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New Host Record of *Amblyomma pakhtunensis* on the Indian Pangolin (*Manis crassicaudata*) with detection of a distinct *Borrelia* Lineage

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Abstract

Pangolins are mammals of the order Pholidota, with most species listed as endangered or critically endangered. They are distributed in both Asia and Africa, with the Indian pangolin (*Manis crassicaudata*) found in South Asia, including Pakistan. The tick genus *Amblyomma*, in the family Ixodidae, is widely distributed in tropical and subtropical regions and infests a variety of terrestrial and semi terrestrial vertebrates. The bacterial genus *Borrelia* is Gram-negative and consists of three main groups, one of which is mainly linked to reptiles, some birds, and mammals. This study focuses on investigating the Indian pangolin as a potential host for *Amblyomma pakhtunensis*, a recently described tick species previously reported only on monitor lizards and with no known microbial associations. It also aims to determine whether ticks from pangolins contain *Borrelia* species and assesses their phylogenetic relationships. Ticks collected from a pangolin were morphologically identified as *A*. *pakhtunensis*. DNA from a subset of ticks was extracted to confirm their molecular identity and to detect *Borrelia* spp. in these ticks using conventional PCR. For the former, a partial fragment of tick 16S rRNA was targeted, while for the latter, partial fragments of the *Borrelia* 16S rRNA, *flaB*, and *glpQ* genes were targeted for amplification. A single live pangolin, conventionally identified as *M*. *crassicaudata*, was found. Twenty-seven ticks were collected (16 males, 11 females), all morphologically identified as *A*. *pakhtunensis*. BLAST analysis of *A*. *pakhtunensis* 16S rDNA sequence showed 99.75% identity with the same tick species. BLAST analysis of *Borrelia* 16S rDNA and *flaB* sequences revealed highest identities of 99.25% and 95.91%, respectively, with *Candidatus* Borrelia javanense. The overall *Borrelia* infection rate was 50% (5/10), with infections detected in 4/5 female ticks and 1/5 male tick. In both 16S rDNA and *flaB* based phylogenetic analyses, the detected *Borrelia* species clustered with *Candidatus* B. javanense from China. In addition to expanding the vertebrate host diversity of *A*. *pakhtunensis*, this study also expands knowledge of the third group of *Borrelia* by extending their known vertebrate host range and genetic diversity. This study can enhance our understanding of the regional epidemiology of tick-borne diseases and may specifically aid pangolin conservation strategies by taking into account the control of ticks and their associated pathogens.

Keywords: Pangolin, Tick, *Amblyomma pakhtunensis*, *Borrelia*, Phylogeny, Pakistan

Introduction

Pangolins (class Mammalia and order Pholidota) include eight extant species, the majority of which are endangered or critically endangered (Gaudin et al., 2009; IUCN, 2019). They are distributed in both Asia and Africa, with Indian pangolin (*Manis crassicaudata*) found in South Asia, including Pakistan (Gaudin et al., 2009; Waseem et al., 2020). Some of their unique features are their external covering made of overlapping scales and highly modified feeding apparatus (Nowak, 1999). Pangolins help increase soil fertility through burrowing and turnover of organic matter, and they also contribute to pest's control by consuming them (Chao et al., 2020; Sun et al., 2025). Six of their species, including *M. crassicaudata*, are known to host 20 tick species from 8 genera, including *Amblyomma* (Mohapatra et al., 2016).

Amblyomma Koch 1844, which comprises approximately 140 species in nine subgenera, is the third most diverse genus of Ixodidae family (Guglielmone et al., 2023; Barker and Barker, 2023; Uribe et al., 2024). Long anterior mouthparts and pale patches on the scutum are their main features (Labruna et al., 2009; Khan et al., 2022; Barker and Barker, 2023). With their main distribution across much of the tropics and subtropics, they infest all classes of terrestrial and semiterrestrial vertebrates (Guglielmone et al., 2014). As three-host ticks, immature stages of *Amblyomma* often parasitize small wild animals, whereas their adults tend to prefer medium to large wild and domestic animals, as well as humans (Guzmán Cornejo et al., 2011; Eisen, 2022; Guglielmone et al., 2023). They can harm their hosts directly (e.g., through skin damage and anemia) and indirectly (e.g., through the transmission of pathogens) (Eisen, 2022; Dupuis et al., 2023; Molaei et al., 2024; Alasmari et al., 2025). One of nine subgenera of *Amblyomma* is *Adenopleura*, which includes around 16 species (Camicas et al., 1998; Barker and Barker, 2023; Ali et al., 2024). With few exceptions, such as *Amblyomma javanense*, which is mainly associated with pangolin, they are generally considered reptile specific (Duan et al., 2020). Within this subgenus, *Amblyomma pakhtunensis*, closely related to *A. javanense*, which mainly infests pangolin, was recently described from Pakistan on monitor lizards based on morphology and mt-genome (Ali et al., 2024).

Borrelia is a genus of *Gram*-negative bacteria that include 43 known species, along with many others that are potentially new and not yet described (Dworkin et al., 2008; Gofton, et al., 2025; Enferadi et al., 2025; Rahman et al., 2026). Of these, 22 species belong to the relapsing fever group (RF), 20 species to the Lyme disease group (LD), and one species to a recently described third group, which so far mainly includes *Borrelia* from reptiles, some birds, and mammals (Takano et al., 2010; Panetta et al., 2017; Gupta et al., 2019; Fesler et al., 2020; Binetruy et al., 2020; Kaenkan et al., 2020; Margos et al., 2022; Gofton et al., 2023; Gofton et al., 2025). Owing a considerable diversity in the genus *Borrelia*, some studies have suggested splitting into additional groups, but this is yet controversial (Adeolu and Gupta, 2014; Margos et al., 2017; Estrada-Peña and Cabezas-Cruz, 2019; Barbour and Gupta, 2021). They can move back and forth between arthropod vectors, including *Amblyomma* ticks and vertebrates like pangolins (Dworkin et al., 2008; Takano et al., 2010; Sprong et al., 2018: Jiang et al., 2021).

As vectors, *Amblyomma* ticks have well established connection with several tick-borne pathogens, including *Borrelia* from all three major groups (Eisen, 2022; Tang et al., 2024; Gofton et al., 2025). Despite this, they have been neglected in South Asia, likely due to their main association with wild animals, which are not considered economically important. Given that *A. pakhtunensis* is closely related to *A. javanense* (Ali et al., 2024), which mainly infest pangolins across its geographical region (Duan et al., 2020), this study aimed to investigate the pangolin as a possible host for *A. pakhtunensis* in Pakistan. In addition, it seeks to determine whether *Borrelia* species are present in *A. pakhtunensis* and to provide insight into its phylogenetic position.

Materials and Methods

Ethical Considerations and Study Area

The Advanced Studies and Research Board (ASRB) Sub Committee-1 Faculty of life and environmental sciences (FLES) University of Peshawar granted approval for this study. Additionally, and all methods were carried out in accordance with the relevant guidelines and regulations. The present study was performed in the District Swabi (34◦7′26.76″ N, 72◦ 27′ 40.68″ E), Province Khyber Pakhtunkhwa (KP), Pakistan. The map for this study was designed using ArcGIS Desktop v 10.3.1 (ESRI, Redlands, CA, United States) (https://www.esri.com/en-us/arcgis/products/arcgis-desktop/overview) (Figure 1).

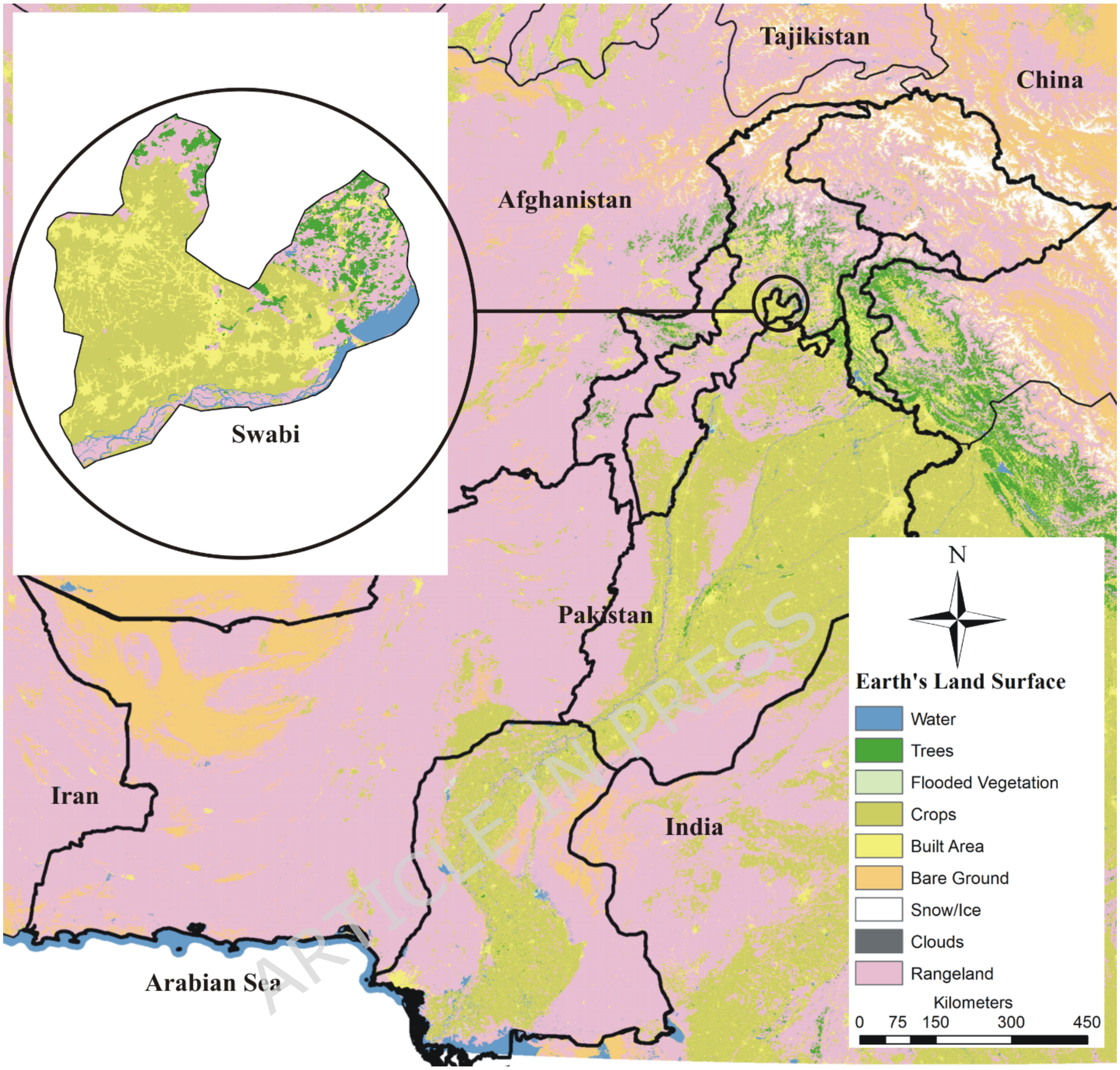

Figure1. Map showing land use and land cover of the study area, created using ArcGIS Desktop v 10.3.1 (ESRI, Redlands, CA, United States) (https://www.esri.com/en-us/arcgis/products/arcgis-desktop/overview). The district where the pangolin host was examined for ticks is highlighted with a circle.

Tick Collection and Identification

On November 07, 2023, the Wildlife Department recovered an Indian Pangolin from poachers in the Swabi subdivision of KP province, Pakistan. The animal was examined and ticks were collected carefully using sterile forceps, washed with distilled water and absolute ethanol. The animal was released by Wildlife officials into the wild on November 10, 2023 by the Wildlife department after necessary procedures. Herein, the specific locality of capture and release is not mentioned due to the potential misuse of this information by poachers. Building on previous studies on *Amblyomma* species in the region, including Khan et al. (2022) and Ali et al. (2020; 2024) from Pakistan, ticks were subjected to morphological identification through stereo-zoom microscope (Luxeo 6Z, LABOMED, USA). For future molecular confirmation and detection of microorganisms, the collected ticks were stored in 70% ethanol.

DNA Extraction, PCR, and Sequencing

Overall, ten unengorged adult ticks, comprising 5 females and 5 males, were subjected to DNA extraction. Using a sterile scissor, each tick was individually powdered in 1.5 mL microtube. The phenol-chloroform method (Sambrook et al., 1989), was used for DNA extraction. Conventional PCR (model GE-96G, BIOER, Hangzhou, China) was performed on genomic DNA samples to target partial fragments of the tick 16S rRNA gene, as well as the associated borrelial 16S rRNA, *glpQ*, and *flaB* genes (Table 1). Tick and borrelia 16S rRNA, *Borrelia flaB* (first round), and *glpQ* PCRs were carried out in 20 µL reaction mixture containing 2µL of DNA (~ 50 ng/µL), 1µL of each primer (10 pmol/µL), 4µL of nuclease-free PCR water, and 12µL of DreamTaq green MasterMix (2X) (Thermo Scientific,Waltham, MA, USA). The second-round *flaB* PCR used 1µL of the first-round *flaB* PCR product as template, with 4.5µL of PCR water. All PCRs were conducted under experimental conditions previously described; Stromdahl et al. (2003), Halperin et al. (2006), Radulović et al. (2010), Khan et al. (2023a), Zahid et al. (2023) for *Borrelia*, and Mangold et al. (1998) and Ullah et al. (2024) for ticks. Nuclease-free PCR water was used the negative control, while DNA of *Borrelia theileri* (for *Borrelia*) and *Amblyomma gervaisi* (for *A. pakhtunensis*) were used as positive controls. Amplicons were separated on a 2% agarose gel and visualized using a Gel Documentation system (BioDoc-It™ Imaging Systems, Upland, CA, USA). The PCR products were sequenced in both directions using Sanger method on ABI 373XL system (Macrogen, Inc., Seoul, South Korea).

Table 1. Primers for amplifying partial fragments of 16S rRNA, *flaB*, and *glpQ* genes of *Borrelia* species via conventional PCR.

Species	Gene	Primer Name	Primer sequence (5′ to 3′)	Amplicon size (bp)	Reference
Tick	16S rRNA	16S+1	CCGGTCTGAACTCAGATCAAGT	460 bp	Mangol et al. (1998) & Ullah et al. (2024)
		16S□1	GCTCAATGATTTTTTAAATTGCTG		
Borrelia	16S rRNA	Forward	GGCTTAGAACTAACGCTGGCAGTGC	552	Radulović et al. (2010)
		Reverse	CCCTTTACGCCCAATAATCCCGA		
	flaB	Fla LL	ACATATTCAGATGCAGACAGAGGT	665	Stromdahl et al. (2003)
		Fla RL	GCAATCATAGCCATTGCAGATTGT		
		Fla LS*	AACAGCTGAAGAGCTTGGAATG	354	Stromdahl et al. (2003)
		Fla RS*	CTTTGATCACTTATCATTCTAATAGC		
	glpQ	128f	CAGAACATACCTTAGAAGCTCAAGC	212	Halperin et al. (2006)
		340r	GTGATTTGATTTCTGCTAATGTG		

*used in a nested reaction

Sequence and Phylogenetic Analyses

To remove impurities and identify potential consensus sequences, the obtained sequences of partial fragments of tick 16S rRNA, *Borrelia* 16S rRNA and Borrelia *flaB* were trimmed through SeqMan (DNASTAR, Inc., Madison, WI, USA). Cleaned and consensus sequences for each partial fragment were then submitted to BLAST (Altschul et al. 1990) at NCBI. However, only *Borrelia* sequences were subjected to phylogenetic analysis, as a comprehensive phylogeny of the tick had been conducted recently (Ali et al., 2024). In addition to the top identical sequences, lower-identity sequences belonging to other *Borrelia* groups were also downloaded. All these sequences, along with those from the current study, were compiled into a single file. Each file was individually subjected to alignment in Bio-Edit V.7.0.5, USA (Hallet al. 2011) using Clustal W multiple alignment method (Thompson et al. 1994). Phylogenetic trees were then constructed in MEGA 12 using the Neighbor Joining (NJ) approach, with 1,000 bootstrap replicates and the Kimura 2 model (Kumar et al., 2024). The analyses were also repeated using the Maximum Likelihood (ML) approach with same model and bootstrap setting to assess the consistency of tree topology.

Search of Literature

Published studies on the detection of third group of *Borrelia* in ticks were searched through electronic databases, including Scopus, ScienceDirect, Web of Science, and PubMed. Manual searches were also conducted on other academic search engines, including Google Scholar and by screening the reference list of relevant studies and reviews. Various search terms, such as “tick”, “*Borrelia*”, “reptile-associated *Borrelia*”, “monotremes-associated *Borrelia*”, and “bird-associated *Borrelia*”, were used. The search was started on 05 December 2024 and finalized on 14 May 2025.

Results

Tick and Host Description

A single pangolin, conventionally identified as *Manis crassicaudata*, was found. A total of 27 ticks were collected, all of which were morphologically identified as *A. pakhtunensis*, comprising 16 males and 11 females. Genomic DNA was successfully extracted from all ten ticks selected for the process.

Sequence Analysis and Detection of *Borrelia* Species

Ticks were molecularly confirmed through successful amplification of partial fragments of 16S rRNA, with sequence showing 99.75% identity to *A*. *pakhtunensis* (sequences available in GenBank as *Amblyomma pakhtunense* to match the neuter gender of *Amblyomma*). Initially, both partial fragments of borrelial 16S rRNA and the longer *flaB* were successfully amplified in 5 ticks. In total, 20 sequences were obtained, 10 for 16S rDNA and 10 *flaB,* each including a forward and a reverse sequence per positive tick sample. Within each set, all sequences were identical, which resulted in two consensus sequences: 534 bp (16S rDNA) and 579 (*flaB*). In addition to the long *flaB* sequences, 10 shorter *flaB* sequences were obtained from the corresponding positive long *flaB* PCR products. Attempts to amplify the *glpQ* gene were unsuccessful for all samples. By BLAST analysis of 16S rDNA and *flaB*, the detected *Borrelia* species showed the highest identities of 99.25% and 95.91%, respectively, with *Candidatus* B. javanense. Other top matches based on 16S rDNA showed 97-98% identities with an array of *Borrelia* from relapsing fever group (e.g., *Borrelia crocidurae* and *Borrelia recurrentis*) and third group (e.g., *Candidatus* Borrelia tachyglossi associated with monotremes). Based on *flaB*, other top matches revealed 94-95% identities with an array of *Borrelia* from the third group (e.g., *Borrelia* species associated with reptiles and monotremes). With 4/5 female and 1/5 male ticks infected, a total infection rate of 50% (5/10) was recorded. Additionally, the overlapping sections of the short and long *flaB* sequences were identical, which supported the BLAST results of each other. However, only the long sequences were selected for constructing phylogenetic trees. Given the recent description of *A*. *pakhtunensis*, which includes mitogenome-based phylogenetic analysis, the tick 16S rDNA sequence was not analyzed phylogenetically in this study. The sequences are available in GenBank with accession numbers PX387109 for tick 16S rDNA, PX387536 for borrelial 16S rDNA and PX394402 for borrelial *flaB*.

Third-group of *Borrelia* in Ticks

Apart from the present study, eight *Borrelia* species (including those with *Candidatus* status) from the third group, along with various undetermined *Borrelia* species, have been reported in 19 different tick species worldwide (Table 2).

Table 2. *Borrelia* species of the third group, with their tick and vertebrate hosts and location details.

Borrelia species	Tick Host	Vertebrate Host of Tick	Country of Origin	Reference
Borrelia turcica	*Hyalomma aegyptium*	Tortoises (*Testudo graeca*)	Turkey	Guner et al., 2003
	Hyalomma aegyptium	Tortoise (*Testudo horsfieldii* or *Testudo graeca*)	Jordan	Takano et al., 2010
	Hyalomma aegyptium	Tortoise (*Testudo graeca*)	Romania	Kalmar et al., 2015
	Hyalomma aegyptium	Tortoise (*Testudo graeca*)	Greece and Turkey	Hepner et al., 2020
	Hyalomma aegyptium	Tortoise (*Testudo graecanabeulensis*)	Algeria	Benyahia et al., 2022
	Ixodes ricinus	Environment	Poland	Wodecka and Kolomiiets 2023
	Hyalomma aegyptium	Tortoises (*Testudo graeca*)	Morocco	Norte et al., 2024
	Hyalomma aegyptium	Tortoise (*Testudo graeca*)	Algeria, Tunisia, Turkey, Georgia and Iran	Kautman et al., 2025
	Hyalomma aegyptium	Tortoises (*Testudo graeca*)	Iran	Enferadi et al.,2025
Borrelia salvatorii	*Amblyomma varanense*	Lizard (*Varanus salvator*)	Indonesia	Gofton et al., 2023
Candidatus Borrelia javanense	*Amblyomma javanense*	Pangolins (*Manis javanica*)	China	Jiang et al., 2021
Candidatus Borrelia mahuryensis	*Amblyomma longirostre*	Bird *(Xenops minutus*, *Dendroplex picus*, *Ramphocelus carbo*, *Pipra aureola*, *Glyphorynchus spirurus*, *Percnostola rufifrons*, *Chiroxiphiapareola*, *Turdus leucomelas)*	French Guiana	Binetruy et al., 2020
	Amblyomma geayi	Bird (*Chiroxiphia pareola, Turdus leucomelas, Percnostolarufifrons, Glyphorynchus spirurus)*	French Guiana	Binetruy et al., 2020
Candidatus Borrelia rubricentralis	*Amblyomma calabyi*	Lizard (*Varanus giganteus*)	Australia	Gofton et al., 2023
Candidatus Borrelia *tachyglossi*	*Bothriocroton concolor*	Echidna (*Tachyglossus aculeatus*)	Australia	Loh et al., 2017
Candidatus Borrelia undatumii	*Bothriocroton undatum*	Lizard (*Varanus varius*)	Australia	Gofton et al., 2023
Borrelia spp.	*Amblyomma trimaculatum*	Snake (*Boiga forsteni*)	Sri Lanka	Takano et al., 2010
	Amblyomma sparsum	Tortoise (*Geochelone pardalis*)	N/A	Takano et al., 2010
	Amblyomma latum	Snake (*Python regius*)	Ghana	Takano et al., 2010
	Amblyomma transversale	Snake (*Python regius*)	N/A	Takano et al., 2010
	Amblyomma geoemydae	Turtle (*Cuora flavomarginata orMauremys mutica*)	Japan	Takano et al., 2011
	Ixodes holocyclus	Echidna *(Tachyglossus* sp.*)*	Australia	Gofton et al., 2015

	Bothriocroton undatum	Lizard (*Varanus varius*)	Australia	Panetta et al., 2017
	Amblyomma varanense	Lizard (*Varanus bengalensis* and *Varanus salvator*), Snake (*Python bivittatus*)	Thailand	Kaenkan et al., 2020
	Amblyomma geoemydae	Tortoise (*Indotestudo elongata*)	Thailand	Kaenkan et al., 2020
	Amblyomma helvolum	Lizard (*Varanus salvator*)	Thailand	Kaenkan et al., 2020
	Amblyomma dissimile	Toad (*Rhinella horribilis*)	Mexico	Colunga‐Salas et al., 2021
	Amblyomma gervaisi	Lizard (*Varanus bengalensis*)	Pakistan	Khan et al., 2022
	Amblyomma sparsum	Tortoise (*Geochelone sulcata*)	Indonesia	Sophia et al., 2023
	Amblyomma helvolum	Reticulated python (*Malayopython reticulatus*)	Indonesia	Supriyono et al., 2025
	Amblyomma pakhtunensis	Pangolin (*Manis crassicaudata*)	Pakistan	This study

Phylogenetic Analysis

NJ and ML approaches produced identical major groupings. Therefore, only the NJ phylogenetic trees are presented in the main figures (Figures 2 and 3), while the ML trees are provided in the supplementary material (Supplementary Figures S1 and S2). Based on 16S rDNA phylogeny, the *Borrelia* species detected in *A. pakhtunensis* in the current study clustered with *Candidatus* B. javanense from China (Figure 2). This monophyletic clade, associated with pangolin as vertebrate hosts, was found to be sister to the monophyletic clade of RF *Borrelia*. Together, these groups formed a clade that was sister to the monotreme-associated clade. In the *flaB*-based phylogeny, *Borrelia* species detected in *A. pakhtunensis* in the present study clustered with *Candidatus* B. javanense from China (Figure 3). This monophyletic clade, associated with pangolin as vertebrate hosts, found to be sister to *Borrelia* of unknown hosts from Brazil and China. Together, these groups formed a clade that was sister to the reptile-associated and monotreme-associated clade.

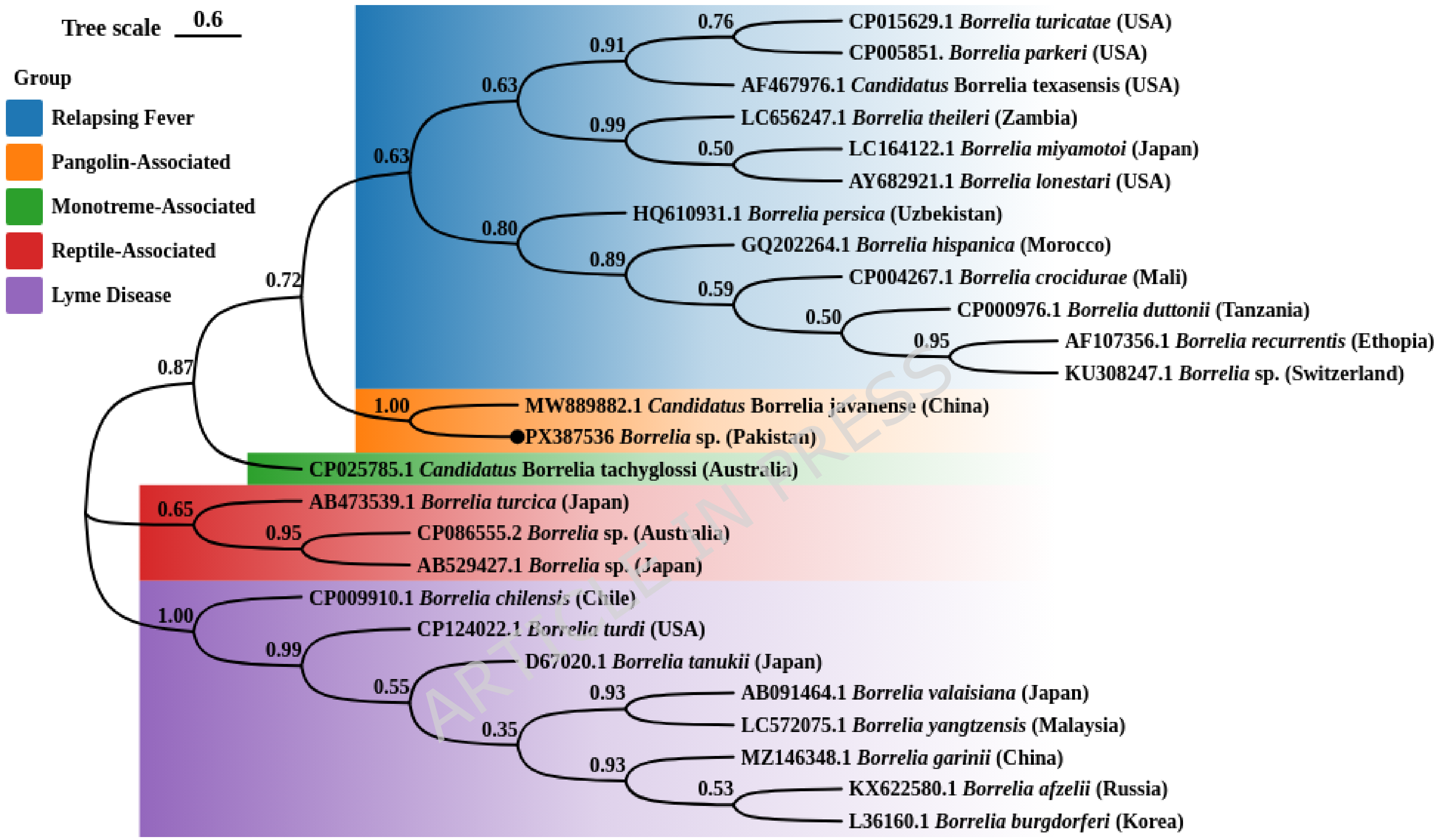

Figure 2. Neighbor-Joining phylogenetic tree of *Borrelia* species based on 16S rDNA sequences. GenBank accession numbers, species names, and geographic origins are shown for all sequences. The *Borrelia* species detected in this study is marked with a black circle. Numbers at the nodes indicate bootstrap support values based on 1,000 replicates.

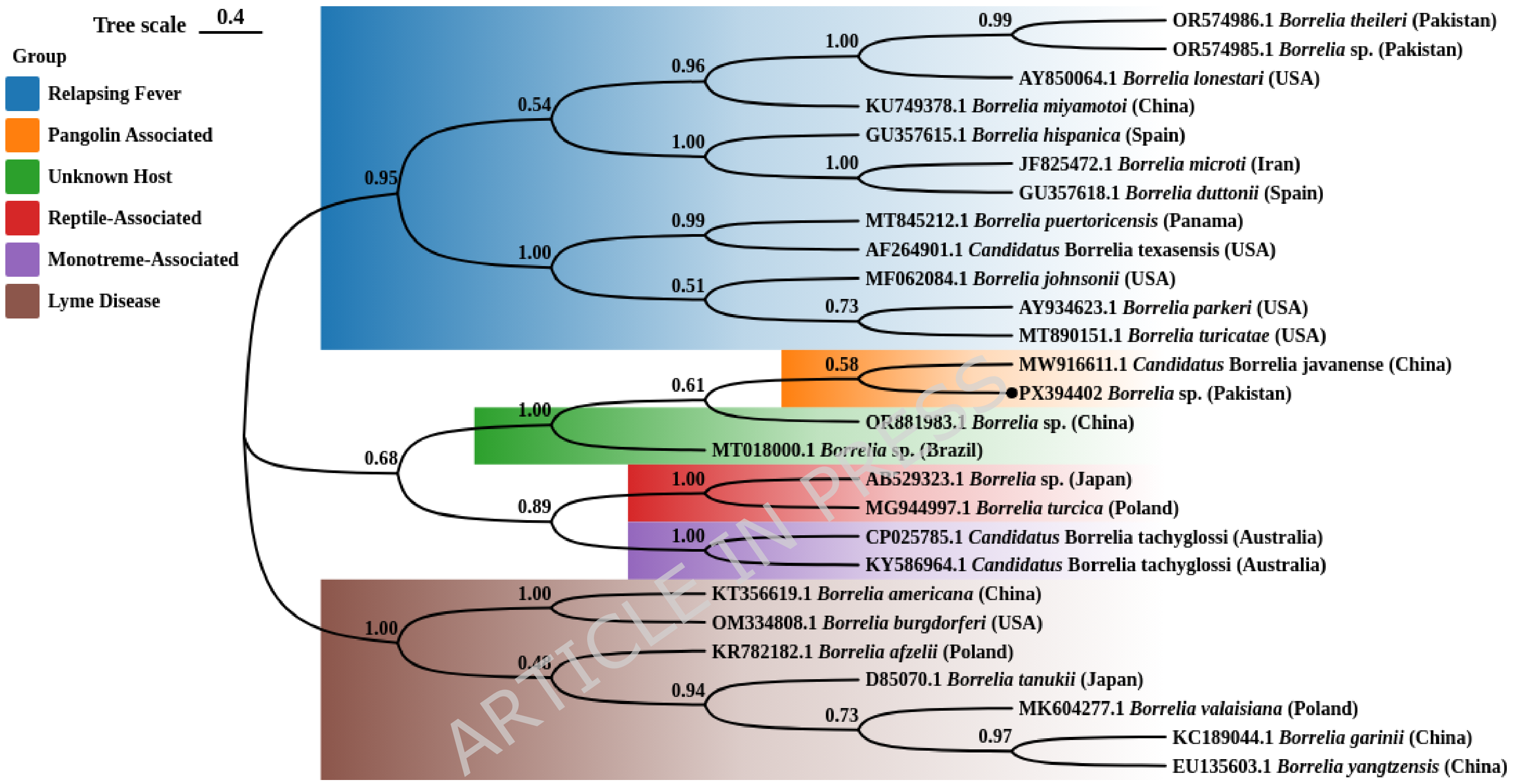

Figure 3. Neighbor-Joining phylogenetic tree of *Borrelia* species based on *flaB* rDNA sequences. GenBank accession numbers, species names, and geographic origins are shown for all sequences. The *Borrelia* species detected in this study is marked with a black circle. Numbers at the nodes indicate bootstrap support values based on 1,000 replicates.

Discussion

As *Amblyomma* ticks are efficient parasites and vectors, neglecting their impacts can adversely affect the health of wildlife, domestic animals, and humans. This study extends our earlier work on *Amblyomma* ticks and associated *Borrelia* species in the region (Khan et□al., 2022; 2023a; Ali et□al., 2024) by providing the first evidence of a *Borrelia* species in *A. pakhtunensis*. It also reports the pangolin as a newly recognized host for this tick and sheds light on the phylogenetic position of the *Borrelia* species associated with pangolin-associated ticks.

Pangolins have suffered a significant population decline, driven primarily by climate change and illegal wildlife trade (Waseem et al., 2020; Ahmad et al., 2025). A pangolin rescued by Wild life department from poachers indicates the occurrence of such activity in the study area. Moreover, various misconceptions among the local people, including the belief that they dig up graves and eat away dead bodies, are other possible reasons for their killing (WWF-Pakistan; Mahmood et al., 2012; Waseem et al., 2020). In fact, they feed on insects (eating around 70 million ants and termites per year) (Wang et al., 2022; Born Free USA Foundation, 2025), which is why they live near old trees close to graveyards.

Various tick species, including *A. javanense* as the primary one in Asia, have been reported on pangolins across their distribution (Mohapatra et al., 2016; Guglielmone et al, 2014). This study confirms the presence of another tick species, *A. pakhtunensis*, parasitizing pangolins. This association had previously been suggested only from ecological and phylogenetic considerations when the species was originally described from a monitor lizard (Ali et al., 2024). However, based on our decade's field observation for various ticks in Pakistan, this species has been found rarely, especially on hosts other than pangolins. This rarity is likely suggesting a potentially co-endangered status of this tick species in the region. No larval and nymphal ticks were collected, which is likely due to three host life cycle of *A. pakhtunensis*, similar to its closest *A. javanense* (Hassan et al., 2013; Apanaskevich and Oliver, 2014). In such life cycles, immature stages may feed on different hosts and/or occur in different seasons than adults.

Currently, the systematics and phylogeny of *Borrelia* species are predominantly based on molecular data, with 16S rRNA and *flaB* among the most widely used markers (Guner et al., 2004; Fingerle et al., 2016; Colunga□Salas et al., 2021). Although 16S rRNA and *flaB* were successfully amplified in this study, *glpQ* could not be, which may be due to various reasons, including primer mismatches or to the gene being absent or highly divergent in pangolin-associated *Borrelia* (Green et al., 2015; Ali et al., 2022; Khan et al., 2023b). Phylogenetic trees constructed based on these partial fragments displayed pangolin-associated as unique evolutionary lineage in between RF and LD. Based on the 16S rDNA, it clustered with RF borreliae in a monophyletic clade, whereas in the *flaB* phylogeny it grouped with borreliae of unknown host origin. Although these analyses demonstrated that pangolin-associated borreliae form a distinct monophyletic lineage, the slightly different sister relationships inferred from the 16S rRNA and *flaB* trees can likely be attributed to the short sequence fragments and limited taxon sampling. Similar conflicts in the placement of novel *Borrelia* clades relative to relapsing fever and Lyme borreliae have been reported in specific host-associated lineages in previous studies (Barbour, 2014; Binetruy et al., 2020; Muñoz-Leal et al., 2021). Therefore, additional genomic data is required to clarify the taxonomic status and confirm their phylogenetic status (Gofton et al., 2023).

Bacterial species, including *Rickettsia*, *Anaplasma*, *Ehrlichia*, as well as protozoan such as *Babesia* have been detected in pangolins (Khatri-Chhetri et al., 2016; Li et al., 2024). Moreover, viruses (e.g., Pestivirus), bacteria (e.g., *Anaplasma* and *Ehrlichia*), and protozoan (e.g., *Trypanosoma* and *Babesia*) have previously been detected in *A. javanense* infesting pangolins (Shi et al., 2022; Chong et al., 2023; Dao et al., 2024). Given that the present study detected *Borrelia* sp. in *A. pakhtunensis* from pangolin in Pakistan, closely related to *Candidatus* B. javanense detected in *A. javanense* from pangolin in China (Jiang et al., 2021), it suggests that pangolins may be reservoir hosts for such *Borrelia* species, facilitating a stable enzootic cycle within *Amblyomma* tick-pangolin system. Additionally, this may indicate potential host-associated specificity of those *Borrelia* across the wide range of pangolin, similar to what has been observed for *Candidatus* Borrelia tachyglossi in echidnas and *Borrelia turcica* in chelonians (Takano et al., 2010; Gofton et al., 2025).

Besides health importance of endangered or critically endangered pangolins, *A. pakhtunensis* may also have complications for domestic animals and human health, similar to their closest *A. javanense* (Hassan et al., 2013). Therefore, future studies should further explore other aspects of this tick species, including its host range, life cycle, and potential role as a reservoir or vector for various pathogens. With most records from Asia and Australia, *Borrelia* spp. from the third group have now been recorded on all inhabited continents (Guner et al., 2003; Kalmar et al., 2015;

Binetruy et al., 2020; Gofton et al., 2023; Kautman et al., 2025). Initially detected in reptile-associated *Hyalomma* ticks, they have since been detected in ticks from four other genera parasitizing hosts from all semi-terrestrial and terrestrial vertebrate classes (Guner et al., 2003; Binetruy et al., 2020; Colunga‐Salas et al., 2021; Jiang et al., 2021). Given this diversity in distribution and hosts, it is now inevitable to undertake genomic characterization and multi-locus sequence typing (Gofton et al., 2023; Parragué-Migone et al., 2025) to improve the systematic and taxonomy of the third group of *Borrelia*, including those associated with *Amblyomma* ticks from Pangolin. It remains to be determined whether *Borrelia* species outside the LD and RF groups, including the one identified here, are pathogenic, and any inference regarding reservoir competence, transmission dynamics, or population-level epidemiological significance should be considered preliminary.

Conclusion

In addition to confirming the ecological closeness between *A. pakhtunensis* and *A. javanense*, this study also extends the vertebrate host range of the former tick species. It also extends the vertebrate host and genetic diversity of the third group of *Borrelia*. The current study may assist future efforts to mitigate tick-borne diseases in general and may specifically support pangolin conservation strategies by taking into account ticks and their associated pathogens. Additionally, it may help inform assessment of the co-endanger status and conservation needs of *A. pakhtunensis*. Given the close resemblance between *A. pakhtunensis* and *A. javanense*, earlier records of the latter tick species on pangolins in this region should be interpreted with caution.

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Authors contributions

A.A: Conceptualization, Project administration, Supervision, Data curation, Methodology, writing original draft, Writing—review and editing, Visualization; M.E.K.: Data curation, Software,

Methodology, Writing original draft, Writing—review and editing, Visualization, Morphology; M.U.K: Data curation, Software, Methodology, Writing original draft, Writing—review and editing, visualization, Morphology; A.M: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization; B.F: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization; M.A: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization; M.M.A: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization; A.F.A: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization; A.T.A: Data curation, Methodology, Writing original draft, Writing—review and editing, Visualization.

Consent to Publish declaration

Not applicable.

Consent to Participate declaration

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

Data availability statement

All DNA sequences generated in this study are publicly available in GenBank under the following accession numbers: PX387109, PX387536, and PX394402.

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