** Head; and **(D)** *Nosopon lucidum* ♂.

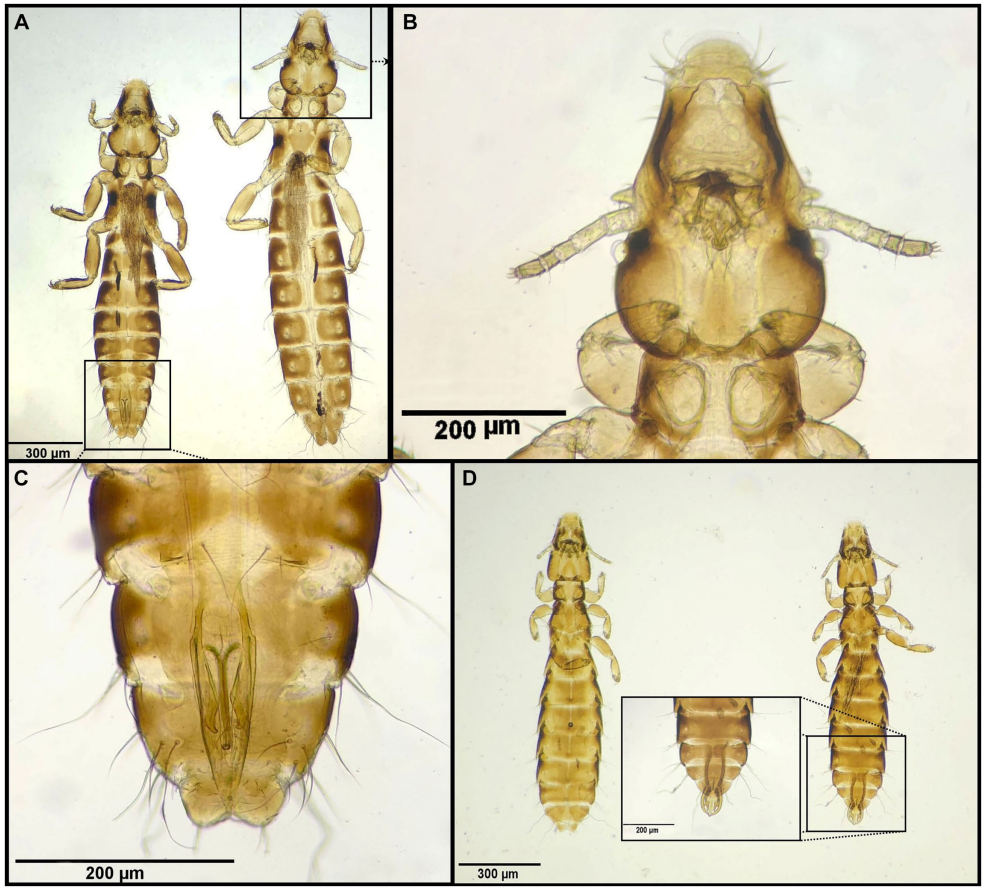

FIGURE 7
Chewing lice identified in this study part VI: **(A–C)** *Anaticola crassicornis* left ♂, right ♀; **(B)** ♀, Head; **(C)** ♂, posterior end; and **(D)** *Quadraceps obscurus* left ♀, right ♂.

It is known that both amblyceran and ischnoceran lice can act as vectors or intermediate hosts of helminths, bacteria, and viruses, so it was suggested to delouse the wild birds with insecticides (1) however, we disagree with manipulating host–parasite interactions in the wildlife. Additionally, from the conservation point of view some authors expressed their concerns about co-extinction of the lice with their hosts, e.g., *Rallicola extinctus* (64) and their extinction during the conservation efforts to save the host, e.g., *Rallicola pilgrimi* (Clay, 1972) and *Colpocephalum californici* (31, 64, 65). According to International Union for Conservation of Nature (IUCN), there are concerns regarding decreasing population of several predators such as *Aquila nipalensis* Hodgson, 1833 steppe eagle (endangered) and

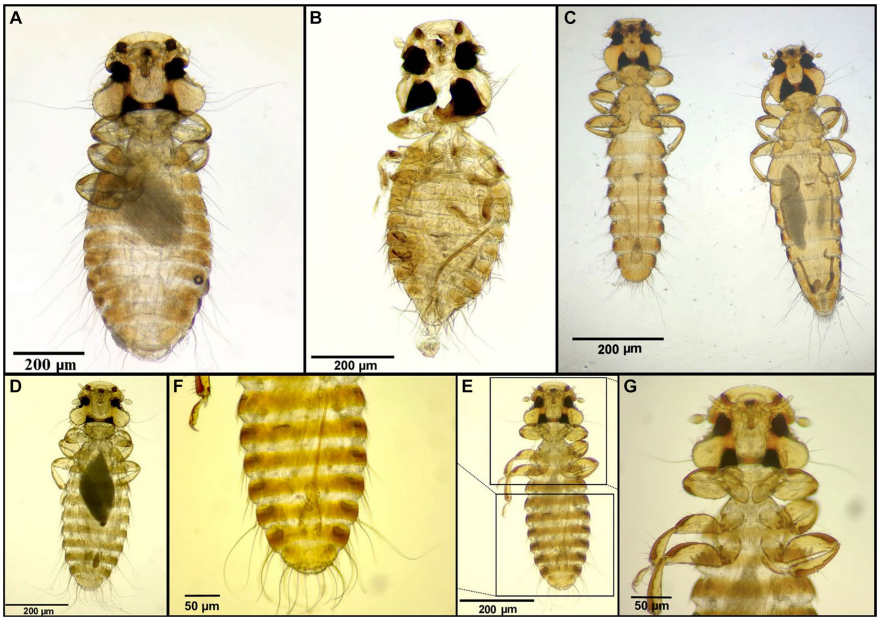

FIGURE 8
Chewing lice identified in this study part VII: **(A)** *Colpocephalum nanum* ♀; **(B)** *Colpocephalum gypsi* ♂; **(C)** *Colpocephalum eucarenum* left ♂, right ♀; **(D–G)** *Colpocephalum impressum*; **(D)** ♀; **(E)** ♂; **(F)** ♂, posterior end; and **(G)** ♂, head.

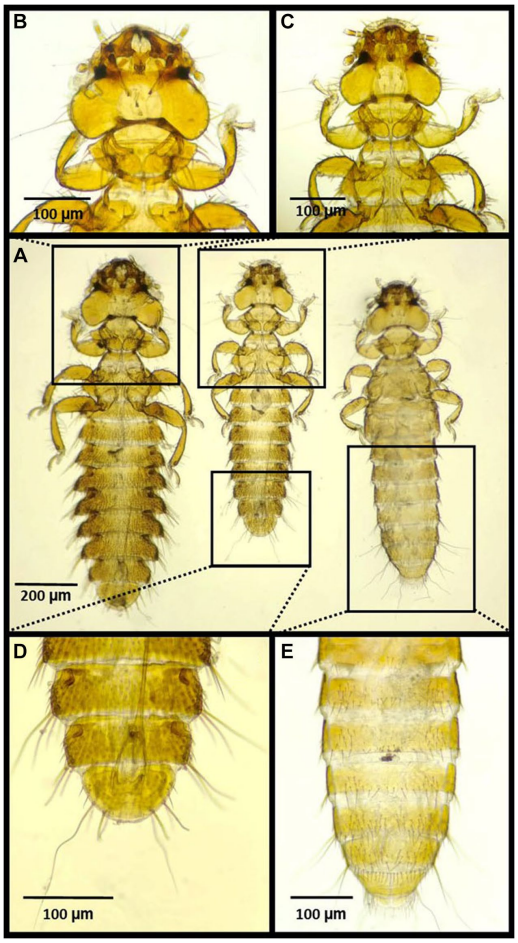

FIGURE 9
Chewing lice identified in this study part VIII: **(A–E)** *Colpocephalum heterosoma*; **(B)** Head of large specimen ♂; **(C)** Head of small specimen, ♂; **(D)** Posterior end of small specimen ♂, and **(E)** Posterior end of small specimen ♀.

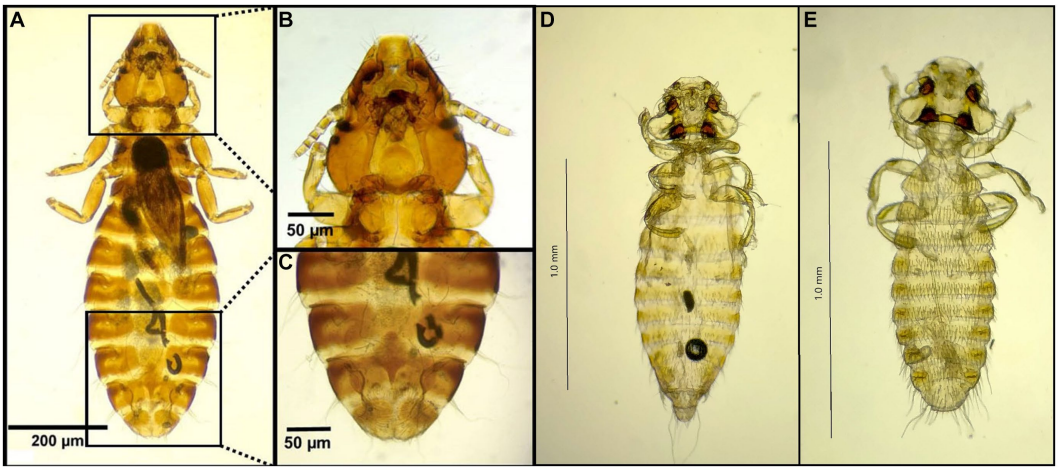

FIGURE 10
Chewing lice identified in this study part IX: **(A–C)** *Pectinopygus* spp. ♀; **(B)** Head; **(C)** Posterior end; **(D)** *Colpocephalum turbinatum* ♀; and **(E)** *Colpocephalum turbinatum* ♂.

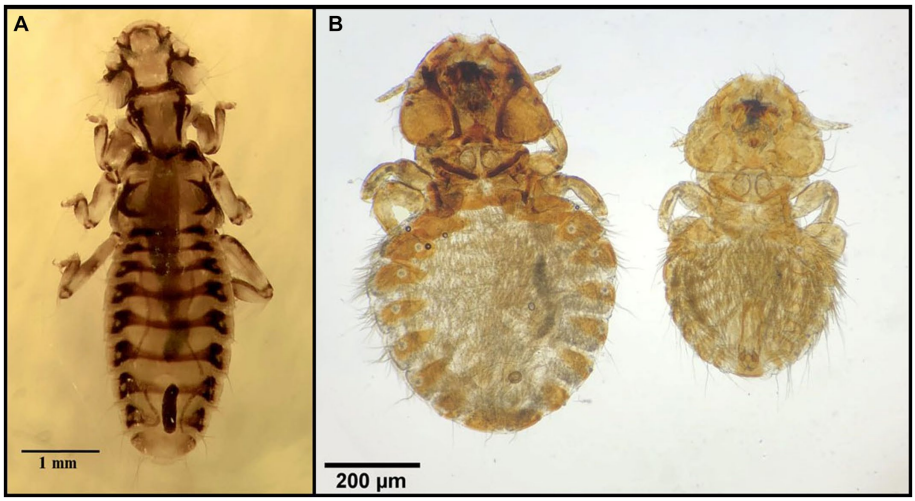

FIGURE 11
Chewing lice identified in this study part X: **(A)** *Trinoton querquedulae* ♀; **(B)** *Aegypoecus trigonoceps* left ♀, right ♂.

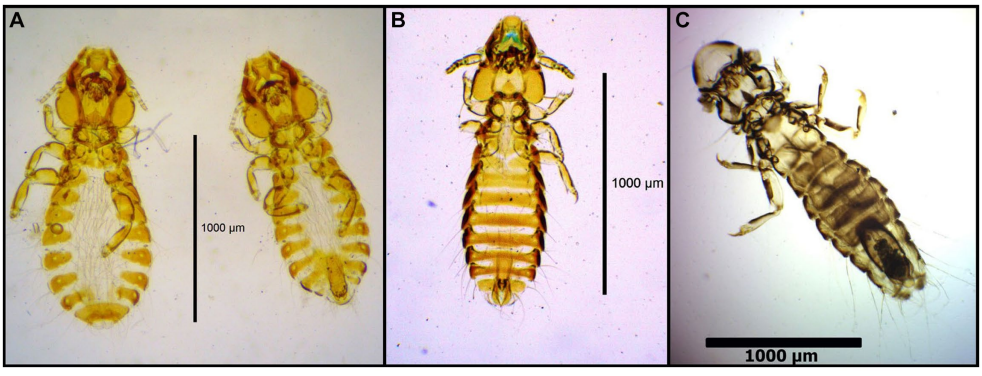

FIGURE 12
Chewing lice identified in this study part XI: **(A)** *Strigiphilus strigis* left ♀, right ♂; **(B)** *Rallicola cuspidatus* ♂; and **(C)** *Cuclotogaster heterographus* ♂.

FIGURE 13
Chewing lice identified in this study part XII: **(A)** *Lunaceps holophaeus* left ♂, right ♀; **(B)** *Laemobothrion* spp. nymph.

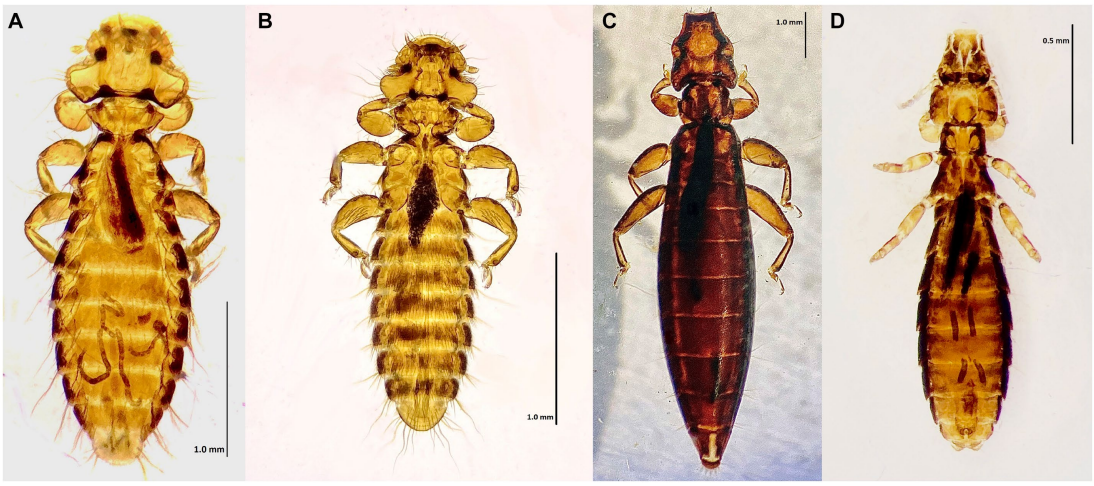

FIGURE 14
Chewing lice identified in this study part XIII: **(A)** *Actornithophilus uniseriatus* ♂; **(B)** *Actornithophilus cornutus* ♂; **(C)** *Lamobothrion atrum* ♀; and **(D)** *Quadraceps* spp. ♂.

Aquila heliaca Savigny, and 1809 Asian imperial eagle (vulnerable) (66). Hence, it is suggested that conservationists consider preserving host-specific lice as part of their efforts to save vertebrate hosts (65).

This study provides the first information about lice infestation of wild birds in different regions of Iran and reports *Craspedorrhynchus platystomus*, *Colpocephalum nanum*, *Colpocephalum gypsi*, *Colpocephalum eucarenum*, *Colpocephalum heterosoma*, *Degeeriella fulva*, *Degeeriella fusca*, *Nosopon chanabense*, *Nosopon lucidum*, *Falcolipeurus quadripustulatus Falcolipeurus suturalis*, *Aegypoecus trigonoceps*, *Trinoton querquedulae*, *Anaticola crassicornis*, *Quadraceps obscurus*, *Rallicola cuspidatus*, and *Strigiphilus strigis* for the first time from the country. Review of the published data on avian lice fauna of Iran shows that the information is available for almost 14% of the bird species. In contrast, researchers from the neighboring country Turkey have identified over 150 lice species from more than half of the bird species inhabiting the country (21). As Iran and Turkey share many bird species, it seems that many louse species remain to be discovered. Molecular phylogenetic analysis of avian lice from Iran will bring clearer understanding of the role of migratory birds in biogeographic distributions.

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TABLE 2 Louse species reported from Iran according to their avian hosts until December 2023.

Avian host scientific name	Avian host vernacular name	Lice species	Reference
ACCIPITRIFORMES			
Accipitridae			
Aquila chrysaetos (Linnaeus, 1758)	Golden eagle	*Craspedorrhynchus aquilinus* (Denny, 1842)	This study (37, 38)
		Laemobothrion maximum (Scopoli, 1763)	
		Laemobothrion sp.	
Aquila fasciata (Vieillot, 1822)	Bonelli's eagle	*Laemobothrion maximum* (Scopoli, 1763)	Reported the bird as *Hieraaetus fasciatus* (39)
Aquila heliaca (Savigny, 1809)	Asian imperial eagle	*Laemobothrion maximum* (Scopoli, 1763)	This study
Aquila nipalensis (Hodgson, 1833)	Steppe eagle	*Colpocephalum impressum* (Rudow, 1866)	This study (40)
		Falcolipeurus suturalis (Rudow, 1869)	
		Laemobothrion maximum (Scopoli, 1763)	
		Craspedorrhynchus sp.	
Aquila rapax (Temminck, 1828)	Tawny eagle	*Laemobothrion vulturis* (Fabricius, 1775)	This study
		Colpocephalum impressum (Rudow, 1866)	
		Nosopon chanabense (Ansari, 1951)	
Buteo buteo (Linnaeus, 1758)	Buzzard	*Degeeriella fulva* (Giebel, 1874)	This study
		Degeeriella fusca (Denny, 1842)	
		Cuclotogaster heterographus (Nitzsch, 1866)	
		Craspedorrhynchus platystomus (Burmeister, 1838)	
		Colpocephalum nanum (Piaget, 1890)	
		Colpocephalum turbinatum (Denny, 1842)	
		Laemobothrion maximum (Scopoli, 1763)	
Buteo rufinus (Cretzschmar, 1829)	Long-legged buzzard	*Trinoton* sp.×	This study (17)
		Laemobothrion maximum (*Scopoli, 1763*)	
Circus aeruginosus (Linnaeus, 1758)	Eurasian marsh-harrier	*Nosopon lucidum* (Rudow, 1869)	This study
Gyps fulvus (Hablizl, 1783)	Eurasian griffon vulture	*Trinoton* sp.×	This study (16)
		Laemobothrion vulturis (Fabricius, 1775)	
		Colpocephalum gypsi (Eichler & Zlotorzycka, 1971)	
		Colpocephalum spp.	
		Falcolipeurus quadripustulatus (Burmeister, 1838)	
		Aegypoecus trigonoceps (Giebel, 1874)	
Neophron percnopterus (Linnaeus, 1758)	Egyptian vulture	*Laemobothrion vulturis* (Fabricius, 1775)	(15)
ANSERIFORMES			
Anatidae			
Anas clypeata (Linnaeus, 1758)	Northern shoveler	*Anaticola crassicornis* (Scopoli, 1763)	This study (38)
		Pectinopygus spp.	
Anas crecca (Linnaeus, 1758)	Common Teal	*Trinoton querquedulae* (Linnaeus, 1758)	This study
		Anaticola crassicornis (Scopoli, 1763)	
Anas platyrhynchos (Linnaeus, 1758)	Mallard	*Lipeurus squalidus* (Piaget, 1880)×	This study (41, 42)
		Menacanthus stramineus (Nitzsch, 1818)×	
		Trinoton anserinum (Fabricius, 1805)×	
		Anatolica crassicornis (*Scopoli*, 1763)	
Anas penelope Linnaeus, 1758	Eurasian wigeon	*Laemobothrion* spp.	This study

(Continued)

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TABLE 2 (Continued)

Avian host scientific name	Avian host vernacular name	Lice species	Reference
Anas querquedula (Linnaeus, 1758)	Garganey	*Trinoton querquedualea* (Linnaeus, 1758)	This study
Anser anser (Linnaeus, 1758)	Greylag goose	*Anaticola anseris* (Linnaeus, 1758)	(15, 41, 43)
		Trinoton anserinum (Fabricius, 1805)	
		Cuclotogaster heterographus (Nitzsch, 1866) [×]. Also reported as *Liperus heterographus*[×]	
		Lipeurus caponis (Linnaeus, 1758)[×]	
		Menopon gallinae (Linnaeus, 1758)[×]	
BUCEROTIFORMES			
Upupidae			
Upupa epops (Linnaeus, 1758)	Hoopoe	*Upupicola upupae* (Schrank, 1803)	(38)
CHARADRIIFORMES			
Laridae			
Chroicocephalus ridibundus (Linnaeus, 1766)	Black-headed gull	*Austromenopon transversum* (Denny, 1842)	Reported the bird as *Larus ridibundus* (13)
Sterna hirundo (Linnaeus, 1758)	Tern	*Quadraceps legatus* (Timmermann, 1952)	This study (12)
		Saemundssonia meridiana (Timmermann, 1950)	This study
Tringa stagnatilis (Bechstein, 1803)	Marsh sandpiper	*Quadraceps obscurus* (Burm, 1838)	This study
Recurvirostridae			
Himantopus himantopus (Linnaeus, 1758)	Black-winged stilt	*Actornithophilus uniseriatus* (Piaget, 1880) *Quadraceps* spp.	This study
Scolopacidae			
Philomachus pugnax (Linnaeus, 1758)	Ruff	*Lunaceps holophaeus* Burmeister, 1838	This study
		Actornithophilus cornutus (Giebel, 1866)	This study
COLUMBIFORMES			
Columbidae			
Columba livia subsp. *domestica* (Gmelin, 1789)	Domestic pigeon	*Campanulotes compar* (Burmeister, 1838). Also reported as *Goniocotes bidentatus*	(41, 44–46)
		Columbicola columbae (Linnaeus, 1758)	
		Columbicola tschulyschman (Eichler, 1942)	
		Lipeurus caponis (Linnaeus, 1758)[×]	
		Menopon gallinae (Linnaeus, 1758)[×]	
		Menacanthus stramineus (Nitzsch, 1818)[×]. Also reported as *Menopon stramineum*[×]	
Columba livia subsp. *livia* (Gmelin, 1789)	Rock dove	*Campanulotes compar* (Burmeister, 1838)	(13, 47)
		Colpocephalum turbinatum Denny, 1842	
		Columbicola columbae (Linnaeus, 1758)	
		Hohorstiella lata (Piaget, 1880)	
		Menacanthus stramineus (Nitzsch, 1818)[×]	
		Menopon gallinae (Linnaeus, 1758)[×]	
		Goniodes sp.	
Streptopelia senegalensis (Linnaeus, 1766)	Laughing dove	*Columbicola columbae* (Linnaeus, 1758)	(48)
Streptopelia turtur (Linnaeus, 1758)	European turtle dove	*Colpocephalum pectinatum* (Osborn, 1902)	(38)
		Strigiphilus sp.[×]	

(Continued)

TABLE 2 (Continued)

Avian host scientific name	Avian host vernacular name	Lice species	Reference
CORACIIFORMES			
Alcedinidae			
Alcedo atthis (Linnaeus, 1758)	Kingfisher	*Alcedoecus annulatus* (Ansari, 1955)	(38)
Meropidae			
Merops apiaster (Linnaeus, 1758)	Bee-eater	*Meromenopon meropis* Clay & Meinertzhagen, 1941	(49)
		Meropoecus meropis (Denny, 1842)	
		Meropsilla apiastri (Denny, 1842). Reported as *Brueelia apiastri*	
Merops persicus (Pallas, 1773)	Blue-cheeked bee-eater	*Meromenopon meropis* Clay & Meinertzhagen, 1941	(49)
		Meropoecus meropis (Denny, 1842)	
		Meropsiella erythropteri (Piaget, 1885). Reported as *Brueelia erythropteri*	
CUCULIFORMES			
Cuculidae			
Cuculus canorus (Linnaeus, 1758)	Cuckoo	*Cuculoecus latifrons* (Denny, 1842). Reported as *Philopterus latifron*	(11)
FALCONIFORMES			
Falconidae			
Falco cherrug (Gray, JE, 1834)	Saker falcon	*Colpocephalum* sp.	(38)
Falco tinnunculus (Linnaeus, 1758)	Kestrel	*Laemobothrion maximum* (Scopoli, 1763)[×]	This study (38)
GALLIFORMES			
Phasianidae			
Coturnix coturnix (Linnaeus, 1758)	Quail	*Amyrsidea fulvomaculata* (Denny, 1842)	(38)
Gallus gallus domesticus (Linnaeus, 1758)	Chicken	*Cuclotogaster heterographus* (Nitzsch, 1866). Also reported as *Lipeurus heterographus*	(18, 41, 46, 50–59)
		Goniodes dissimilis Denny, 1842	
		Goniocotes gallinae (de Geer, 1778)	
		Goniodes gigas (Taschenberg, 1879). Also reported as *Goniocotes gigas*	
		Lipeurus caponis (Linnaeus, 1758)	
		Menacanthus pallidulus (Neumann, 1912). Also reported as *Menopon pallidulum*	
		Menacanthus stramineus (Nitzsch, 1818). Also reported as *Menopon stramineum*	
		Menopon gallinae (Linnaeus, 1758)	
		Goniodes sp.	
		Lipeurus sp.	
		Menopon sp.	
Meleagris gallopavo (Linnaeus, 1758)	Turkey	*Chelopistes meleagridis* (Linnaeus, 1758)	(41, 46, 60)
		Goniocotes gallinae (de Geer, 1778)	
		Goniodes gigas (Taschenberg, 1879)	
		Menacanthus stramineus (Nitzsch, 1818)	
		Menopon gallinae (Linnaeus, 1758)	

(Continued)

TABLE 2 (Continued)

Avian host scientific name	Avian host vernacular name	Lice species	Reference
Phasianus colchicus (Linnaeus, 1758)	Pheasant	*Amyrsidea perdicis* (Denny, 1842). Reported as *Amyrsidea hexapilosus*	(38)
Pavo cristatus (Linnaeus, 1758)	Peafowl	*Goniodes pavonis* (Linnaeus, 1758)	(61)
Perdix perdix (Linnaeus, 1758)	Grey partridge	*Lipeurus* sp.	(62)
		Menacanthus sp.	
		Menopon sp.	
GRUIFORMES			
Rallidae			
Rallus aquaticus (Linnaeus, 1758)	Water rail	*Rallicola cuspidatus* (Scopoli, 1763)	This study
Fulica atra Linnaeus, 1758	Coot	*Laemobothrion atrum* (Nitzsch, 1818)	This study
PASSERIFORMES			
Acrocephalidae			
Acrocephalus stentoreus (Hemprich & Ehrenberg, 1833)	Clamorous reed warbler	*Brueelia* sp.	(14)
Alaudidae			
Calandrella rufescens (Vieillot, 1819)	Lesser short-toed lark	*Menacanthus* sp.	(14)
Galerida cristata (Linnaeus, 1758)	Crested lark	*Brueelia* sp.	(14)
		Ricinus sp.	
Corvidae			
Corvus corax (Linnaeus, 1758)	Raven	*Myrsidea anaspila* (Nitzsch, 1866)	(11, 15)
		Philopterus corvi (Linnaeus, 1758)	
		Cuclotogaster heterographus (Nitzsch, 1866)×	
Corvus corone (Linnaeus, 1758)	Carrion crow	*Philopterus ocellatus* (Scopoli, 1763)	(11, 14, 63)
		Brueelia sp.	
		Cuculoecus latifrons (Denny, 1842). Also reported as *Philopterus latifron*×	
Pica pica (Linnaeus, 1758)	Black-billed magpie	*Philopterus picae* (Denny, 1842)	(38)
Emberizidae			
Emberiza bruniceps (Brandt, 1841)	Red-headed bunting	*Sturnidoecus rostratus* (Mey, 1982)	(14)
		Menacanthus sp.	
Emberiza calandra (Linnaeus, 1758)	Corn bunting	*Sturnidoecus rostratus* (Mey, 1982)	(14)
		Brueelia sp.	
Fringillidae			
Chloris chloris (Linnaeus, 1758)	European greenfinch	*Myrsidea* sp.	(14)
Rhodospiza obsolete (Lichtenstein, 1823)	Desert finch	*Brueelia gobiensis* Mey, 1982	Reported the bird as *Carduelis obsoleta* (14)
		Philopterus sp.	
Fringilla coelebs (Linnaeus, 1758)	Chaffinch	*Philopterus fringillae* (Scopoli, 1772)	(14)
		Brueelia sp.	
Muscicapidae			
Saxicola torquatus (Linnaeus, 1766)	African stonechat	*Brueelia* sp.	(14)
Luscinia megarhynchos (Brehm, 1831)	Nightingale	*Brueelia* sp.	(14)

(Continued)

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TABLE 2 (Continued)

Avian host scientific name	Avian host vernacular name	Lice species	Reference
Oenanthe lugens (Lichtenstein, 1823)	Mourning wheatear	*Philopterus* sp.	(14)
Paridae			
Parus major (Linnaeus, 1758)	Great tit	*Philopterus pallescens* (Denny, 1842)	(14)
Passeridae			
Gymnoris xanthocollis (Burton, 1838)	Yellow-throated sparrow	*Philopterus fringillae* (Scopoli, 1772)	Reported the bird as *Petronia xanthocollis* (14)
Passer domesticus (Linnaeus, 1758)	House sparrow	*Brueelia cyclothorax* (Burmeister, 1838). Reported as *Brueelia subtilis* (Nitzsch, 1874)	(14)
		Philopterus fringillae (Scopoli, 1772)	
		Sturnidoecus refractariolus (Zlotorzycka, 1964)	
Passer montanus (Linnaeus, 1758)	Eurasian sparrow	*Brueelia cyclothorax* (Burmeister, 1838). Reported as *Brueelia subtilis* (Nitzsch, 1874)	(14)
		Philopterus montani (Zlotorzycka, 1964)	
		Sturnidoecus ruficeps (Nitzsch, 1866)	
		Campanulotes compar (Burmeister, 1838)×	
Petronia petronia (Linnaeus, 1766)	Rock petronia	*Sturnidoecus refractariolus* (Zlotorzycka, 1964)	(14)
		Brueelia sp.	
		Philopterus sp.	
Phylloscopidae			
Phylloscopus collybita (Vieillot, 1817)	Chiffchaff	*Brueelia* sp.	(14)
		Menacanthus sp.	
		Philopterus sp.	
		Sturnidoecus sp.	
Phylloscopus nitidus (Blyth, 1843)	Green warbler	*Brueelia* sp.	(14)
		Menacanthus sp.	
Sturnidae			
Acridotheres tristis (Linnaeus, 1766)	Myna	*Brueelia chayanh* Ansari, 1955	(14, 40)
		Myrsidea invadens (Kellogg & Chapman, 1902)	
Sturnus vulgaris (Linnaeus, 1758)	Starling	*Brueelia nebulosa* (Burmeister, 1838)	(14)
Sylviidae			
Sylvia communis (Latham, 1787)	Whitethroat	*Sturnidoecus* sp.	(14)
Turdidae			
Turdus ruficollis (Pallas, 1776)	Black-throated Thrush	*Philopterus* sp.	(38)
Turdus merula (Linnaeus, 1758)	Blackbird	*Ricinus* sp.	(14)
PELECANIFORMES			
Ardeidae			
Ardea purpurea (Linnaeus, 1766)	Purple heron	*Menacanthus* sp.×	(13)
Egretta garzetta (Linnaeus, 1766)	Little egret		
		Ardeicola sp. Probably *Ardeicola expallidus* Blagoveshtchensky, 1940	(13)
		Ciconiphilus decimfasciatus (Boisduval & Lacordaire, 1835)	
Pelecanidae			
Pelecanus onocrotalus (Linnaeus, 1758)	Great white pelican	*Piagetiella titan* (Piaget, 1880)	(4), this study

(Continued)

TABLE 2 (Continued)

Avian host scientific name	Avian host vernacular name	Lice species	Reference
Pelecanus crispus (Bruch, 1832)	Dalmatian pelican	*Colpocephalum eucarenum* (*Burmeister, 1838*)	
PHOENICOPTERIFORMES			
Phoenicopteridae			
Phoenicopterus ruber (Linnaeus, 1758)	American flamingo	*Colpocephalum heterosoma*, (Clay, 1951)	This study
PODICIPEDIFORMES			
Podicipedidae			
Podiceps cristatus (Linnaeus, 1758)	Great crested grebe	*Aquanirmus podicipis* (Denny, 1842)	(13)
		Pseudomenopon dolium (Rudow, 1869)	
Scolopacidae			
Numenius arquata (Linnaeus, 1758)	Curlew	*Cummingsiella ovalis* (Scopoli, 1763)	(11, 12, 15)
		Quadraceps obtusus (Kellogg & Kuwana, 1902)	
		Saemundssonia scolopacis phaeopodis subsp. *humeralis* (Denny, 1842)	
Scolopax rusticola (Linnaeus, 1758)	Eurasian woodcock	*Lipeurus* sp.	(19)
		Philopterus sp.	
STRIGIFORMES			
Strigidae			
Asio otus (Linnaeus, 1758)	Long-eared owl	*Strigiphilus* sp.	This study
Athene noctua (Scopoli, 1769)	Little owl	*Colpocephalum pectinatum* (Osborn, 1902)	(38)
		Philopterus ocellatus (Scopoli, 1763)[×]	
Bubo bubo (Linnaeus, 1758)	Eagle owl	*Strigiphilus strigis* (Pontoppidan, 1763)	This study
SULIFORMES			
Phalacrocoracidae			
Phalacrocorax carbo (Linnaeus, 1758)	Cormorant	*Pectinopygus gyricornis* (Denny, 1842)	(13)

Names of orders are capitalized, and names of families are showed in bold. [×]The louse species is not normally found on this bird. Its report is probably due to contamination or misidentification.

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.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because examined birds Hamedan province were euthanized by a certified veterinarian of the Provincial Department of Environment because of general health failure prior to transfer to the Faculty of Veterinary Medicine, Bu-Ali Sina University. Birds in other provinces were dead animals.

Author contributions

ZB: Methodology, Writing – original draft. AS: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. JK: Methodology, Writing – original draft. MB: Methodology, Writing – original draft. EM: Methodology, Writing – original draft. BD: Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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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.

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The Tengmalm's owl *Aegolius funereus* (Aves, Strigidae) as the definitive host of *Sarcocystis funereus* sp. nov. (Apicomplexa)

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Background: Owls have been reported as definitive hosts, whereas wild small mammals (naturally and experimentally) as intermediate hosts of several species of *Sarcocystis*. Recently, dead fledglings were found infected by an unnamed species of *Sarcocystis* since its intermediate host was unknown. After collecting additional samples of owls and wild small mammals, the present study focused on elucidating the identity, potential intermediate host, and complete life cycle of the found *Sarcocystis* through experimentally infected rodents. The developmental stages' morphological and molecular characterizations (*28S* rRNA gene, ITS1 region) are presented herein.

Methods: In total, 21 Tengmalm's owl carcasses (15 nestlings, 5 fledglings, and 1 adult male) were collected in Kauhava (west-central Finland) and parasitologically examined by wet mounts. Intestinal mucosa scrapings were used to isolate oocysts/sporocysts and employed for experimental infections in dexamethasone-immunosuppressed BALB/cOlaHsd mice. Additionally, sarcocysts were searched in the skeletal muscle of 95 samples from seven wild small mammal species. All these developmental stages were molecularly characterized by the *28S* rRNA gene and ITS1 region. Experimental infections were carried out by using immunosuppressed female 8-week-old BALB/cOlaHsd mice, divided into three groups: (1) water with 15 μg/mL of dexamethasone, (2) water with 30 μg/mL of dexamethasone, (3) no dexamethasone treatment. Each group consisted of four individuals. In each group, two mice were infected with 1,000 sporocysts each, and the remaining two with 10,000 sporocysts each. All mice were euthanized on specific days post-infection.

Results: The intestinal mucosa of 11 nestlings and 5 fledglings of the Tengmalm's owl were positive for *Sarcocystis funereus* sp. nov. The adult male owl and all owls' breast and heart muscles were negative for *Sarcocystis*. Two dexamethasone-immunosuppressed BALB/cOlaHsd mice (group 2) were positive to *S. funereus* sp. nov. in diaphragm and leg muscles after 22- and 24-day post-infection. Some sarcocysts were found in the wild small mammals. Molecular identification at *28S* rRNA revealed sequences from naturally infected

Tengmalm's owls, as well as sarcocysts of dexamethasone-immunosuppressed BALB/cOlaHsd mice were 99.87–100% similar to *Sarcocystis* sp. isolate Af1 previously found in the Tengmalm's owl. At the ITS1 region, the *S. funereus* sp. nov. isolates Af2 haplotype B and Af3 haplotype A were 98.77–100% identical to *Sarcocystis* sp. isolate Af1. The sequences from sarcocysts of naturally infected wild small mammals were 75.23–90.30% similar at ITS1 region to those of *S. funereus* sp. nov.

Conclusion: The morphological and molecular characterizations and phylogenetic placement of *S. funereus* sp. nov. are presented here for the first time and support the erection of the new species.

KEYWORDS

rodents, birds of prey, Europe, sarcocysts, morphology, molecular characterization, oocysts and sporocysts, phylogeny

Introduction

Species of *Sarcocystis* (Apicomplexa) have an indirect, two-host life cycle in which mammals, birds, reptiles, and humans are involved. Due to the wide variety of hosts acting as intermediate or definitive hosts, it is not easy to know the natural life cycle of many species. Birds represent a group of vertebrates commonly utilized as either intermediate and definitive hosts by various members of the genus *Sarcocystis*. Particularly, several species of owls have been reported as definitive hosts, while wild small mammals (rodents and shrews) serve as (natural and experimental) intermediate hosts of *Sarcocystis*. Such a relationship was, for instance, described in the case of the barn owl *Tyto alba* and the house mouse *Mus musculus* (1, 2) and the masked owl *Tyto novaehollandiae* (3) with *Sarcocystis dispersa*, the northern saw-whet owl *Aegolius acadicus* and the eastern deer mouse *Peromyscus maniculatus* with *Sarcocystis espinosai* (4), the tawny owl *Strix aluco* and *M. musculus* with *Sarcocystis scotti* [this species is considered invalid by Dubey et al. (5)] (3, 6, 7), *St. aluco* and the wood mouse *Apodemus sylvaticus* with *Sarcocystis sebeki* (8, 9), as well as the snowy owl *Bubo scandiacus* (reported as *Nyctea scandica*) and Richardson's collared lemming *Dicrostonyx richardsoni* with *Sarcocystis rauschorum* (10, 11). Wiesner (12) further described an unnamed *Sarcocystis* species in the Tengmalm's owl *Aegolius funereus* and experimentally found the bank vole *Clethrionomys* (=*Myodes*) *glareolus* to be its intermediate host. Thus, the involvement of wild small mammals in the life cycles of *Sarcocystis* parasitizing owls appears to be mandatory and obligate.

A recent analysis of a 45-year breeding data set on Tengmalm's owl population in the Kauhava study area (Finland) revealed a decreasing trend in fledgling production corresponding with the long-term decline of the whole population (13). Additionally, we have documented long-term decline in the body condition of both male and females parent owls (14). Though the primary reasons for the observed trends regarding the local population are inappropriate forest management, we know practically nothing about the possible detrimental effects of different internal or external parasites on the individual Tengmalm's owl's long-term survival. The recent findings, indicating that *Sarcocystis* sp. isolate Af1 [see (15)] infected 100% of dead fledglings, have raised doubts about the presumed harmlessness of the mentioned parasite. Thus, the main aim of the present study was to determine the identity, potential intermediate host/s, and complete life cycle of the *Sarcocystis* sp. isolate Af1, through experimentally infecting rodents with newly collected oocysts and sporocysts isolated from the Tengmalm's owls inhabiting the same study area. The morphological and molecular characterizations (*28S* rRNA gene, ITS1 region) of the developmental stages are presented herein.

Methods

The Tengmalm's owl carcasses were collected in the Kauhava study area (west-central Finland) throughout the breeding season 2021 during regular nest box visits starting in early April and later during radio-tracking of fledged young [see details in Kouba et al. (16)]. A total of 21 specimens (15 nestlings, 5 fledglings, and 1 adult male) were sent frozen to the State Veterinary Institute (SVI) Prague, Czech Republic, where parasitological examinations of intestine and muscles (breast, legs, and heart) were carried out by wet mounts. Intestinal mucosa scrapings were used to isolate oocysts/sporocysts under light microscopy with an optical microscope (Leica DM2500 LED), a digital camera (Leica DMC5400), and Leica Application Suite X microscope software (both Leica Microsystems, Wetzlar, Germany). Prior to experimental infections, 25 whole dead small mammals and 70 hind leg samples of seven wild species [i.e., the short-tailed field vole *Microtus agrestis* (2 whole bodies, 2 legs), the sibling vole *Microtus rossiaemeridionalis* (5 bodies), the European water vole *Arvicola amphibius* (1 body), the Eurasian harvest mouse *Micromys minutus* (5 bodies, 23 legs), the bank vole (4 bodies, 30 legs), the common shrew *Sorex araneus* (5 bodies, 15 legs), and the Eurasian pygmy shrew *Sorex minutus* (3 bodies)] were collected from owl nests and examined by wet smear of skeletal muscle. The prey items/bodies were exchanged for frozen newly hatched chickens not to deprive the owls of food.

Oocysts/sporocysts isolated from 2 birds were used to experimentally infect dexamethasone-immunosuppressed BALB/cOlaHsd mice (see below). At the same time, other parasite developmental stages were stored in Eppendorf tubes for DNA extraction under −20°C until further use. All measurements are given in micrometers unless otherwise specified. The molecular analysis of the oocysts/sporocysts isolates ($n = 16$) from the intestinal mucosa of

owls and sarcocyst isolates from wild small mammals ($n = 9$) was carried out following that of Máca et al. (15), with minor changes. Genomic DNA of oocysts/sporocysts was extracted by the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), while that of sarcocysts by using NucleoSpin tissue XS kit (Macherey-Nagel, Düren, Germany).

All isolates were characterized by the *28S* rRNA gene and ITS1 region by using the following primers: KL_P1F/KL_P2R, KL_P2F/P1R, and ITSR/ITSF, respectively (17). PCR procedures were performed in reaction mixtures consisting of 12.50 µL of GoTaq® G2 Hot Start Green Master Mix (Promega, Madison, WI, United States), 0.4 µM of each primer, and 5 µL DNA template. RNase/Dnase-free water was used to top up the reaction mixture to a final volume of 25 µL. PCR amplification of negative controls was also conducted simultaneously. PCR conditions were as follows: 95°C for 3 min, 5 cycles of 94°C for 45 s, 64°C for 60 s, 72°C for 90 s; followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, with a final elongation step of 72°C for 10 min. Amplified products were checked on 1% agarose gel electrophoresis and visualized on a UV transilluminator. Positive PCR products were purified with the ExoSAP-IT™ Express PCR Product Cleanup Reagent Kit (Thermo Fisher Scientific) and sent for sequencing on both strands (using the same forward and reverse primers as for the PCR) to the commercial company Eurofins Genomics (Ebersberg, Germany).

The reference nucleotide sequences used were selected based on similarities using the Basic Local Alignment Search Tool (BLAST) for sequence analysis[1]. The most similar sequences were downloaded and compared with the newly obtained sequences, aligned using the MAFFT software version 7 online server[2] (18) for phylogenetic analysis using the MEGA 11 software version 11.0.13 (19). The phylogenetic trees were inferred by using the Maximum Likelihood (ML) with evolutionary distances calculated by the best-fitting model based on the lowest Bayesian Information Criterion (BIC) scores and resulted as Hasegawa-Kishino-Yano model (20) for the *28S* rRNA gene (analysis involved 38 nucleotide sequences with a total of 1,409 positions). The Hasegawa-Kishino-Yano model (20) was also the best model based on BIC scores for the ITS1 region (involving 25 nucleotide sequences with a total of 1,610 positions in the final dataset), both modeled with a Gamma distribution and invariant sites, with 1,000 bootstrap replications.

Immunosuppression of 8-week-old BALB/cOlaHsd female mice (ENVIGO) was required to establish a successful infection with *Sarcocystis* sporocysts. The mice were divided into three groups, each consisting of four individuals. Two of these groups received water-soluble dexamethasone sodium phosphate (Sigma-Aldrich) dissolved in their drinking water. The first group ($n = 4$) received water with a concentration of 15 µg/mL of dexamethasone continuously starting 2 weeks before the infection and continuing until the end of the experiment. The second group ($n = 4$) received water with a concentration of 30 µg/mL of dexamethasone 1 day before the infection until the end of the experiment. The third group ($n = 4$) did not receive any dexamethasone treatment. All mice were orally inoculated with oocysts/sporocysts delivered with food. In each group, two mice were infected with 1,000 sporocysts each, and the other two were infected with 10,000 sporocysts each. The data for parasitological examination was collected as follows: all mice were euthanized by intraperitoneal injection of ketamine (Narkamon 5%, Bioveta; 1.2 mL/kg) in combination with xylazine (Rometar 2%, Bioveta; 0.6 mL/kg) on specific days (22, 24, 52, 58, 69, and 77 days) after the infection with oocysts/sporocysts (Table 1). Subsequently, muscles containing sarcocysts were fixed in 10% formalin, embedded in paraffin, and sectioned. The histological sections were stained with hematoxylin and eosin and examined under the microscope (Leica DM2500 LED).

Owls were tagged and radio-tracked, and the carcasses were transported to Czechia under the approval of the Centre for Economic Development, Transport, and the Environment (Varsinais-Suomen Elinkeino-, Liikenne-ja Ympäristökeskus: permit numbers VARELY/1389/2018 and VARELY/5933/2019, respectively). All experiments and the maintenance of experimental animals were consistent with current animal welfare laws of the Czech Republic and were approved by the Animal Welfare Committee of the Czech University of Life Sciences Prague (permit number: MSMT-15824/2023-4).

Results

The intestinal mucosa of 11 out of 15 (73%) Tengmalm's owl nestlings and 5 out of 5 (100%) fledglings were positive for *Sarcocystis*. The only adult male owl available was negative to the presence of the parasite. The breast and heart muscles of all 21 examined owls were negative for *Sarcocystis*. No macroscopic lesions were observed in the organs of infected birds. No other protozoan gastrointestinal parasites were found. Sarcocysts from wild small mammals were found in 9 (1 *M. rossiaemeridionalis*, 7 *S. araneus*, 1 *S. minutus*) out of 95 samples (9.5% prevalence). All samples from *A. amphibius*, *C. glareolus*, *M. agrestis*, and *Mi. minutus* were negative for sarcocysts.

Developmental stages were described as follows:

Family Sarcocystidae Poche, 1913.

Sarcocystis funereus sp. nov. (Figure 1).

Description: Thin-walled sporulated oocysts, 16.7–17.0 × 11.5–12.2 ($n = 5$), and sporocysts (Figure 1A) were 11.3–12.9 × 8.1–9.3 ($n = 50$). Sarcocysts were microscopic, the largest 219.5 long and 30.7 wide (found at 24 days post-infection), elongate, ribbon-shaped, with both ends rounded (Figures 1B–D). Wall was 3.1 thick, characterized by dense finger-shaped villar protrusions, 2.6 long (Figure 1B).

TABLE 1 Experimental design for establishing the *Sarcocystis* infection in 12 dexamethasone-immunosuppressed BALB/cOlaHsd mice (2 mice per treatment).

Treatment	Infection dose	Day of sacrifice
15 µg/mL	1,000 sporocysts	22, 58
15 µg/mL	10,000 sporocysts	58, 69
30 µg/mL	1,000 sporocysts	22, 22
30 µg/mL	10,000 sporocysts	22, 24
Without treatment	1,000 sporocysts	69, 77
Without treatment	10,000 sporocysts	52, 58

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2 http://mafft.cbrc.jp/alignment/software/

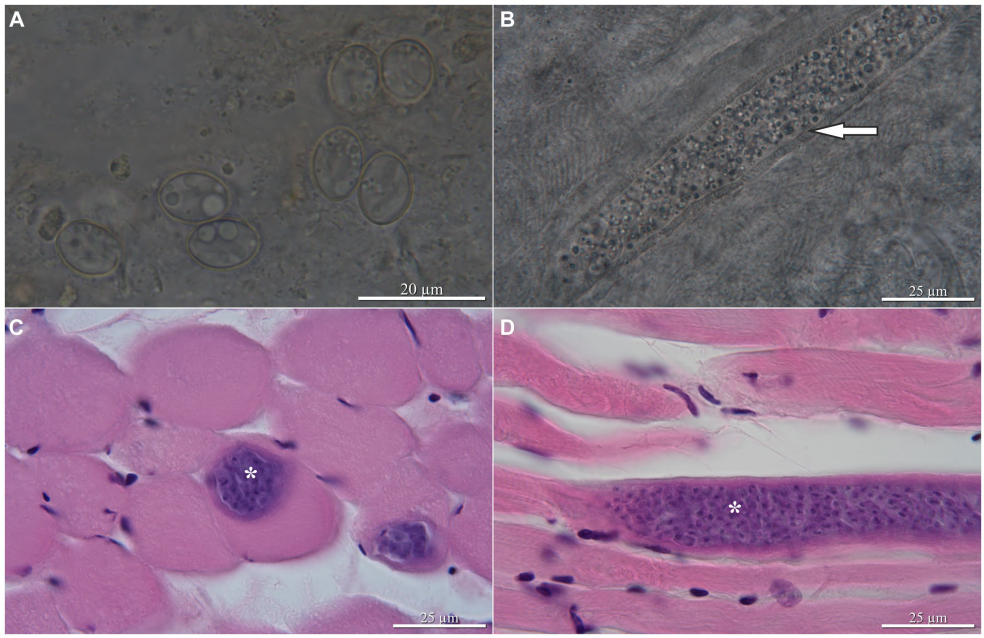

FIGURE 1
Sarcocystis funereus sp. nov., light micrographs. Oocysts/sporocysts from the intestinal mucosa of the Tengmalm's owl *Aegolius funereus* from Finland; arrow indicates cyst wall with finger-like protrusions **(A)**, sarcocyst in wet mount **(B)** and hematoxylin and eosin staining **(C,D)** from the skeletal muscles of experimentally infected dexamethasone-immunosuppressed BALB/cOlaHsd mice 24 days postinfection, longitudinal and transversal sections, respectively. Asterisks indicate sarcocysts.

Taxonomic summary

Definitive host: Tengmalm's owl *Aegolius funereus* Linnaeus, 1758 (Strigiformes: Strigidae).

Natural Intermediate host: Unknown.

Experimental intermediate host: Dexamethasone-immunosuppressed BALB/cOlaHsd mouse.

Distribution: Kauhava region, west-central Finland (~63° N, 23° E).

Site of infection: Small intestine (definitive host), skeletal muscle (experimental intermediate host).

Deposited material: Symbiotype (oocysts/sporocysts in 2.5% potassium dichromate) and genomic DNA in an Eppendorf tube were stored at SVI Prague. GenBank sequences OR725602 and OR726006 (*28S* rRNA gene), OR726007 and OR726008 (ITS1 region). Positive mice were frozen at −20°C, and histology slides were stored in lab SVI Prague.

Sequences obtained from the experimental study: OR725602 (*28S* rRNA gene), and OR726007 (ITS1 region).

ZooBank registration: To comply with the regulations in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (21), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Sarcocystis funereus* sp. nov. is urn:lsid:zoobank.org:pub:536F1351-1157-4C09-999A-D41078EE3CBC.

Etymology: The specific epithet is derived from the species name of its definitive host, i.e., *funereus*.

Molecular identification at *28S* rRNA revealed that 12 sequences [isolate Af3 haplotype A (OR726006) and isolate Af2 haplotype B (OR725602), both 1,509 bp] obtained from the 16 oocyst/sporocyst isolates (4 failed sequencing) from the naturally infected Tengmalm's owls, as well as the 6 sequences (haplotype B) of the 6 sarcocyst isolates in the skeletal muscles of dexamethasone-immunosuppressed BALB/cOlaHsd mouse were 99.87–100% similar to *Sarcocystis* sp. isolate Af1 (MW349707), 97.59% similar to *Sarcocystis strixi* (MF162316) and 97.46–97.53% to *Sarcocystis lari* (MF946611), in the white-tailed sea eagle *Haliaeetus albicilla* from Norway; and 97.42–97.49% similar to *Sarcocystis lutrae* (KM657771) in the Eurasian otter *Lutra lutra* from Norway. Haplotypes A and B were 99.87% similar each other and showed single cases of double peaks at nucleotide positions 666 and 667 (TT/CC), especially in those samples from owls and experimental mice that resulted in TT (haplotype A) or CC (haplotype B) peaks or double peaks at these positions. Isolates used for experimental infections and those of all sarcocysts resulted in CC dominant peak at this position and represent *S. funereus* sp. nov. isolate Af2 haplotype B (OR725602). *Sarcocystis* cf. *strixi* isolate LTAfl120 (OQ557459) in the tawny mouse *Apodemus flavicollis* from Lithuania and *Sarcocystis* sp. isolate No. 5 (AF513497) in *S. araneus* from Czech Republic were 95.37 (47% query cover; 733 bp) and 96.22% (35% query cover; 554 bp), respectively, similar to haplotypes A and B of *S. funereus* sp. nov., although both have short sequences and were not used in the phylogenetic analysis.

All isolates were successfully sequenced at the ITS1 region. Like at the *28S* rRNA gene, the isolates *S. funereus* sp. nov. isolate Af2 (OR726007, 1,300 bp) and *S. funereus* sp. nov. isolate Af3 (OR726008, 1,297 bp) were used for infection and all sequences obtained from sarcocysts (OR726007) showed 10 SNPs and 3 nucleotide insertions of GTG in position 1,011–1,013. Those insertions were not found in other ITS1 region sequences that Máca et al. (15) obtained. Similarly, there was only one SNP (T/C) at nucleotide position 466 in newly

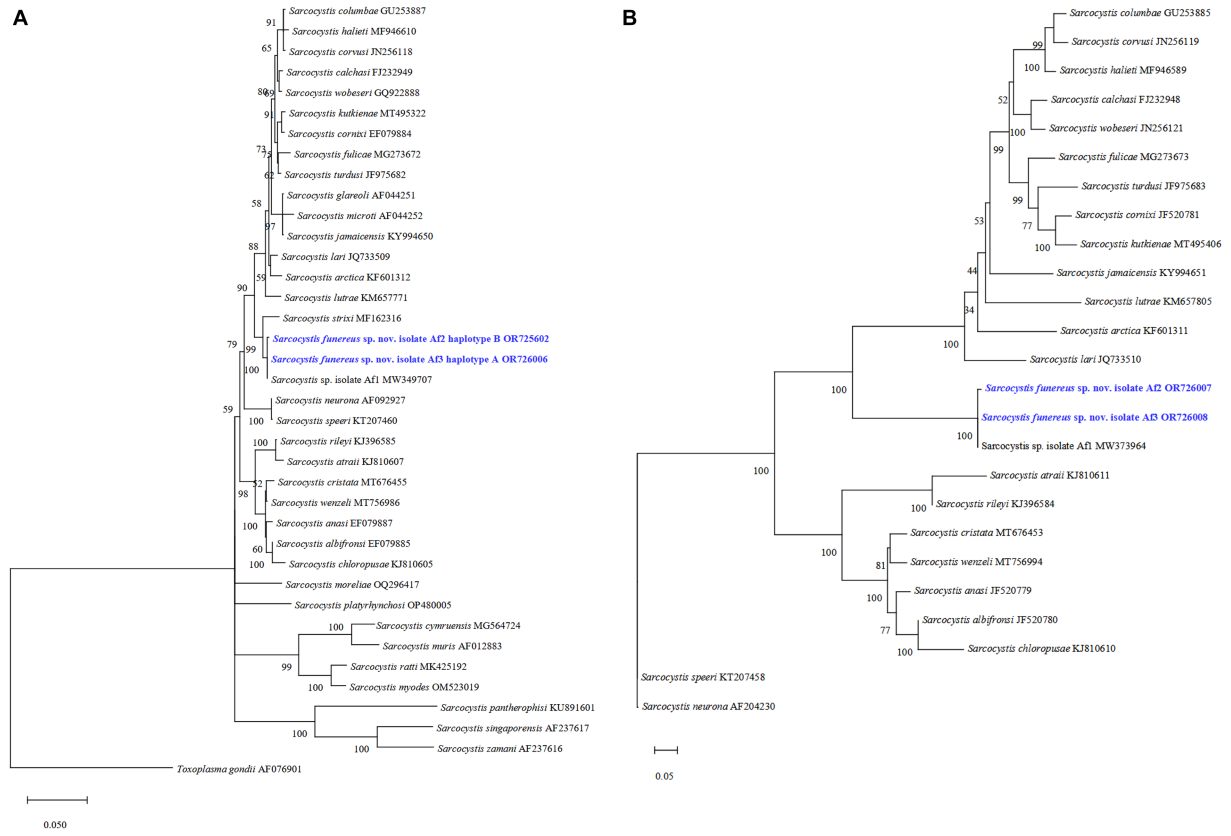

FIGURE 2
Phylogenetic trees of the related species of *Sarcocystis* from various hosts based on sequences of the *28S* rRNA gene **(A)** and the ITS1 region **(B)**. The numbers on phylogenetic trees represent bootstrap values based on 1,000 replications. GenBank accession numbers follow *Sarcocystis* taxa.

obtained sequences. Moreover, *S. funereus* sp. nov. isolate Af2 (OR726007) and *S. funereus* sp. nov. isolate Af3 (OR726008) were 98.77–100% identical to *Sarcocystis* sp. isolate Af1 (MW373964), and 88.43–89.57% (26–45% query cover) to *S. lutrae* (MG372108) in the European badger *Meles meles* from the Czech Republic and *Sarcocystis halieti* (MF946596) in the white-tailed sea eagle from Norway.

The phylogenetic tree showed that isolates Af3 haplotype A and Af2 haplotype B of *S. funereus* sp. nov. at the *28S* rRNA gene grouped with *Sarcocystis* sp. Af1, which was previously reported in *A. funereus*. Moreover, *S. strixi* appears as a sister species in a close clade (Figure 2A). At the ITS1 region, sequences of haplotypes A and B are also grouped in a single clade with *Sarcocystis* sp. Af1 (Figure 2B).

Out of the 8 dexamethasone-immunosuppressed BALB/cOlaHsd mice used for the experimental infections, only 2 of the second group, treated with 30 µg/mL dexamethasone and dosed with 10,000 sporocysts were positive to *S. funereus* sp. nov. after 22- and 24-day post-infection. Both mice showed sarcocysts in the skeletal muscles (diaphragm, leg), but no in the brain nor heart. Infected mice were asymptomatic during the whole infection process, from inoculation to euthanasia.

The 6 sequences (5 from *S. araneus*, 1 from *S. minutus*) successfully obtained from the sarcocysts of naturally infected small mammals were molecularly identified by using the ITS1 region marker (unpublished data). Those sequences from *S. araneus* and *S. minutus* were 75.23–90.30% (13–26% query cover) and 90.30% (19% query cover), respectively, similar to *Sarcocystis* sp. isolate Af1 (MW373964) and *S. funereus* sp. nov. isolate Af3 (OR726008). Negative PCR resulted in the samples of *M. rossiaemeridionalis* and 2 of *S. araneus*.

Discussion

While recently reporting the finding of oocysts/sporocysts in the intestine of the Tengmalm's owl, Máca et al. (15) refrained from specifically naming the *Sarcocystis* sp. isolate Af1, since its intermediate host and sarcocysts were unknown. This unnamed *Sarcocystis* was fully characterized by using four loci (*18S* rRNA, *28S* rRNA, ITS1, and *CO1*), so we decided to only use two of them (*28S* rRNA and ITS1) because both, but especially the ITS1 region, clearly delimite species using avian hosts than the other two loci (22). Máca et al. (15) mentioned that the ITS1 region is more sensitive to the genetic differences among *Sarcocystis* species from birds and carnivores as intermediate hosts, while CO1 are considered of limited taxonomic help.

After the experimental infections of mice, the presence of sarcocysts in the skeletal muscles elucidates the determination of such species as new to science and the possible route of the life cycle. The current finding supports the rodent-owl life cycle reported in our previous study (15). The phylogenetic position of *S. funereus* sp. nov. inferred separately at the *28S* rRNA gene and ITS1 region was the same than that obtained by Máca et al. (15); thus, *Sarcocystis* sp. isolate Af1 should be considered to belong to *S. funereus* sp. nov.

There is only one previous record of an unnamed *Sarcocystis* in the Tengmalm's owl, whose sporocysts and oocysts were not morphologically nor molecularly described [see (12)]; additionally, this unnamed species was experimentally transmitted to the bank vole, while the new species to immunosuppressed mice. Considering that *Sarcocystis* are more specific to their rodent intermediate hosts [see (5)] and that those bank voles examined during this study were free of sarcocysts, they most probably represent two different species. Our opinion supports the finding that *S. sebeki* of the house mouse was not transmissible to the wood mouse, bank vole or meadow vole (*Microtus arvalis*) (9).

The Tengmalm's owl acts as the definitive host for *Sarcocystis* sp. Wiesner, 1980 [as named by Levine and Ivens (23)] and *S. funereus* sp. nov., with bank voles and mice, respectively, are its intermediate hosts. However, more information on the former unnamed species is required. The finding of *S. halieti* and *S. lari* in the white-tailed sea eagle showed that more than one *Sarcocystis* species might infect a particular bird of prey species [see (22)]. As stated by Máca et al. (15), more Tengmalm's owls and other birds of prey species should be examined to determine the presence of other parasite species or morphospecies of *Sarcocystis*.

Corticosteroids, such as dexamethasone, have proven to be useful in developing animal models for studying coccidian parasites (24). Dexamethasone induces the depletion of CD4+ T lymphocytes (25) and suppresses T- and natural killer (NK) cell-mediated immunity (26). Similarly, Interferon-gamma (IFN-gamma) gene knockout (KO) mice, often employed to establish *Sarcocystis neurona* infection in laboratory mice, lack a CD4+ Th1 response. The immunity of gamma-IFN KO mice is either deleted or reduced, allowing the establishment of infection and the development of clinical disease in mice (27). Therefore, we selected dexamethasone immunosuppression to set up *Sarcocystis* infection. The optimal concentration of dexamethasone in the drinking water was determined to be 30 μg/mL, as a lower concentration likely did not sufficiently suppress the immune response for successful infection establishment.

Máca et al. (15) found *S. strixi* to cluster with *Sarcocystis* sp. isolate Af1 (now recognized as *S. funereus* sp. nov.) and regarded them as distinct species. Interestingly, both have a densely covered wall of the sarcocyst, although *S. strixi* has knob-like blebs and a thinner wall (< 2 μm). In contrast, *S. funereus* sp. nov. has longer finger-shaped blebs and a thicker wall (> 2 μm). Additionally, the ends of the sarcocyst (pointed vs. rounded) differ from each other.

Six sequences from wild small mammals were positive for *Sarcocystis*, but none of them could be considered as *S. funereus* sp. nov. They probably belong to one or several different species, although more analyses are needed to fully understand whether they represent various species and find their natural definitive hosts. The natural intermediate host of *S. funereus* sp. nov. is still unknown, but it is very likely that the Eurasian harvest mouse, one of the most common small mammals used as prey by the Tengmalm's owls (28, 29), plays that important role. Interestingly, after examining several samples of this host ($n = 28$), no sarcocysts were found. It does not mean that they cannot be parasitize by *Sarcocystis*, but that the density of intermediate hosts, visiting of sites with contaminated feces, and the climatic seasons are important factors to determine the infection of the definitive hosts, as stated by Hoogenboom and Dijkstra (30). Therefore, further investigations of potential intermediate hosts (wild small mammals) should be done to warrant the identification of the real host. As in other species of *Sarcocystis* using owls as definitive hosts, mice are essential in the life cycle, as *S. espinosai* in the northern saw-whet owl and eastern deer mouse [see (4)].

The present findings show the role of the Tengmalm's owl in the life cycle of a *Sarcocystis* species and increase our knowledge of rodent predators as part of the life cycle of parasites. Many more studies are needed to understand the individual relationships between parasites and their intermediate and final hosts. As in other parasite/birds of prey relationships worldwide, we know nothing about the impact of *S. funereus* sp. nov. on the body condition of fledglings, dispersal, and probability of recruitment to the breeding population and particularly long-term survival of the Tengmalm's owl. It is important to understand if it is possible to have intestines full of parasites without adversely affect the health of individuals, as in the present study. Thus, more focus and studies are needed to determine how parasites and their hosts interact and influence each other's lives, either negatively or positively.

Conclusion

This work elucidates the specific identity of the *Sarcocystis* infecting the Tengmalm's owl and its experimental intermediate host, although more prey and potential intermediate hosts should be examined to determine their role in the life cycle of the new species. The morphological and molecular characterizations and phylogenetic placement of *S. funereus* sp. nov. are presented here for the first time and support the erection of the new species.

Data availability statement

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

Ethics statement

The animal study was approved by the Varsinais-Suomen Elinkeino-, Liikenne-ja Ympäristökeskus (permit numbers VARELY/1389/2018 and VARELY/5933/2019), respectively, and welfare laws of the Czech Republic and were approved by the Animal Welfare Committee of the Czech University of Life Sciences Prague (permit number: MSMT-15824/2023-4). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

OM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MK: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. IL: Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. LP: Investigation, Methodology, Writing – review & editing. EK: Investigation, Methodology, Writing – review

& editing. DG-S: Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

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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.

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A revision of the trichostrongylid nematode *Cooperia* Ransom, 1907, from deer game: recent integrative research confirms the existence of the ancient host-specific species *Cooperia ventricosa* (Rudolphi, 1809)

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The trichostrongylid roundworms of the genus *Cooperia*, which are important in veterinary medicine, currently comprise 19 valid species that parasitize the small intestine of both free-living and domestic ruminants. Only four *Cooperia* spp. have been reported in Europe, namely *C. oncophora, C. punctata, C. curticei* and *C. pectinata*. In 2018–2022, 25 red deer (*Cervus elaphus*) and 30 sika deer (*Cervus nippon*) of both sexes and various ages from several remote locations in the Czech Republic were parasitologically examined. Intestinal nematodes of the genus *Cooperia* were found only in two northern regions. Using the globally recognized key book on trichostrongylid nematodes, they were preliminarily identified as *C. pectinata*. However, a molecular analysis of *cox*2 and ITS rDNA gene sequences revealed that *Cooperia* sp. parasitizing Czech deer is a separate taxon that is more closely related to *C. oncophora* than to *C. pectinata*. A subsequent morphological analysis and literature survey confirmed the independence of deer *Cooperia* sp., which is similar but not identical to bovid *C. pectinata*. Previous long-term correct identifications of bovid *C. pectinata* and misidentifications of deer *Cooperia* species were caused by a fundamental error in the key book mentioned above. Interestingly, the ancient trichostrongylid nematode *Strongylus ventricosus* from the type host red deer (*Cervus elaphus*) shot near Greifswald (Germany) was described by Rudolphi in 1809. Rudolphi's type material (one male and four females) was deposited in the Museum für Naturkunde (Berlin). Later, the ancient species *S. ventricosus* was taken as a synonym for various *Cooperia* spp. Our current re-examination of the type male indicated that there is a relatively good agreement with our new material from Czech deer regarding the most important characteristics of *S. ventricosus* (i.e., the shape and size of the male spicules); however, Rudolphi's type material is in rather poor condition. The suggested resurrection of the deer *Cooperia* sp. in this study as *Cooperia ventricosa* (Rudolphi, 1809) requires verification by collecting and analyzing new nematode material from the type locality near Greifswald.

KEYWORDS

***Cooperia ventricosa*, *Cooperia pectinata*, deer game, mitochondrial DNA, ribosomal DNA, redescription, gastrointestinal nematodes**

1 Introduction

Nematodes of the genus *Cooperia* Ransom, 1907 (Strongylida, Trichostrongyloidea: Cooperiidae) are gastrointestinal parasites of many wild and domestic ruminants, and some of these nematodes are distributed worldwide (1). There is a rich species spectrum, predominantly in Africa and other tropical and subtropical regions, where up to 40% of cattle and goats are infected with *Cooperia* spp. (2–5). Although *Cooperia* spp. are not highly pathogenic parasites, a high nematode burden can substantially reduce host production, as infestation has been associated with loss of appetite and poor weight gain (6).

Cooperia spp. are monoxenous parasites with free-living pre-parasitic larval phases. Adult worms that reside in the small intestine of the ruminant host produce eggs that are passed in the host's feces. The first-stage larvae hatch in the so-called "fecal pat" where they feed on soil/fecal bacteria. Two subsequent molts are completed within 2 days (7, 8). The third-stage larva remains enclosed in the second-stage cuticle sheath and becomes infective to the host in 1–6 weeks (9). The larvae migrate to the grass and can survive for up to 1 year until swallowed by a ruminant host (10). In the host's small intestine, the larvae shed their sheath, undergo the last two molts, and become sexually mature males or females. When the fertilized females produce eggs, the cycle is complete (11, 12). However, under unfavorable environmental conditions, a life-cycle variation can occur that involves a slowing of development. This strategy involves larval L_4 hypobiosis (developmental restriction) within the host digestive tract for up to several months (13, 14).

The latest taxonomic revision confirmed that there are 19 valid species of the genus *Cooperia* (1). So far, only four congeners have been reported in Europe, namely *C. curticei*, *C. oncophora, C. pectinata,* and *C. punctata.* All of them have been reported almost worldwide in both wild and domestic ruminants [e.g., (15, 16)]. It is not entirely certain that all previous morphological identifications were reliable, as *Cooperia* congeners are morphologically very similar to each other. The only morphological traits with a high discriminatory value are the size and shape of the male spicules, the characteristics (shape, length, and spatial arrangement) of the rays of the male bursa, and partly the synlophe morphology (longitudinal cuticular ridges) [e.g., (17, 18)]. However, even these traits can sometimes be problematic, as has been the case with the *C. pectinata* species.

Cooperia pectinata was first described in a paper by Ransom (19) as a parasite of bovids (*Bos taurus*) from Texas, USA. However, this paper only included a short verbal description without any images. Moreover, Ransom noted that "*C. pectinata* might be identical with *Strongylus ventricosus* Rudolphi, 1809 from red deer (*Cervus elaphus*); additionally, the determination of this point necessitates the restudying of the type specimens of Rudolphi". Later, in 1911, the same author (20) published drawings of the male spicules and bursa, the female vulva, and the exact measurements of *C. pectinata* (Figure 1). After two decades, Baylis (21) published new drawings of *C. pectinata* from Australian cattle, which were later copied into a key Russian monograph on the superfamily Trichostrongyloidea by Skrjabin et al. (15) (Figure 2, left). More recent drawings and photographs are now available of the typical morphological features of *C. pectinata* from cattle and sheared sheep in Brazil (6, 23), from long-term bred alpacas in Australia (24), and from impalas in Africa (16).

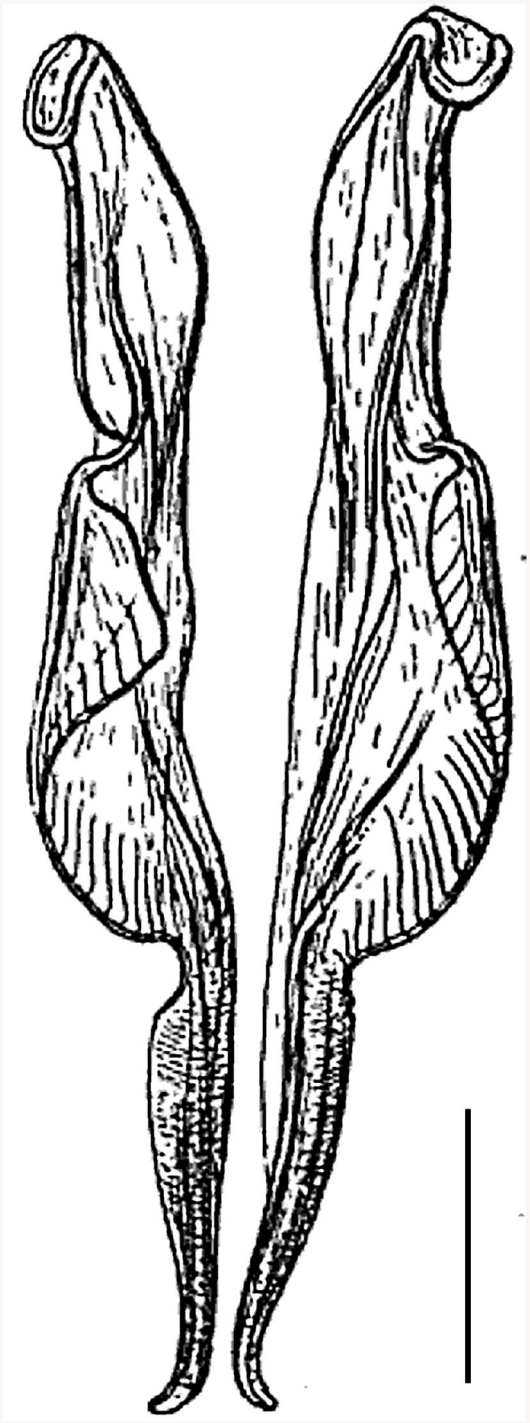

FIGURE 1
Drawings of spicules of *Cooperia pectinata* Ransom, 1907, which parasitizes bovids (*Bos taurus*), published by Ransom (20). Scale bar = 50 μm.

However, a confusing incident occurred in the 1930s, when another very similar *Cooperia* nematode was dissected by

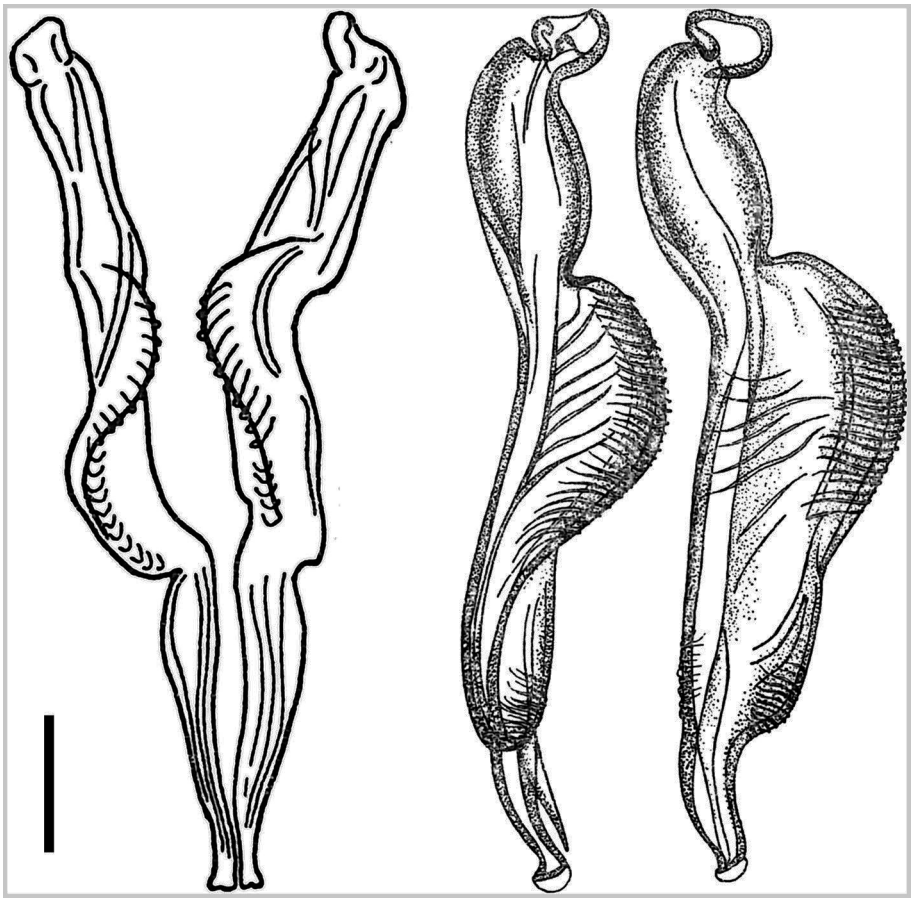

FIGURE 2
Details of hitherto recognized variants of spicule types of the species *Cooperia pectinata* Ransom, 1907 **(left, right)**, published together in the monograph by Skrjabin et al. (15). According to information by Skrjabin et al. (15), **(left)** is a copy from Baylis (21) and the host species of *C. pectinata* was cattle, while **(right)** was copied from Skrjabin and Orlov (22) and the host of *C. pectinata* was deer. Scale bar = 50 μm.

R. S. Schulz from a sika deer (*Cervus nippon*) in the Russian Far East. This deer *Cooperia* was mistakenly suggested to be *C. pectinata* by I. V. Orlov, who drew the original images of the morphologically important features (22). Although the spicules of these deer nematodes (Figure 2, right) differed slightly from the spicules of the bovid *C. pectinata* (Figure 2, left), both were together erroneously published under the name *C. pectinata* in the Russian monograph by Skrjabin et al. [(15), p. 321], using Ransom's bovid nematode measurements. No original measurements of the deer parasites were published in the Russian monographs (15, 22), so numerical data can only be estimated from the figures.

Unfortunately, the two drawings of male *Cooperia* spicules from bovids and deer (Figure 2), however different they may be, have been widely used to represent alternative characteristics of *C. pectinata*, and they seem to have been mistakenly thought to be its morphotypes.

In summary, it is apparent that deer *Cooperia* differ morphologically from *C. pectinata* (typically found in bovids), to which they have hitherto been assigned for a long time. The aim of the present study was to assess the phylogenetic position of deer parasites in the genus *Cooperia* and to update the morphological and molecular characteristics of *Cooperia* sp. parasitizing red deer (*Cervus elaphus*) or sika deer (*Cervus nippon*) from the Czech Republic. Subsequent aims will involve taxonomic anchoring of the redefined taxon in the context of the current zoological nomenclature rules (25).

2 Materials and methods

2.1 Collection of nematodes

In 2018–2022, 25 red deer (*Cervus elaphus*)—10 males (M) and 15 females (F) and 30 sika deer (*Cervus nippon*) (10 M + 15 F) were caught in nine official hunts across the Czech Republic. Deer that tested positive for *Cooperia* nematodes were relatively rare, being found in only two regions of northern Bohemia: the Doupov Mountains (Valeč and Doupov hunting regions), and Mimon (the Ralsko hunting ground).

Freshly discarded deer entrails were transported to the Czech University of Life Sciences, Prague (Czech Republic). They were parasitologically examined either immediately or after temporary freezing. For the detection of *Cooperia*, the contents of the small intestine were thoroughly removed, placed on a fine sieve, and

washed with running tap water. The residues left on the sieve were transferred to a saline solution on a Petri dish and examined under a stereomicroscope.

2.2 Processing of nematodes for morphological and molecular analyses

The collected nematodes were numbered, sexed, and preserved in 70% ethanol. Specimens used for morphological analyses were cleared in a glycerol–ethanol solution by evaporation of the ethanol, and then mounted on glass slides with a 50% glycerol solution and measured using an optical microscope (BX51 light microscope, Olympus). Additional specimens were cleared in a lactophenol solution (26) and then photographed and measured using Quick PHOTO MICRO 3.0 software (Promicra). Special attention was paid to the morphological parameters of the male spicules.

The specimens used for molecular analysis were divided into three body parts: cephalic, middle, and caudal. Only the middle parts were used for molecular analyses, with the cephalic and caudal parts being used for morphological analyses.

2.3 Deposited material

The collected specimens were deposited at the Faculty of Agrobiology, Food, and Natural Resources of the Czech University of Life Sciences, Prague (Czech Republic). Additionally, four collected specimens (two males and two females) were deposited in the "Vermes (worm-like animals)" museum collection in the Museum für Naturkunde of the Leibniz Institute for Evolution and Biodiversity (Berlin, Germany).

2.4 Molecular analyses

Total DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) using spin column purification according to the manufacturer's protocol. A partial mitochondrial gene sequence (cytochrome oxidase subunit 2, *cox* 2) and a nuclear segment of rDNA (ITS1-5.8S-ITS2) were amplified by PCR.

Cox 2 was amplified by PCR as described by Ramünke et al. (6) with slight modifications. The 25-µl PCR mixture contained 1.6 U Top-Bio Taq DNA Polymerase, PCR Blue Buffer, and 1.2 µM of each primer [COII_deg_for (5′-ATKGARTAYCARTTTGGIGGARTT-3′) and COII deg_rev (5′-CTRTGRTTIGCICCRCARATYTC-3′)]. The cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 49°C for 20 s, and extension at 68°C for 30 s.

The ITS1-5.8S-ITS2 region was amplified by PCR using a 25-µl PCR mixture prepared as described by Callejón et al. (27) with the exception that the following primers were used: forward primer NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and reverse primer NC2 (5′-TTAGTTTCTTTCCTCCGCT-3′) (28), corresponding to the conserved 3′-5′ ends of the ITS1-5.8S-ITS2 region flanking the 18S and 28S regions. The cycling conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a single period at 72°C for 10 min.

The obtained sequences were compared with those of the most closely related species published by Ramünke et al. (6) along with *C. oncophora* from Australian sheep (GQ888713) (29) and *Cooperia* sp. from China (KY769271.1) (30). The trichostrongylid species *Haemonchus contortus* (EU346694.2) and *Teladorsagia circumcincta* (KT428386) were used as outgroups.

The sequences were visually inspected for the accuracy of base calls and the presence of potential heterozygotes. Homologous sequences were aligned using the ClustalW program with the default settings in BioEdit (31). The presence of stop codons was checked using the MEGA software (32).

To estimate the phylogenetic relationships of *Cooperia* spp. with respect to the divergence of *Cooperia oncophora*, Bayesian inference using StarBEAST2 (33) implemented in BEAST v2.7.4 was utilized (34). A phylogenetic analysis was run separately for each partial gene sequence (ITS1-5.8S-ITS2 and *cox 2*) and both simultaneously, generating three independent phylogenetic trees.

Sequence substitution models were estimated in W-IQ-Tree (35) and fitted to a Hasekawa-Kishino-Yano 1985 (HKY85) model with gamma distribution in four categories. The trees were reconstructed under the Strict clock and the Yule model of coalescent evolution. The log files of the three independent runs with 5×10^7 iterations were checked for convergence in Tracer v1.7.2 (36) with 10% burn-in. Combined and annotated trees were graphically generated in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

3 Results

3.1 Occurrence of *Cooperia* in deer in the Czech Republic

Despite the relatively high number of deer examined from various parts of the Czech Republic in 2018–2022 (116 deer belonging to seven ruminant species, not shown), *Cooperia* nematodes occurred occasionally. They were present in only three out of 25 red deer (prevalence = 12.0%) and four out of 30 sika deer (prevalence = 13.3%) and were found only in two regions of northern Bohemia (Table 1).

The first site (50°39′32″ S, 14°43′29″ E) was Mimon (the Ralsko hunting ground). One red deer was positive out of the two examined (prevalence = 50%). The intensity of nematode infection was 141 worms.

The second site (50°10′30″ S, 13°2′48″ E) was the hunting ground in the Doupov Mountains directly adjacent to the territory around the village of Valeč. Two red deer were positive out of the 17 examined (prevalence = 11.8%) and they had four and 11 worms, respectively. Four sika deer were positive out of the 20 examined (prevalence = 20%), and the intensity of infection ranged from three to 24 worms.

TABLE 1 Occurrence of *Cooperia* nematodes in seven infected deer in two Czech localities.

Examined deer	Locality	No. (sex) of examined deer	Sex/age (year) of individual positive deer	No. (sex) of *Cooperia* worms
Red deer (*Cervus elaphus*)	Mimoň	2 (2 F)	F/2	141 (70 M + 71 F)
	Doupov (Valeč)	17 (7 M + 10 F)	F/4	11 (4 M + 7 F)
	Doupov (Valeč)		F/3	4 (1 M + 3 F)
Sika deer (*Cervus nippon*)	Doupov (Valeč)	20 (6 M + 14 F)	F/1	3 (2 M + 1 F)
	Doupov (Valeč)		F/2	3 (1 M + 2 F)
	Doupov (Valeč)		M/6	24 (9 M + 15 F)
	Doupov (Valeč)		F/1	16 (6 M + 10 F)

M, male; F, female for both deer and worms.

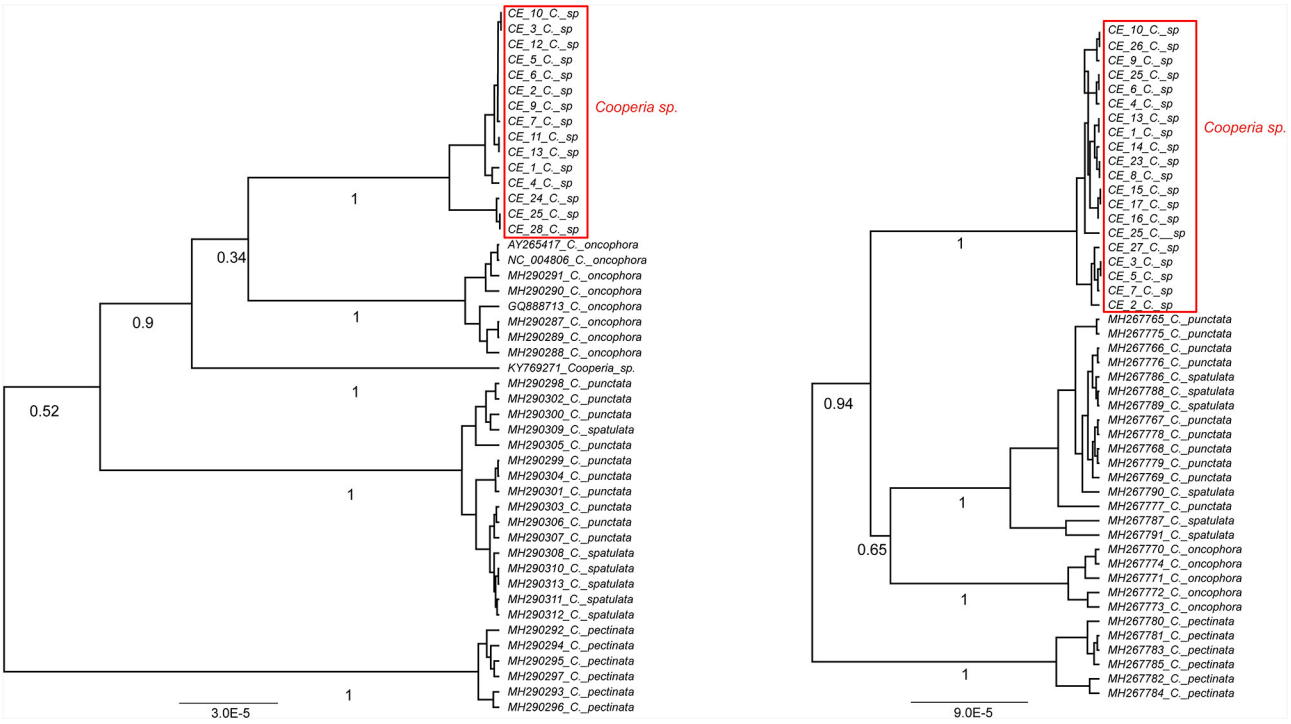

FIGURE 3
Ultrametric Bayesian phylogenetic trees of *Cooperia* spp. based on the *cox2* gene **(left)** and the ITS1-5.8S-ITS2 region **(right)** of *Cooperia* spp. Branch support values (posterior probability) are displayed under the branches. The trees were rooted with *Teladorsagia circumcincta* (KT428386) and *Haemonchus contortus* (EU346694.2) (not shown), respectively. "*C*" stands for *Cooperia*. Congruently, both trees show *Cooperia* sp. to be an independent lineage with a branch support value of 100%.

3.2 Bayesian phylogenetic trees based on two partial gene sequences

The phylogenetic analysis of *Cooperia* sp. from Czech deer based on 18 sequences of *cox* 2 and 20 sequences of the ITS1-5.8S-ITS2 region resulted in six haplotypes (accession numbers OR879242-7) and three haplotypes (accession numbers OR804235, OR804236, and OR804237), respectively. Our data were compared with published data, mainly by Ramünke et al. (6), who compared *C. pectinata, C. punctata, C. spatulata*, and *C. oncophora* and also indicated that *C. spatulata* is most likely only a morphotype of *C. punctata* and its name should be considered a synonym (Figures 3, 4).

Our Bayesian phylogenetic analyses indicated that *Cooperia* sp. from the Czech deer represents a new lineage. The *cox* 2 phylogenetic tree indicated the clustering of this new lineage in the clade containing *C. oncophora*, despite the low branch support (Figure 3, left). The ITS1-5.8S-ITS2 phylogenetic tree showed that this new lineage represents a sister lineage to *C. punctata/spatulata, C. oncophora*. Finally, the *C. pectinata* branch represents the sister

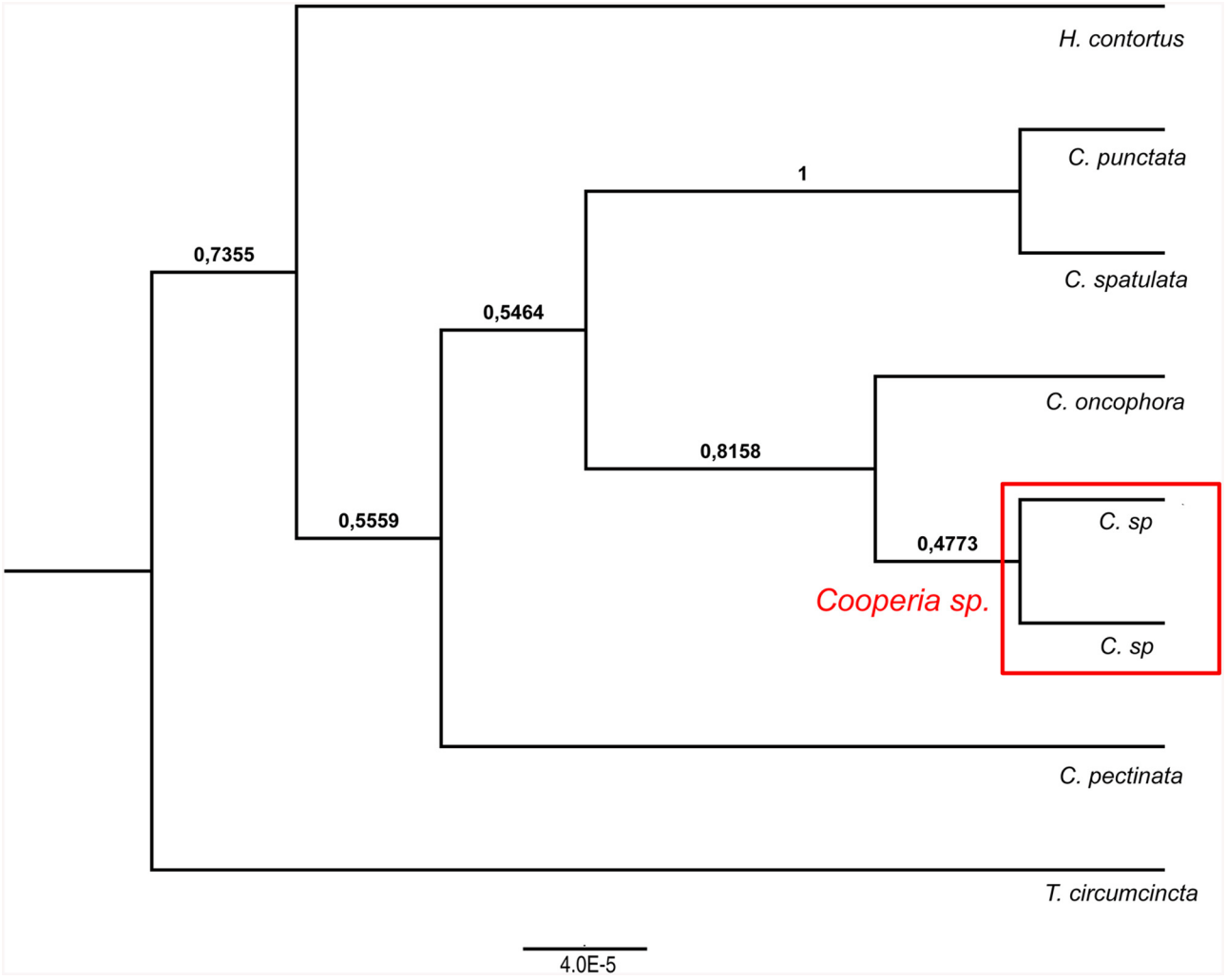

FIGURE 4
Ultrametric Bayesian phylogenetic tree of *Cooperia* spp. based on combined analysis of mitochondrial DNA (*cox*2) and nuclear ITS1-5.8S-ITS2 rDNA (ITS region). Branch support values (posterior probability) are displayed above the branches. The tree was rooted with *Haemonchus contortus* (EU346694.2) and *Teladorsagia circumcincta* (KT428386). *"C"* stands for *Cooperia*.

lineage to the common cluster described above (Figure 3, right). Also, the phylogenetic tree based on both loci (ITS1-5.8S-ITS2 region and *cox* 2) (Figure 4) agreed with the *cox* 2 phylogenetic tree. Thus, the lineage of *Cooperia* sp. from Czech deer constitutes a new sister lineage to *C. oncophora* and this common branch is a sister lineage to *C. punctata/spatulata*. *Cooperia pectinata* represents the sister lineage to the above cluster.

In conclusion, the Bayesian phylogenetic analysis of combined mitochondrial and nuclear markers (*cox*2 and ITS1-5.8S-ITS2 region) supported the existence of a new independent lineage of *Cooperia* sp. from Czech deer. This analysis confirmed that specimens parasitizing deer game represent a sister lineage to *C. oncophora* while the congener *C. pectinata* is more distantly related. These results indicate a high probability that *Cooperia* sp. that parasitizes deer game does not belong to the *C. pectinata* species that parasitizes bovids.

3.3 Morphological description

The measurements of *Cooperia* sp. parasitizing deer are expressed in micrometers (μm) unless otherwise noted, based on 30 males and 30 females.

Male: Body 5.98–10.24 mm long, 117–189 wide just anterior to bursa, head diameter 32–40, cephalic vesicle up to 105 wide, esophagus 380–515. Bursa 277–400 wide, spicules 265–348 long, 67 maximum spicule width, with four parts (length × width): "short head" 19 × 32, "barrel neck" 62 × 40, "bulky belly" 174 × 67, and "thin tail" 53 × 18. The butterfly-shaped genital cone is situated in the middle of the bursa, 64–222 behind the posterior end of the spicules. The next important morphological characteristics are the shape and size of the dorsal ray of the male bursa: it is a double-branched fork with a total length of 180–208 (196 on average), with the main bifurcation at 56% of the total length (Figures 5, 6). The number of ventrally oriented rays is six on the left and six on

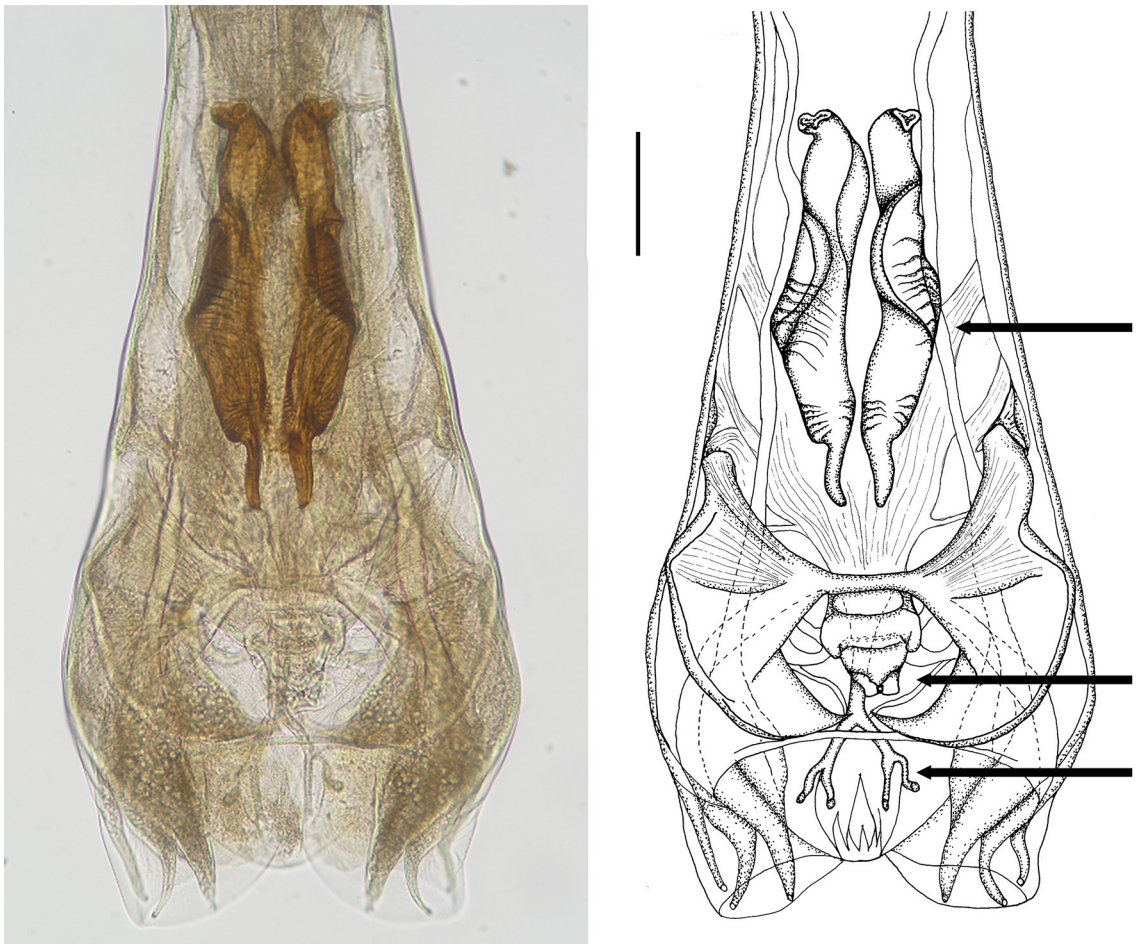

FIGURE 5
Male genital bursa of deer *Cooperia* sp. Photomicrograph **(left)** and drawing **(right)** outlining spicules (up arrow), genital cone (middle arrow), and dorsal ray (down arrow). Scale bar = 50 μm.

the right side, while four are always long and the remaining two are shorter.

Female: Body 7.57–12.74 mm long, its widest part reaching 190–255 behind the vulva, head diameter 33–42, cephalic vesicle 70–77 wide, esophagus 342–622. Vulva opens 2.11–2.70 mm from the posterior end. Anus opens 172–217 from the body end, tail 43–50 wide at the anus level, narrowing sharply to the terminal tip. Eggs in the uterus are 55–61 long and 21–30 wide.

4 Discussion

There are no significant differences in the majority of the morphological characteristics between *Cooperia* sp. parasitizing deer (this paper) and *C. pectinata* parasitizing bovids (20), especially when larger sets of individuals are measured and compared. The original description of *C. pectinata* by Ransom (20) was as follows: "Measurements based on few specimens. Male: Body about 7 mm long, by 130–160 μm wide at base of the bursa. Head about 32 μm or more if cuticle is expanded. Esophagus 400 μm long. Dorsal ray including its terminal branches at least 180 μm long. Spicules 240–280 μm long, distal third much more slender than remainder, middle third with a prominent curved ventrally-projecting edge corrugated on the inner surface. Female: 7.5–9 mm long, 110–135 μm wide close to the vulva. Head 35–50 μm wide, esophagus 360–400 μm long. Posterior body end gradually attenuated backward, terminating in a slender sharply pointed tail. Vulva opens 1.6–2 mm from the tip of the tail, its opening with projecting vesicular lips. Anus about 175 μm from the body end. Eggs 70–80 μm long and 36 μm wide." **[sic]** Moreover, Gibbons (16) provided figures of the dorsal ray of *C. pectinata* from impalas in Africa (original Figure 78), which measured 269 μm in total, and its proximal unbranched part represented 45% of its length.

Although deer and bovid *Cooperia* females do not exhibit significant differences, two male species-specific characteristics clearly distinguish these lineages: the morphology of the spicules and, to some extent, the shape of the dorsal ray of the male bursa (37).

First, spicular morphology differs between the *Cooperia* lineages of bovids and deer (though the length and width of the spicules are comparable between the two lineages). Both lineages have four morphologically distinctive parts, typical of the genus *Cooperia*: a short anterior head, a barrel neck, a noticeable central

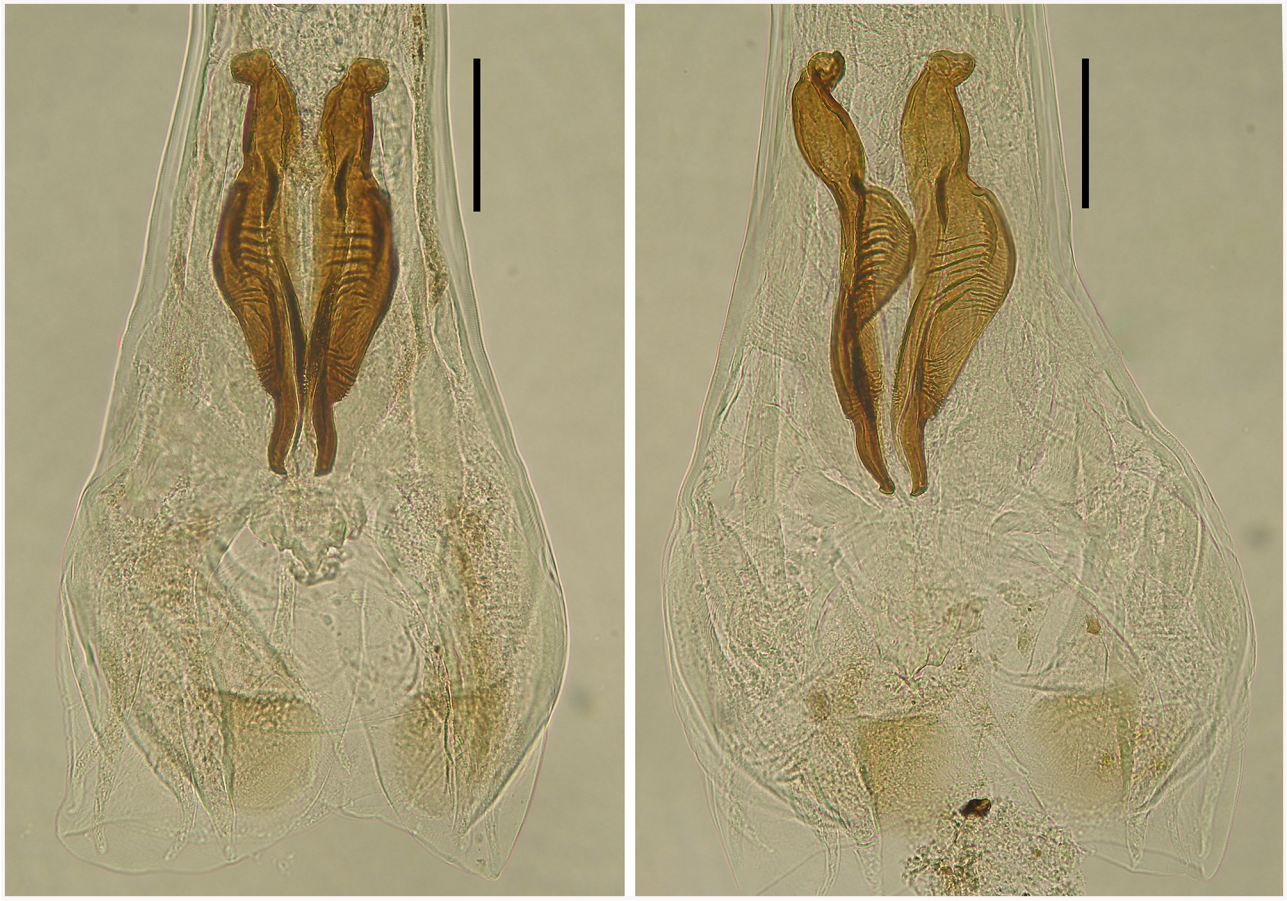

FIGURE 6
Spicules of two male deer *Cooperia* sp. in dorsal position **(left)** and lateral position **(right)**. Scale bar =100 μm.

edge with well-demarcated corrugations (a bulky belly), and a thin tail. The species-specific characteristic lies in the length of the distal thin tail part, which is significantly longer in bovid *C. pectinata* (one third of the total spicule length according to the original Figure 1, by Ransom (19) or Figure 2, left, by Baylis (21), while this thin tail part represents less than one fifth (∼14%) in deer *Cooperia* sp. (Figures 2, right, 5, 6).

Second, the dorsal ray of the male bursa differs slightly in shape. Its total length is similar between bovid and deer *Cooperia*, more than 180 μm in bovids according to Ransom (21) and 180–208 in deer (this work), and it is twice forked in its posterior half. However, the main bifurcation is located before half of its total length (∼45%) in bovid *C. pectinata* (16) but closer to the end of the bursa at 56–60% of the total ray length, in deer *Cooperia* sp. (Figure 5).

Nevertheless, these discrepancies have not prevented *Cooperia* from deer from being mistakenly identified as *C. pectinata* in various parts of Europe, such as the Czech Republic (38), most likely Austria (39, 40) Norway (41), and certainly New Zealand (42). This was mainly caused by the dubious species characteristics published in the key monograph by Skrjabin et al. (15), with the subsequent widespread acceptance of this erroneous information in the past (15, 22, 38) and more recently (39–42).

The overall morphological results clearly confirmed the results of our phylogenetic analysis. It is therefore certain that the *Cooperia* sp. parasitizing the Czech deer does not belong to the species *C. pectinata*.

4.1 History of deer *Cooperia* spp.

Our molecular and morphological characterization of the nematodes from Czech deer led to a review of historical information on deer nematodes. The first scientific description of a strongylid species from a deer, caught near Greifswald (Germany) was provided by Rudolphi (43), who used the name *Strongylus ventricosus*. Over the years, this ancient deer nematode has been reassigned twice (as *C. curticei* or *C. oncophora*) to the newer genus *Cooperia* Ransom, 1907. However, both suggestions are currently invalid (17).

Original description of *Strongylus ventricosus* Rudolphi, 1809 (translation from Latin, https://www.biodiversitylibrary.org/item/50353#page/5/mode/1up), [(43), p. 222]:

"*Strongylus ventricosus*, R."

"*Strongylus*: with a thin, winged head, a male blunt bursa behind, and a female tail awl-shaped.

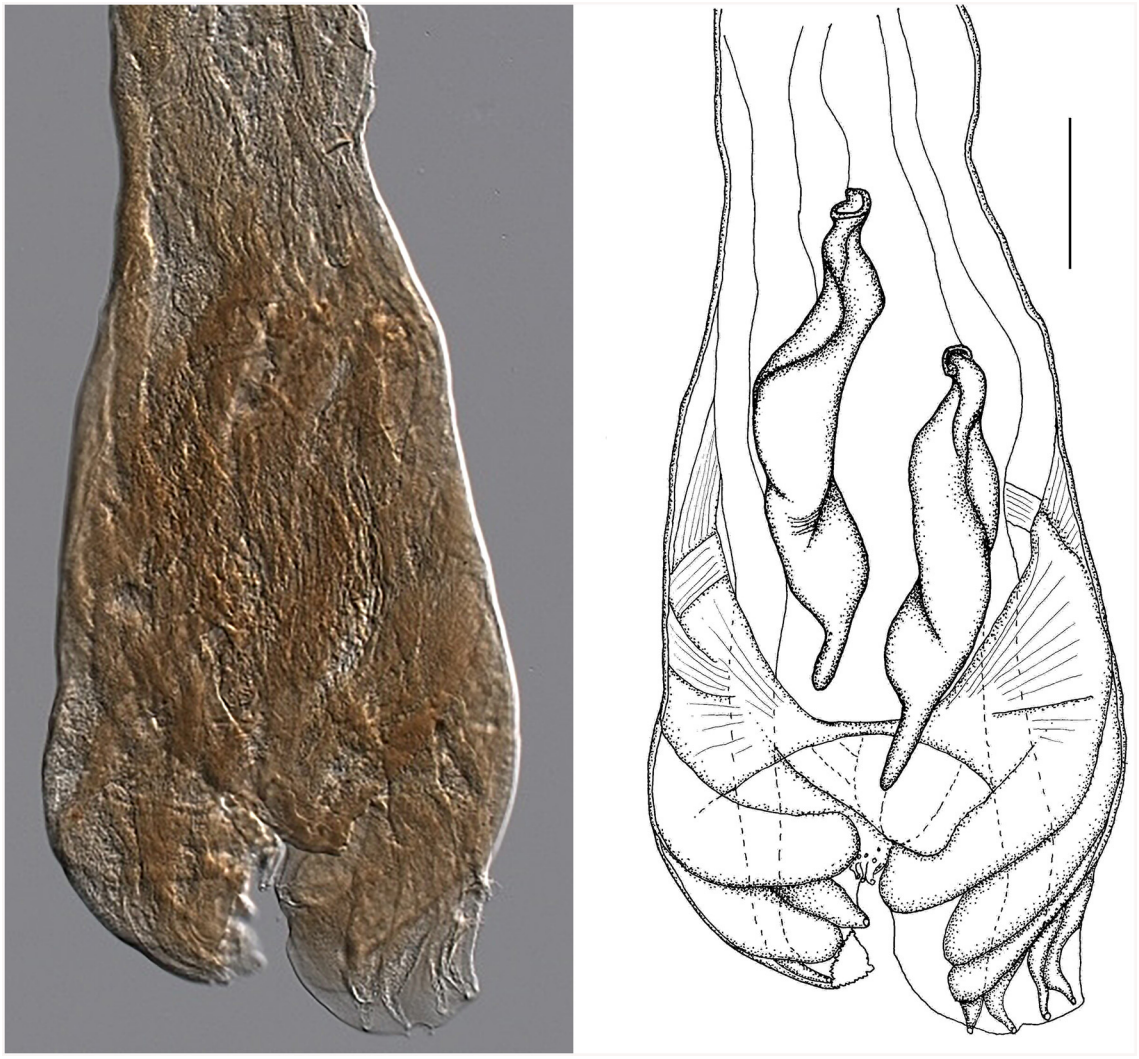

FIGURE 7
Male genital bursa of the type specimen *Strongylus ventricosus* Rudolphi, 1809 (current reconstruction) from the Museum für Naturkunde (Berlin). Drawing **(left)** and photomicrograph **(right)**. Scale bar = 50 μm.

Hab: four specimens found in the upper part of the intestines of the *Cervus elaphus*, February (1809, current note)

Description: worms six to eight "lines" long, very thin, reddish.

Male: the head is thin and winged by a thin membrane on both sides. The body is thin and almost linear toward the middle, and then it gradually thickens and forms the genital bursa at the end. This is obtuse, radiating, with thinly folded membranes, so that I cannot tell the number of lobes. A thin feeding tube, running through the middle of the body, gives the worm a striated face.

Female: the head as in male, but in another specimen the wing-shaped membrane is wider. The body is linear anteriorly, in the third part of the worm it is initially very thick, as if knotted, then thins again, the tail is awl-shaped. The vulva is partially protruding" [sic].

The type material of the species *S. ventricosus* Rudolphi, 1809, was deposited in the Museum für Naturkunde (Berlin) under no. AHC 49508. It is in the form of permanent slides made from five voucher specimens of deer trichostrongylid parasites (one male and four females). As a final step to clarify the species affiliation of *Cooperia* sp. from Czech deer, our new material was compared to the five voucher specimens of deer trichostrongylid parasites. Unfortunately, all five specimens are in poor condition, and only the male bursa is intact, although it is also considerably damaged (Figure 7).

Morphology of the bursa and spicules of the type specimen *Strongylus ventricosus* Rudolphi, 1809 (measurements in mm from Figure 7): bursa 306 wide, spicules 308 long on average, maximum spicule width 61, Each spicule has four parts: short head, two middle parts (barrel neck and bulky belly) which are difficult to distinguish, and clearly visible characteristic thin tail 51 long on average which represents 16.5% of the spicule length.

Although all structures are quite damaged, the size of the spicules matches well with *Cooperia* sp. males from Czech deer (in the Morphological description, subsection of the Results sections). Some of the slightly larger dimensions of the spicule parts were probably caused by flattening during the preparation of permanent slides of the museum material. The data from the original description of *S. ventricosus* also concur with the morphology of *Cooperia* sp. from Czech deer.

As there is a good agreement between our new material (*Cooperia* sp. from Czech deer) and the voucher specimens of the deer trichostrongylid parasite *S. ventricosus*, which were registered in the Museum für Naturkunde (Berlin), the transfer of the ancient species *S. ventricosus* Rudolphi, 1809, into the genus *Cooperia,* and the creation of the species *Cooperia ventricosa* comb. nov. should be seriously considered. The ideal solution to the problem would be to obtain new material for nematodes of the genus *Cooperia* from the type host *Cervus elaphus* from the type locality near Greifswald (Germany), and to redescribe *C. ventricosa* according to the stricter ICZN rules (25).

Taxonomic summary of *Cooperia ventricosa* (Rudolphi, 1809) comb. nov. (Figures 5–7) from deer game

Class Chromadoria (Order Rhabditina, Superfamily Strongyloidea, Family Trichostrongylidae, Tribe Cooperiinii, genus *Cooperia* Ransom, 1907) (44, 45).

***Cooperia ventricosa* (Rudolphi, 1809), comb. nov.**

Synonym: *Strongylus ventricosus* Rudolphi, 1809.

Type host: red deer *Cervus elaphus* Linnaeus, 1758 (Artiodactyla: Cervidae).

Other hosts: European fallow deer *Dama dama* (Linnaeus, 1758), sika deer *Cervus nippon* Temninck, 1838.

Site of infection: small intestine.

Type locality: vicinity of Greifswald, Germany (40).

***Documented distribution*:** various regions of Europe (38, 46), New Zealand (42), northern regions of the Czech Republic—new geographical record (this paper).

***Type material*:** Museum für Naturkunde Berlin, collection "Vermes," catalog Entozoa, E.258, 6 syntype fragments in deteriorated condition, mounted as 5 glycerol-paraffin slides on Cobb aluminum frames by B. Neuhaus on 16.XI.2021, E.258-1 female, E.258-3 female, E.258-5 male, sex of E.252-2, and E.252-4 unknown.

Morphological descriptions: (43), [(22)—Figure 142], [(15)—Figure 164], this paper

Remarks: A member of the genus *Cooperia* that shares all the morphological characteristics that define the genus *C. ventricosa,* differs from the most similar species *C. pectinata* based on the following features: the shape of the male spicules and that of the dorsal ray of the male genital bursa.

5 Conclusion

In conclusion, this integrative study of the ruminant parasite of the genus *Cooperia* (Nematoda, Trichostrongyloidea) revealed the existence of a separate species found in red and sika deer. According to the morphology, it was quite similar to, but not identical to, *Cooperia pectinata,* which was long erroneously considered to be a parasite of both bovids and deer game. A new comparative analysis of *cox*2 and ITS rDNA partial gene sequences from a spectrum of *Cooperia* spp. revealed that this nematode represents a separate lineage, morphologically nearly identical to the ancient deer nematode *Strongylus ventricosus* Rudolphi, 1809. We, therefore, suggest its resurrection as *Cooperia ventricosa* (Rudolphi, 1809) comb. nov., which should ideally be followed by verification by collecting and analyzing new nematode material from the type deer host and the type locality near Greifswald.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OR804235, OR804236, and OR804237.

Ethics statement

The animal studies were approved by Ethical Committee of Czech University of Life Sciences Prague. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because the game deer owners did not require written informed consent.

Author contributions

MA: Conceptualization, Data curation, Investigation, Writing – original draft. EK: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. IL: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing. VH: Data curation, Investigation, Resources, Supervision, Writing – review & editing. BN: Data curation, Methodology, Resources, Supervision, Validation, Writing – review & editing. IJ: Conceptualization, Investigation, Supervision, Writing – review & editing. MP: Methodology, Visualization, Writing – review & editing. JM: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. MŠ: Conceptualization, Data curation, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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.

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Pterygodermatites (Mesopectines) whartoni (Nematoda: Rictulariidae) encysted larvae in invasive Cuban treefrogs (*Osteopilus septentrionalis*) from Florida, United States

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Species of *Pterygodermatites* are spirurid nematodes that have expanded their geographic distribution worldwide. They infect a variety of mammalian definitive hosts with few reports of potential paratenic infections in amphibian and reptile hosts. In this study, we report *Pterygodermatites* sp. larvae identified in free-ranging, invasive Cuban treefrogs (*Osteopilus septentrionalis*), from central Florida, United States. Encysted larvae were recovered from the skeletal muscle and/or the coelomic cavity of three frogs; molecular characterization of the small subunit (18S) ribosomal RNA and cytochrome oxidase I genes of the parasites matched reported sequences of *Pterygodermatites (Mesopectines) whartoni* (Tubangui, 1931). This is a parasite native to Southeastern Asia and to the best of the authors' knowledge, it is the first report of the species in the New World. The recovery of invasive *Pterygodermatites* from invasive Cuban treefrogs in North America highlights the growing concern regarding the potential impact non-native parasites and invasive species may have on native wildlife populations.

KEYWORDS

paratenic, encysted larvae, amphibian host, nematode, helminth

1 Introduction

Species of *Pterygodermatites* are spirurid nematodes belonging to the family Rictulariidae. The adult worms inhabit the gastrointestinal tract of various mammals including rodents, cats, primates, and bats (1–5). Eggs are shed in the feces of the definitive host, which are ingested by an intermediate host, commonly an arthropod (3, 6). In the arthropod, larvae develop into an infective third stage (L3) and are transmitted to the definitive host following ingestion of the arthropod (3). There are few reports of this nematode in hosts other than mammals and arthropods (3). *Pterygodermatites* (*Multipectines*) *cahirensis* (Jägerskiöld, 1909) (syn. *Rictularia*

cahirensis) has been reported in a species of gecko (*Hemidactylus flaviviridis*) and frog (*Hoplobatrachus tigerinus,* syn. *Rana tigrina*) in Asia as encysted or encapsulated larvae (3, 7). These larvae successfully infected a cat, demonstrating the potential of reptile and amphibian paratenic hosts in the life cycles of these nematodes (7), and that completion of the life cycle required two hosts, one an invertebrate and the second a lizard or snake (4).

Species of *Pterygodermatites* have expanded their geographic distribution worldwide, facilitating interactions with novel definitive, intermediate and paratenic hosts (3). Currently, there are reports of *Pterygodermatites* infections in mice and rats from Florida (8, 9), but none in humans or other primates. While there are currently no reports of amphibian infections in Florida, anuran diet and ecology allow for interactions with known intermediate and definitive hosts. Moreover, the spectrum of potential host amphibians in Florida is ever growing due to the establishment of invasive species that can serve as hosts for both native and invasive parasites. For instance, the invasive Cuban treefrog (*Osteopilus septentrionalis*) is documented as a potential paratenic and intermediate host for invasive *Angiostrongylus cantonensis* (10) and *Raillietiella orientalis* (11) respectively, in Florida. Both of these parasites may pose a threat to native Florida wildlife.

There is limited information regarding the relationship between *Pterygodermatites* spp. and reptile and amphibian paratenic hosts. As many of the invertebrates known to harbor *Pterygodermatites* (7, 9, 12) can be found in the diet of Cuban treefrogs (13), there is the potential that they may serve as intermediate or paratenic hosts in Florida. Species that predate known hosts of *Pterygodermatites* spp., including free-roaming domestic cats, also may predate Cuban treefrogs (14). While many species of *Pterygodermatites* have been shown to infect captive wildlife species in zoos (2, 15), free-ranging predator–prey dynamics outside of the zoo environment could allow for infections of novel intermediate and paratenic hosts.

This report documents three cases of natural *Pterygodermatites (Mesopectines) whartoni* (Tubangui, 1931) infection in invasive Cuban treefrogs (*O. septentrionalis*) in central Florida. This report demonstrates (i) Cuban treefrogs can serve as potential paratenic hosts of *P. whartoni*, (ii) *P. whartoni* is not restricted to southeastern Asia, and (iii) the role invasive species can play in invasive parasite distribution and transmission.

2 Materials and methods

In June and July 2021, *O. septentrionalis* adults were collected from Hillsborough ($n = 2$ female, $n = 8$ male) and Orange ($n = 6$ female, $n = 7$ male) Counties in Florida, United States (Figure 1). Frogs were captured by hand from PVC-pipe refugia deployed in ornamental plant gardens at Hillsborough Community College (Plant City Campus) and the Orange County Extension office. This study was approved by the Institutional Animal Care and Use Committee at the University of Florida IACUC #202011222 and all frogs were collected and evaluated per approved protocols. Frogs were euthanized by immersion in buffered tricaine methanesulfonate (MS-222) prior to exposure of the coelomic cavity and submersed in 70% ethanol for preservation. Ethanol fixed liver, lung, hind leg muscle, and the full gastrointestinal tract tissues were removed and examined grossly and microscopically for parasites; samples of the brain, heart, liver, lung and hind leg skeletal muscle were processed for microscopic examination. Sections were cut at 5 µm, stained with hematoxylin and eosin (H&E), and examined histologically for the presence of parasites.

All nematodes were removed from tissue, transferred to 70% ethanol and examined microscopically for morphological identification. DNA was extracted from each nematode using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) per manufacturer's recommendations.

Portions of both the small subunit (18S) ribosomal RNA [using primers 18SF-342 and 18S-530R as reported in Thomas (16)] and the cytochrome oxidase I [using primers COIintF and COIintR as reported in Hamer et al. (17)] were amplified from extracted nucleic acids as previously described. PCR products were visualized by gel electrophoresis, and amplicons of the correct size (approximately 480 and 690 base pairs [bp], respectively) were excised and gel extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) per manufacturer's recommendations. Gel extracted amplicons were submitted for bidirectional commercial sequencing (Genewiz, Azenta Life Sciences). Sequences were assembled and edited using commercial bioinformatic software (Geneious 11.1.5; Biomatters, Auckland, New Zealand). The determined sequences were compared to known nematode sequences using the NCBI Basic Local Alignment Search Tool (18). Consensus 18S and COI gene sequences were uploaded to GenBank (OR861525 & OR861524, respectively).

3 Results

Multiple nematode larvae consistent with *Pterygodermatites* sp. were recovered encysted in the hind leg muscle of a male *O. septentrionalis* collected from Hillsborough County (Frog OS2021-59) and the leg muscle and adhered to the serosa outside of the stomach of two female *O. septentrionalis* collected from Orange County (Frogs OS2021-64, -65). The recovered nematodes were cleared with lactophenol and examined under a dissecting and compound microscope. The oval cysts contained a single, coiled larva and were approximately 285–288 µm in length (Figure 2A). Once removed from the cyst, the larva (Figure 2B) was approximately 580 µm in length and cuticular spines were visible from the anterior to midbody (Figure 2C); alae were also present. The tail tip had a distinct toothed or ridged end (Figure 2D).

No parasite was observed microscopically within the sections of hind leg skeletal muscle, heart, brain, liver, or lung of the three frogs noted to have nematode larvae. In two (OS2021-59 and -64) frogs, sections of nematodes were present within the liver, associated with a mild granulomatous reaction. In OS2021-59, nematode sections were characterized by a thick cuticle, coelomyarian-polymyarian musculature, prominent basophilic lateral cords, and portions of the intestinal tract lined by large, multinucleate enterocytes with abundant foamy cytoplasm and disorganized nuclei (data not shown). No sublateral alae were present. In OS2021-64, the nematodes in section were degenerate which precluded further characterization (data not shown).

Portions of the 18S rRNA and COI genes were amplified and sequenced directly from nematode larvae collected from the three Cuban tree frogs. After primer editing, a 447 bp fragment of the 18S gene was determined from each parasite. The nucleotide sequence was identical between the three parasites (Genbank OR861525), 100% identical to reported sequences of *P. whartoni* (MG489657,

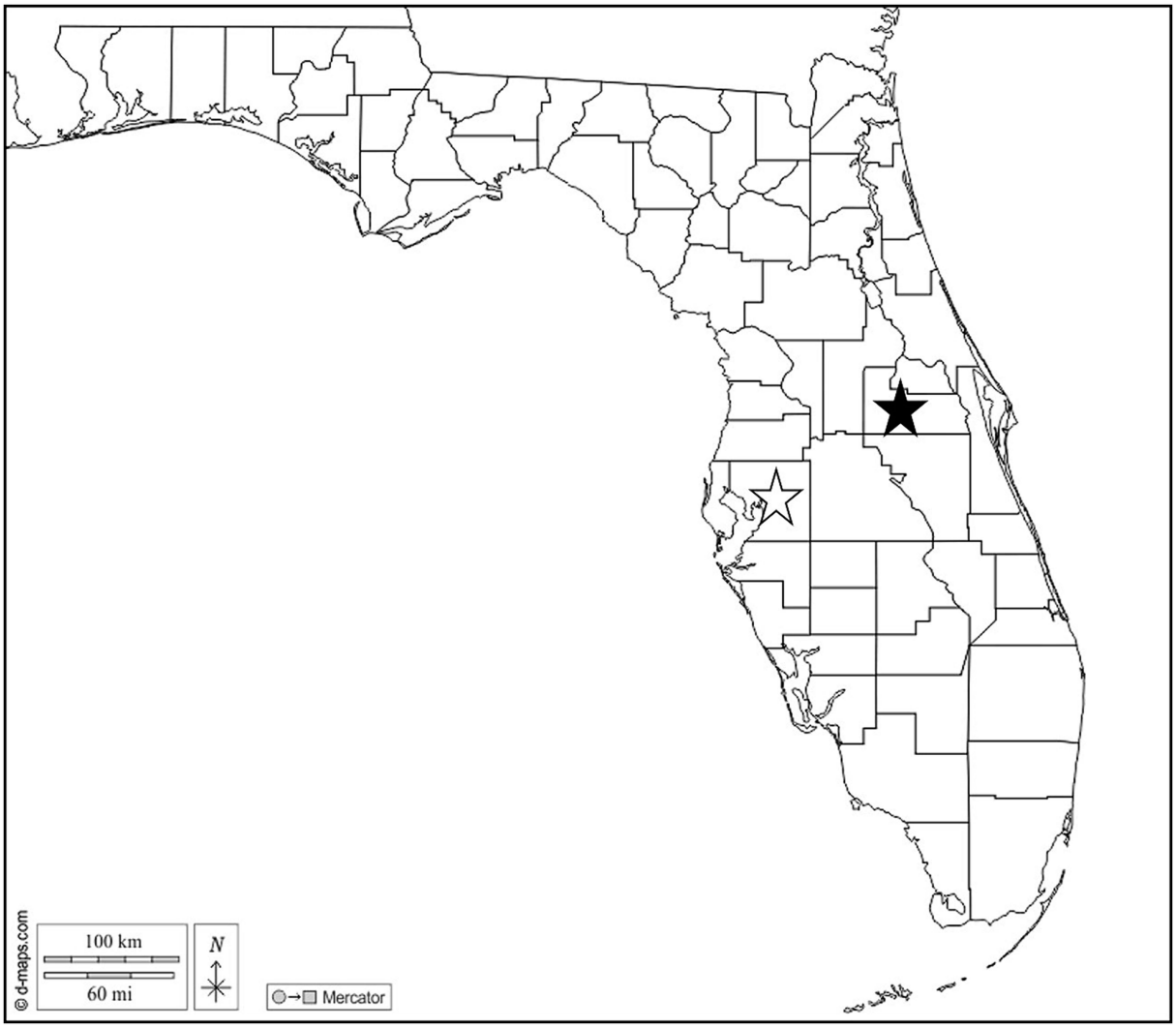

FIGURE 1
Map of Florida, US. Stars indicate Hillsborough (open) and Orange (solid) Counties where Cuban treefrogs were collected. Map courtesy of d-maps.com. https://d-maps.com/carte.php?num_car=20131&lang=en.

MG489658, MG489660), and 99.5% identical to *Pterygodermatites (Mesopectines) nycticebi* (Mönnig, 1920) (MG753548; Supplementary Table S1). For the COI gene, a 649 bp fragment was determined for each parasite; the nucleotide sequence between the three parasites was identical (Genbank OR861524). The parasite COI sequence exhibited 97.0 and 97.2% sequence identity to reference *P. whartoni* sequences (MZ476254 and MZ476257, respectively) and 88.8–89.1% identical to reference *P. nyctecebi* sequences (MZ476253, MG757149, and MZ476258; Supplementary Table S2).

4 Discussion

This report documents the presence of *Pterygodermatites* larvae in three invasive Cuban tree frogs (*Osteopilus septentrionalis*) in Florida. There are 68 species of *Pterygodermatites* described worldwide from mammalian hosts, with all known life cycles utilizing an arthropod intermediate host (3). No paratenic hosts have been described for *P. nycticebi* or *P. whartoni*; intermediate hosts have been suggested to be cockroaches or other arthropods (2, 3, 15). Confident determination of the species of *Pterygodermatites* in these frogs is limited by a general lack of molecular reference sequence for representatives of the genus. The NIH NCBI Nucleotide database contained only 28 *Pterygodermatites* spp. reference sequences at the time of analysis for this report, representing eight assigned and one unassigned species at three different loci: the cytochrome oxidase I ($n = 15$), small subunit (18S) rRNA ($n = 11$), and the large subunit rRNA ($n = 2$) gene. At the 18S gene, the larvae in these frogs were 100% identical to three sequences of *P. whartoni* reported from parasites collected from *Leopoldamys* giant rats in Viet Nam. However, at the COI gene, the frog parasites exhibited only 97.0–97.2% sequence identity to *P. whartoni* also from Viet Nam *Leopoldamys* giant rats. Unfortunately, no peer-reviewed publication is associated with the direct nucleotide submissions for *P. whartoni*, and no other genetic loci are published for additional comparison. When comparing reported COI sequences between different accessions of the same

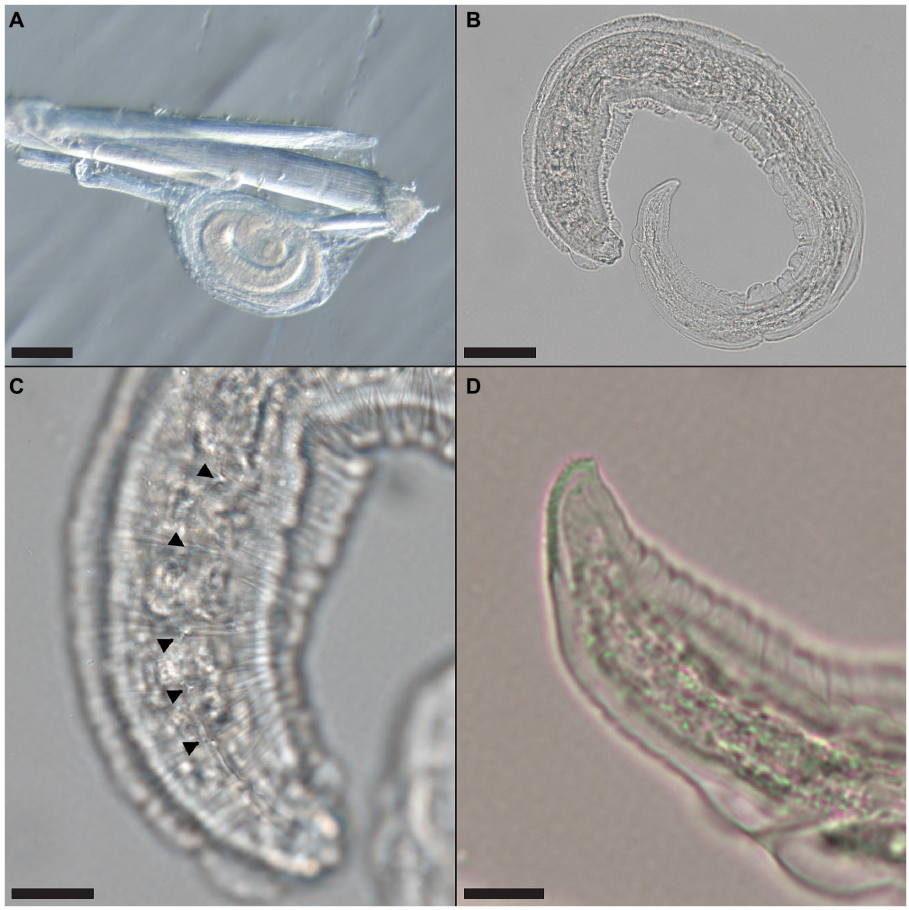

FIGURE 2
Pterygodermatites whartoni larvae recovered from free-ranging, invasive Cuban treefrog (*Osteopilus septentrionalis*) leg muscle. **(A)** Coiled larva attached to a muscle fiber; scale bar = 100 µm. **(B)** Larva removed from cyst capsule; scale bar = 50 µm. **(C)** Cuticular spines (arrowheads) along the cuticle of the larva, anterior end shown; scale bar = 20 µm. **(D)** Toothed or ridged posterior end of the larva; scale bar = 10 µm.

Pterygodermatites spp., differences of up to 3–4% can be seen (Supplementary Table S2; *Pterygodermatites (Paucipectines) jagerskioldi* (Lent & Freitas, 1935) [3.3%]; *Pterygodermatites (Paucipectines) zygodontomis* (Quentin, 1967) [2.8–3.5%]; *P. nycticebi* [1.4–3.9%]), though for the three published *P. whartoni* sequences, variation of only 0.5–0.9% is present. However, all of these sequences originate from the same research group studying *Leopoldamys* giant rats in Viet Nam, and as such may exhibit decreased sequence variation than parasites from more distant geographic locales. Given the 100% nucleotide identity at the 18S gene and COI nucleotide identity differences consistent with those observed for other *Pterygodermatites* species, the larvae found in these invasive frogs in Florida most likely represent *P. whartoni*.

Encysted larvae were identified within the limb skeletal muscle and the coelomic cavity of the frogs in this study. Microscopic examination of select tissues from the affected frogs did not reveal sections of identifiable *Pterygodermatites* spp. While two frogs had nematodes present in their livers, in one frog the degenerate nature of the parasites precluded identification; in the other frog, the features of the hepatic nematode, namely the absence of sublateral alae and the presence of multinucleate enterocytes in the intestinal tract, is inconsistent with previous reports of the histomorphology of *Pterygodermatites* (2). PCR was attempted on formalin fixed paraffin-embedded sections of the affected tissues, but either due to parasites being absent in deeper levels or limitations due to the limited parasite DNA in the extracts, molecular characterization of the hepatic parasites was not successful.

Pterygodermatites nycticebi and *P. whartoni* are found in primates and rodents, respectively (3). While primates infected with *P. nycticebi* have been documented with varying severity of disease that includes weakness, anemia and hypoproteinemia (15), little is known or documented regarding disease in rodents infected with *P. whartoni* (1, 19). *Pterygodermatites whartoni* has been recovered from rodents (*Rattus* spp., *Sundasciurus steerii juvencus*) in Taiwan, Japan and the Philippines (3, 19). Detection of encysted *P. whartoni* in Cuban treefrog tissues suggest that this amphibian is a potential paratenic host, much like the lizard *H. flaviviridis* and frog *H. tigerinus* have been described as paratenic hosts for *P. cahirensis* in cats (4, 7).

The Cuban treefrog is an invasive species in the US and has been documented throughout Florida and into the southeastern US (13). With a diet that includes a variety of insects (13), these frogs are well positioned to serve as paratenic hosts for species of *Pterygodermatites*. Cuban tree frogs are consumed by many species of snakes and birds, as well as free-roaming cats (13, 14). The dynamics of this life cycle and the use of human-associated paratenic hosts can help facilitate geographic spread and transmission of *Pterygodermatites* spp., as well as other pathogenic and potentially zoonotic parasites.

Additional research is needed to better characterize the larval stages of *Pterygodermatites* spp., and the hosts they utilize. The use of paratenic hosts allows the parasite to infect a wider variety of mammalian hosts, which may increase the likelihood of disease in new parasite–host interactions. Additionally, identifying *P. whartoni*, a parasite native to Asia, recovered from invasive Cuban treefrogs in North America highlights the growing concern regarding invasive parasites and their potential impact on native domestic and wildlife populations. As the movement of humans and animals continues to increase, introductions of invasive parasites will follow. Identifying potential health risks are important, as well as identifying ways to minimize transmission and limit the geographic spread of invasive parasites to safeguard vulnerable hosts.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/; OR861525-OR861524.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee at the University of Florida. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KL: Data curation, Investigation, Writing – original draft, Writing – review & editing. RO: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. EC: Data curation, Investigation, Writing – original draft, Writing – review & editing. NT: Data curation, Supervision, Writing – original draft, Writing – review & editing. TF: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. TW: Formal analysis, Writing – original draft, Writing – review & editing. SJ: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. HW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1353975/full#supplementary-material

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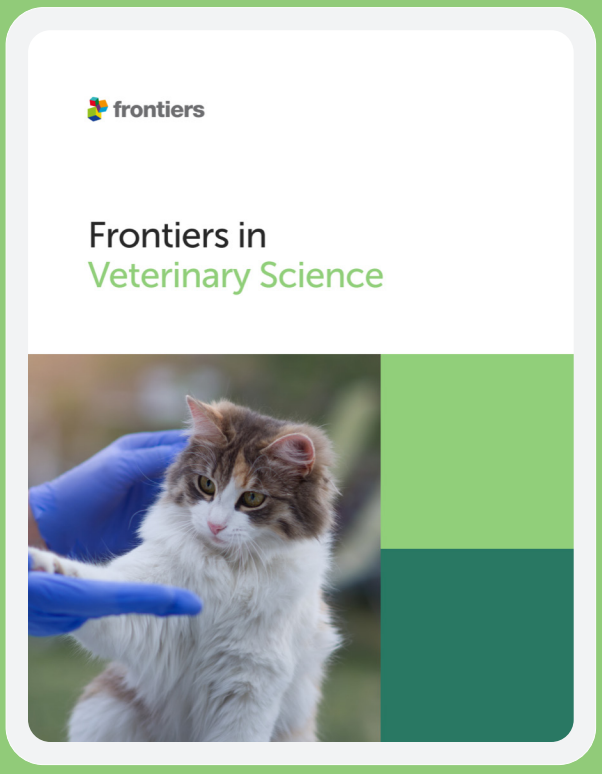

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