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Detection of tick-borne pathogens in the pangolin tick, *Amblyomma javanense,* from Vietnam and Laos, including a novel species of *Trypanosoma*

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ABSTRACT

Two species of Southeast Asian pangolins (the Chinese pangolin, *Manis pentadactyla* and the Malayan or Sunda pangolin, *Manis javanica*) are critically endangered species. Therefore, knowledge on their parasitic infections is very important, especially considering ticks that can transmit which pathogens. In this study, 32 pangolin ticks (*Amblyomma javanense*), that were collected in Vietnam and Laos, were analyzed with molecular methods for the presence of tick-borne pathogens. Two members of the family Anaplasmataceae were shown to be present in 14 pangolin ticks, i.e., *Candidatus* Anaplasma pangolinii and an *Ehrlichia* sp. In three ticks, a single Rickettsia genotype was also detected, and in seven ticks four 18S rRNA sequence variants of a *Babesia* sp. Most importantly, a novel protozoan agent, tentatively called here *Trypanosoma* sp. "PAT14" was detected in one *A. javanense* nymph. These results imply the first molecular finding of any species of *Anaplasma, Ehrlichia* and *Babesia* in pangolin ticks from Vietnam and Laos. On the other hand, detection of a new tick-associated *Trypanosoma* sp. in *A. javanense* from Southeast Asia is not only important from a taxonomic point of view, but it is also the first finding of any trypanosomes in the genus *Amblyomma* in Eurasia and adds pangolins to the potential placental mammalian hosts of any trypanosomes.

Introduction

Pangolins (Pholidota: Manidae) include eight species of toothless, insectivorous placental mammals that are phylogenetically closely related to carnivores (Mohapatra et al., 2016). Four species, the Indian pangolin (*Manis crassicaudata*), the Philippine pangolin (*Manis culionensis*), the Chinese pangolin (*Manis pentadactyla*) and the Malayan or Sunda pangolin (*Manis javanica*) are native to Asia. The last two species are critically endangered (Mohapatra et al., 2016). Because of the latter, knowledge on infectious agents, in particular parasites that may affect the health or even threaten the life of pangolins, is extremely important (Mohapatra et al., 2016).

Amblyomma javanense is a relatively host-specific parasite of Asian pangolins. It was reported from at least 12 species of mammals, i.e. bats, boars, deer, hyenas, and porcupines (from the families Vespertilionidae, Suidae, Cervidae, Hyaenidae and Hystricidae, respectively). Its host range includes humans, and rarely even reptiles (Guglielmone et al., 2014; Kwak et al., 2018). This tick species has a very broad distribution in the Oriental Zoogeographic Region. Thus, *A. javanense* is found in eastern Pakistan and India, Sri Lanka, eastward through Cambodia, Myanmar, Thailand, Vietnam, western Indonesia, Malaysia, Singapore, Southern China, and the Philippines (Voltzit and Keirans, 2002; Guglielmone et al., 2023).

Recently, several tick-borne pathogens have been reported from pangolins and/or *A. javanense*, including members of the genera *Babesia, Anaplasma, Ehrlichia* and *Borrelia* (Koh et al., 2016; Jiang et al., 2021; Yodsheewan et al., 2021; Zhai et al., 2021). These studies focused on Southeastern Asia, in particular southern China, Malaysia, Singapore, Taiwan and Thailand. However, *A. javanense* was not collected and analyzed in a similar context in Vietnam or Laos. Therefore, the aim of

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this study was to compensate for this lack of data, using ticks collected during regular veterinary care from pangolins rescued in Vietnam and Laos.

Materials and methods

Sample collection and DNA extraction

Ticks were originally removed from pangolins in 2023 at the Rescue Center of Cuc Phuong National Park, Vietnam, as part of their regular veterinary care. All ticks were saved in 96 % ethanol for general diagnostic (morphological species identification) purposes. Therefore, the registration numbers of vials containing tick(s) from the same animal were not recorded according to host individuals. It was only decided later to process all ticks for pathogen detection. Data on the origin of samples are shown in Supplementary Table 1.

Ticks were identified morphologically based on standard keys (Voltzit and Keirans, 2002). Pictures were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan). Ticks were disinfected on their surface with sequential washing in 10 % sodium-hypochlorite, tap water and distilled water. DNA was extracted from the whole body of nymphs and males, or the idiosomal contents of female ticks, with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), including an overnight digestion in tissue lysis buffer and Proteinase K at 56 °C. Two extraction controls (tissue lysis buffer) were also processed with the tick samples to monitor cross-contamination.

Molecular-phylogenetic analyses

Data of PCR methods (primers and cycling conditions) used to test all DNA extracts are summarized in Table 1. Sequences longer than 500 bp or from extra genetic markers were only amplified and analyzed further

Table 1
Primers and details for conventional PCR methods used in this study.

Target group	Target gene	Primer name	Primer sequence (5′-3′)	Amplicon length (bp)	Thermocycling profile	Reference
Ixodidae	16S rRNA	16S+1 16S-1	CTG CTC AAT GAT TTT TTA AAT TGC TGT GG CCG GTC TGA ACT CAG ATC AAG T	~460	95 °C for 5 min; 40 × (94 °C for 40 s; 51 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min	Black and Piesman, 1994
Piroplasms	18S rDNS	BJ1 BN2	GTC TTG TAA TTG GAA TGA TGG TAG TTT ATG GTT AGG ACT ACG	~500	95 °C for 10 min; 40 × (95 °C for 30 s; 54 °C for 30 s; 72 °C for 40 s); 72 °C for 5 min	Casati et al., 2006
***Hepatozoon* spp.**	18S rDNS	HepF HepR	ATA CAT GAG CAA AAT CTC AAC CTT ATT ATT CCA TGC TGC AG	~650	95 °C for 5 min; 35 × (95 °C for 40 s; 57 °C for 40 s; 72 °C for 60 s); 72 °C for 7 min	Inokuma et al., 2002
Anaplasmataceae	16S rRNA	EHR16sD EHR16sR	GGT ACC YAC AGA AGA AGT CC TAG CAC TCA TCG TTT ACA GC	~350	95 °C for 10 min; 40 × (95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s); 72 °C for 5 min	Brown et al., 2001
***Rickettsia* spp.**	gltA	RpCs.877p RpCs.1258n	GGG GGC CTG CTC ACG GCG G ATT GCA AAA AGT ACA GTG AAC A	~380	95 °C for 5 min; 40 × (95 °C for 20 s; 48 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min	Regnery et al., 1991
***Rickettsia* spp.**	17 kDa	17kd1 17kd2	GCT CTT GCA ACT TCT ATG TT CAT TGT TCG TCA GGT TGG CG	~480	95 °C for 5 min; 40 × (95 °C for 30 s; 51 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min	Williams et al., 1992
***Rickettsia* spp.**	OmpA	Rr190.70p Rr190.602n	ATG GCG AAT ATT TCT CCA AAA AGT GCA GCA TTC GCT CCC CCT	~530	95 °C for 5 min; 40 × (95 °C for 30 s; 48 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min	Regnery et al., 1991
Borrelia burgdorferi s.l.	5S-23S IGS	B5Sborseq B23Sborseq	GAG TTC GCG GGA GAG TAG GTT ATT GCC TCA GGG TAC TTA GAT GGT TCA CTT CC	~450	94 °C for 5 min; 10 × 'touchdown' (94 °C for 20 s, 70 °C for 30 s (dropping 1 °C per cycle), 72 °C for 30 s); 40 × (94 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s); 72 °C for 7 min	Heylen et al., 2013
***Bartonella* spp.**	16S-23S ITS	BA325s BA1100as	CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG GAA CCG ACG ACC CCC TGC TTG CAA AGC A	~600	95 °C for 5 min; 40 × (94 °C for 30 s; 65 °C for 30 s; 72 °C for 50 s); 72 °C for 5 min	Maggi et al., 2006; Maia et al., 2014
***Trypanosoma* spp.**	ssu	609F 706R 706R+1	CAC CCG CGG TAA TTC CAG C CTG AGA CTG TAA CCT CAA CTG AGA CTG TAA CCT CCA A	~800–1000	95 °C for 5 min; 40 × (94 °C for 40 s; 49 °C for 1,5 min; 72 °C for 1 min); 72 °C for 5 min	da Silva et al., 2004; Ramírez et al., 2012
***Trypanosoma* spp.**	cytB	p18 p20	GAC AGG ATT GAG AAG CGA GAG AG CAA ACC TAT CAC AAA AAG CAT CTG	~520	95 °C for 5 min; 35 × (94 °C for 1 min; 53 °C for 40 s; 72 °C for 1 min); 72 °C for 5 min	da Silva et al., 2004; Ramírez et al., 2012

if preliminary results justified novelty of the results in the context of pangolin ticks (i.e., this was not performed with samples having positivity for piroplasms and Anaplasmataceae, but only in the case of those containing Rickettsiae and trypanosomes). The reaction mixture, in a volume of 25 μl, contained 1 U (0.2 μl) HotStarTaq Plus DNA polymerase, 2.5 μl 10x CoralLoad Reaction buffer (including 15 mM $MgCl_2$), 0.5 μl PCR nucleotide Mix (0.2 mM each), 0.5 μl (1 μM final concentration) of each primer, 15.8 μl ddH_2O and 5 μl template DNA.

In all PCRs, non-template reaction mixture served as negative control. Extraction controls and negative controls remained PCR negative in all tests. Purification and sequencing of the PCR products were done by Eurofins Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences were performed with the BioEdit program. Obtained sequences were compared to GenBank data by the BLASTN program (https://blast.ncbi.nlm.nih.gov). New sequences were submitted to GenBank under the following accession numbers: Ixodidae 16S rRNA gene (PQ062236-PQ062239), Anaplasmataceae 16S rRNA gene (PQ061770-PQ061771), *Rickettsia* sp. 17 kDa, gltA, OmpA genes (PQ069061-PQ069063, respectively) *Babesia* sp. 18S rRNA gene (PQ062244-PQ062247), *Trypanosoma* sp. 18S rRNA gene (PP958817).

Phylogenetic analysis was only performed for trypanosomes, because the corresponding sequence was new both in geographical and taxonomic contexts. Other tick-borne pathogens were either already detected in the region of Vietnam, or they were 100 % identical in all examined genetic markers to those reported from other parts of Asia. Sequences from other studies (retrieved from GenBank) included in the phylogenetic analyses had 99–100 % coverage with sequences from this study. Sequence datasets were resampled 1000 times to generate bootstrap values. Phylogenetic analyses were performed with the Maximum Likelihood method, Kimura 2-parameter model according to the selection of the MEGA 7.0 software (Kumar et al., 2016).

Results

Identification of tick species

All 32 ticks (20 nymphs, 2 females, 10 males) were morphologically and molecularly identified as *Amblyomma javanense*. In brief, the males (Fig. 1.A, B) had inornate scutum with few small and large but shallow punctuations; coxa I with 2 long, broadly rounded spurs, the external spur being slightly larger; genital aperture situated between coxae II; spiracular plates long and oval; postanal groove with short median groove. The females (Fig. 1.C, D) had evenly distributed small punctuations extending into the scapular area; small, caudally directed cornuae; small, oval areae porosae enclosing an acute angle; palpal article I with a small ventral spur. The 16S rRNA sequences from representative samples of adults (n = 3) and nymphs (n = 5) were 99.5–100 % (399–401/401 bp) identical to each other, and to sequences of *A. javanense* reported from China in GenBank (e.g., ON845565).

Molecular detection of tick-borne pathogens

Members of the family Anaplasmataceae were shown to be present in 14 pangolin ticks (nine nymphs, one female, and four males). Two species were verified by sequencing a short fragment of the 16S rRNA gene. One of them occurred in six ticks and had 100 % (304/304 bp)

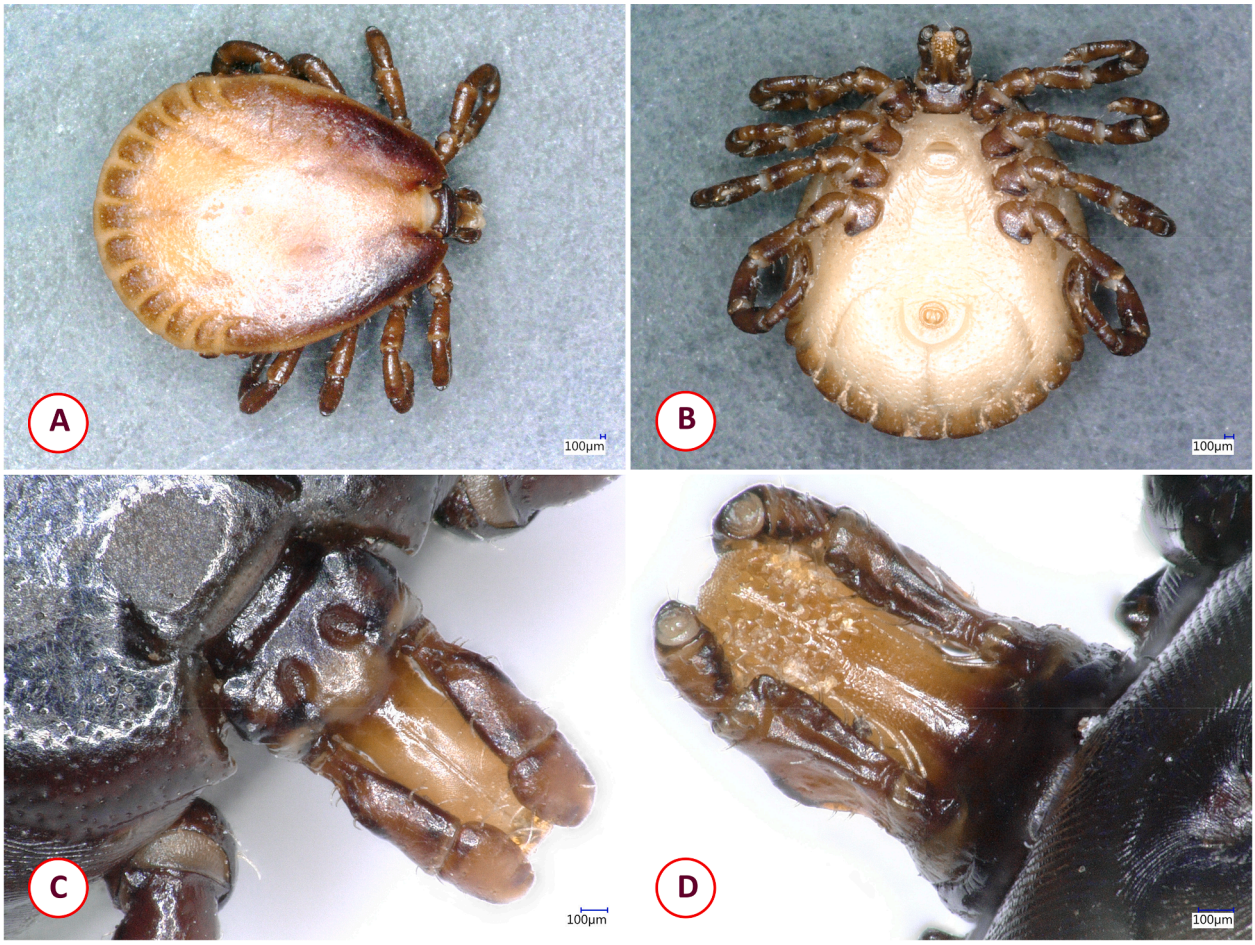

Fig. 1. Morphology of *Amblyomma javanense* collected from pangolins in Vietnam and Laos. (A) Male, dorsal surface. (B) Male, ventral surface. (C) Female capitulum, dorsal surface. (D) Female capitulum, ventral surface.

identity to *Candidatus* Anaplasma pangolinii already reported in the blood of pangolins in Malaysia (KU189193), as well as from pangolin ticks (*A. javanense*) in Thailand (AF497580). The other species, detected in eight ticks, had only 1 bp difference in the amplified part of its 16S rRNA gene from the *Ehrlichia* sp. reported from Malayan pangolins (MK928409) and *Ehrlichia ruminantium* reported from *Amblyomma lepidum* (MN747328) and *Amblyomma variegtaum* (MN747324) in Africa, meaning 99.3 % (140/141 bp) and 99.7 % (303/304 bp) sequence identities, respectively.

A single Rickettsia genotype was also detected in three ticks (two nymphs, one male), identical in the amplified parts of its gltA, 17 kDa and OmpA genes to *Rickettsia* sp. isolate MIVNM6/2018 (MN557223, MN557233 and MK905248, respectively) and several others reported from India. The gltA and OmpA sequences of this *A. javanense*-associated Rickettsia were also identical to those of *Rickettsia* sp. TwKM01 from Taiwan (AY445819, EF219467) and its 17 kDa sequence to that of *Rickettsia* sp. isolate LOI69 from Thailand (MW415893).

In the PCR detecting piroplasma, seven ticks (two nymphs, one female, and four males) were positive. In these, four 18S rRNA sequence variants of a *Babesia* sp. were identified. One of these, obtained from three ticks, showed 100 % (445/445 bp) identity to a *Babesia* sp. reported from pangolin blood in Malaysia (KX168696) and in pangolin liver in Singapore (OR229749). Compared to these as reference sequences, the remaining four 18S rRNA sequences amplified from *A. javanense* in this study had 2 or 4 bp differences in four positions, appearing in the chromatogram as ambiguous (double) peaks (Supplementary Figure 1), or in addition also a single nucleotide polymorphism, meaning 98.9 % (440/445 bp) sequence identity.

Last but not least, a novel protozoan agent, tentatively called here *Trypanosoma* sp. "PAT14" was detected in one *A. javanense* nymph. This trypanosome showed only 87.7 % (845/964 bp) sequence identity to the most closely related species that was reported from *Ixodes ricinus* ticks in Europe (Slovakia: MT482752, France: OQ025411). However, their separate clustering received high (99 %) support, and the evolutionary distance was proportionate to that between other, well established *Trypanosoma* spp., confirming the probable status of *Trypanosoma* sp. "PAT14" as a new species. This new species clustered among the tick-associated trypanosomes which occupied a monophyletic group (Fig. 2).

None of the ticks were positive for bartonellae, borreliae and *Hepatozoon* spp.

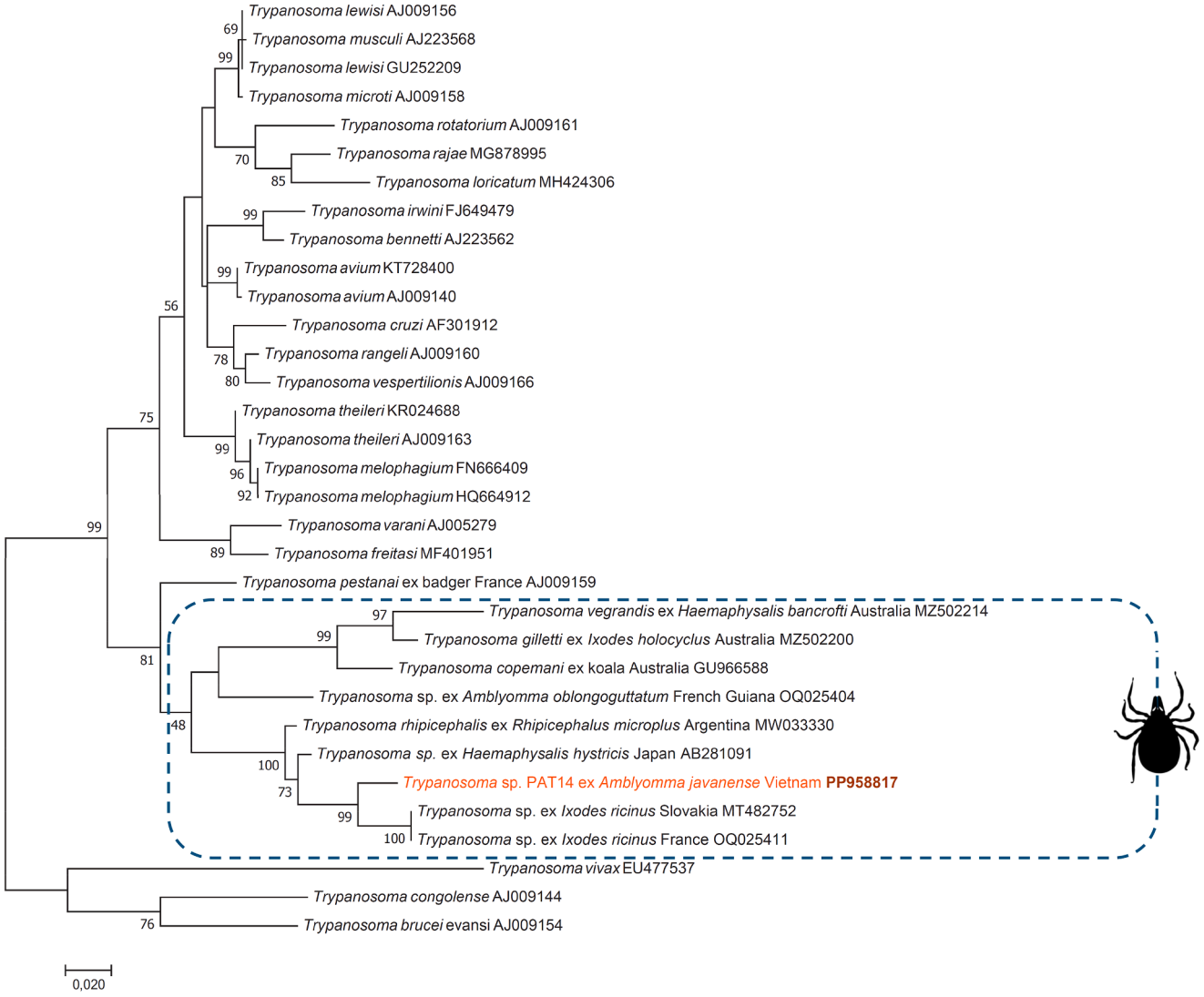

Fig. 2. Phylogenetic tree of trypanosomes based on the 18S rRNA gene. Species were selected to include the most important taxonomic groups (subgenera) of trypanosomes, based on Koual et al. (2023). For tick-associated *Trypanosoma* spp., the isolation source is shown after the species name. The sequence from this study is indicated with red fonts and bold, maroon accession number. The evolutionary history was inferred by using the Maximum Likelihood method and the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences and there were a total of 622 positions in the final dataset.

Discussion

This is the first study on the molecular detection of tick-borne pathogens in pangolin ticks (*A. javanense*) from Vietnam and Laos. Some of the detected pathogens have already been reported from pangolins and/or their ticks in Southeastern Asia, as exemplified by *Candidatus* Anaplasma pangolinii and unnamed *Ehrlichia* spp. and *Babesia* spp. However, to our knowledge, the *Rickettsia* sp. identified from *A. javanense* in this study has only been reported previously in South Asia (India) and Southeastern Asia from *Haemaphysalis* spp. and *Rhipicephalus* sp. ticks. More importantly, the *Trypanosoma* sp. "PAT14" detected in *A. javanense* in this study appears to be a novel finding in a broader, taxonomic context, as outlined below. Importantly, all pangolins from which ticks have been used in this study, originated in the region of Vietnam (Supplementary Table 1), therefore the results are highly relevant to its geographical region.

Considering Anaplasmataceae, *Candidatus* Anaplasma pangolinii was identified here in pangolin ticks. Previously, this species was reported from the blood of Malayan pangolins (Koh et al., 2016), as well as from *A. javanense* collected in Thailand (Parola et al., 2003). Its pathogenicity remains to be clarified. Another tick-borne bacterium detected in pangolin ticks from Vietnam and Laos was closely related to the *Ehrlichia* sp. reported recently from Malayan pangolins (Zhai et al., 2021). This was deemed to be a highly pathogenic species, causing congestion, hemorrhages and inflammation in the lungs, heart and kidneys. This condition may even prove to be fatal in affected pangolins (Zhai et al., 2021). Therefore, demonstration of this *Ehrlichia* sp. in the region of Vietnam raises concerns in the conservation of pangolins and necessitates further studies.

Furthermore, a single Rickettsia genotype was demonstrated here in *A. javanense* ticks, identical in the amplified parts of its gltA, 17 kDa and OmpA genes to *Rickettsia* sp. clone MIVNM6/2018 and several others, detected in various *Haemaphysalis* spp. ticks in India (Babu et al., 2023). Based on the 17 kDa and gltA sequences, this Rickettsia detected for the first time in Vietnam and Laos was also identical to an isolate present in *Rhipicephalus haemaphysaloides* from Taiwan (Tsui et al., 2007) and *Haemaphysalis heinrichi* from Thailand (Hirunkanokpun et al., 2022). This species belongs to the *Rickettsia massiliae* group, named after a species which can cause spotted fever when transmitted by *Rhipicephalus* sp. ticks to humans (Raoult and Roux, 1997), but it also appears to be closely related to the non-pathogenic *Rickettsia rhipicephali* (Hirunkanokpun et al., 2022). To the best of our knowledge, since this Rickettsia was previously reported from *Haemaphysalis* spp. and *Rhipicephalus* spp. in various parts of Asia, this is the first detection of this bacterium in any *Amblyomma* sp. ticks.

The *Babesia* sp. detected in *A. javanense* from Vietnam and Laos was identical to the one reported from Sunda pangolin tissues in Thailand (Yodsheewan et al., 2021), and in Sunda pangolins and their ticks in Singapore (Chong et al., 2023). This novel, unnamed *Babesia* sp. is mildly pathogenic (Yodsheewan et al., 2021), thus does not appear to be very important from the aspects of clinico-pathology and conservation of pangolins. However, phylogenetically it occupies a basal position to other members of the family Babesiidae (Yodsheewan et al., 2021; Chong et al., 2023), therefore deserving extra attention from the point of view of piroplasm systematics. At the same time, in four positions of the 18S rRNA sequences of this *Babesia* sp. in this study, ambiguous nucleotides (double peaks) were present, always as "Y" (cytosine or thymine). The most likely explanation for this phenomenon is that multiple copies (paralogues) of the 18S rRNA gene might be present in the same piroplasm genome, similarly to what was reported in the case of *Babesia canis* and a vast array of other Apicomplexan parasites (Hrazdilová et al., 2019).

Most notably, a novel protozoan parasite, designated here as *Trypanosoma* sp. "PAT14", was identified in one nymph of *A. javanense*. This probably represents a hitherto unknown species which clearly belongs to the phylogenetic group of tick-borne trypanosomes. Apart from fly-, flea- and bug-associated trypanosomes (e.g., *Trypanosoma brucei, Trypanosoma lewisi, Trypanosoma cruzi*, respectively), tick-borne trypanosomes have only recently become a hot topic of research in protozoology (Koual et al., 2023). The pathogenic role of tick-associated trypanosomes is not known, but previous data suggest that these can be transmitted by ixodid ticks (which probably act as biological vectors) to a broad range of mammalian hosts, now potentially including pangolins. This may be an important finding in the context of health-related and conservation issues of pangolins, especially taking into account that tick-associated trypanosomes have hitherto been reported only from marsupials among mammals (Koual et al., 2023). To our knowledge, this is also the first report of a tick-associated trypanosome from the genus *Amblyomma* in Southeast Asia. Nevertheless, it was already shown that phylogenetically related trypanosomes might be present in geographically or taxonomically distantly related tick species (Koual et al., 2023).

In conclusion, this is the first report of DNA from species of *Anaplasma, Ehrlichia* and *Babesia* in pangolin ticks from Vietnam and Laos. The molecular detection of a new tick-associated *Trypanosoma* sp. in *A. javanense* from Southeast Asia is an important finding, implying the discovery of the first *Amblyomma*-associated trypanosome in Eurasia. While all previously reported tick-borne trypanosomes were demonstrated from marsupials, this result adds pangolins to the potential placental mammalian hosts of such protozoan parasites.

CRediT authorship contribution statement

Thanh Thi Ha Dao: Writing – original draft, Data curation, Conceptualization. **Nóra Takács:** Methodology. **Trieu Nam Tran:** Investigation. **Anh Ngoc Truong:** Investigation. **Kelsey Skinner:** Investigation. **Jenő Kontschán:** Methodology. **Róbert Farkas:** Supervision, Formal analysis. **Sándor Hornok:** Writing – original draft, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethical statement

No ethical approval was required for analysis of ticks that were collected from rescued pangolins during their regular veterinary care.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107384.

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