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INTEGRATED SPECIES IDENTIFICATION AND MOLECULAR DETECTION OF TICK-BORNE PATHOGENS IN *Amblyomma javanense* FROM SUNDA PANGOLINS, *Manis javanica* IN PENINSULAR MALAYSIA

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ABSTRACT

The Sunda pangolin (*Manis javanica*), a critically endangered species, is known to host the tick *Amblyomma javanense*. However, limited data exists on the tick-borne pathogens associated with this tick species in Malaysia. In this preliminary study, both morphological and molecular identification of *A. javanense* were performed, alongside molecular screening for tick-borne pathogens, using specimens collected from rescued pangolins in Peninsular Malaysia. Genomic DNA was extracted from individual ticks (n=122) and subjected to polymerase chain reaction (PCR) targeting the COI gene for tick species confirmation (n = 5), general bacterial 16S rRNA gene for bacterial detection (using one representative tick per host; n = 5), as well as conserved genes specific to *Rickettsia* spp. (*gltA*), *Anaplasma* spp. (*16S rRNA*), *Ehrlichia* spp. (*gltA*), *Bartonella* spp. (*gltA*), *Trypanosoma* spp. (*ITS1*), and *Borrelia* spp. (*flaB*). Molecular identification confirmed morphological identification, with all ticks identified as *A. javanense*. General bacterial amplification in one tick revealed dominance of *Staphylococcus* spp., a genus with potentially pathogenic members, though its role in tick-borne transmission is uncertain. No amplification was detected for any of the targeted pathogens. While no known tick-borne pathogens were detected, the presence of potentially relevant bacterial taxa highlights the need for continued surveillance. Given the risk of zoonotic spillover through illegal wildlife trade and close human–pangolin interactions, further studies with larger sample sizes, pathogen isolation, and host blood screening are essential to better understand the epidemiological role of *A. javanense* and its associated microbial communities.

Keywords: *Amblyomma javanense*; Sunda pangolin; tick-borne pathogens; zoonoses; wildlife surveillance

ABSTRAK

Tenggiling Sunda (*Manis javanica*), iaitu spesies yang terancam kritikal, diketahui menjadi perumah kepada kutu *Amblyomma javanense*. Walau bagaimanapun, data mengenai patogen bawaan kutu yang berkaitan dengan spesies kutu ini di Malaysia masih terhad. Dalam kajian awal ini, pengecaman morfologi dan molekul terhadap *A. javanense* telah dijalankan, bersama-sama dengan saringan molekul bagi patogen bawaan kutu, menggunakan spesimen yang dikumpulkan daripada tenggiling yang diselamatkan di Semenanjung Malaysia. DNA genom diekstrak daripada kutu individu (n=122) dan menjalani tindak balas rantai polimerase (PCR) yang mensasarkan gen COI bagi pengesahan spesies kutu, gen bakteria umum 16S rRNA untuk pengesanan bakteria (satu kutu wakil bagi setiap perumah; n=5), serta gen terpelihara khusus bagi *Rickettsia* spp. (*gltA*), *Anaplasma* spp. (16S rRNA), *Ehrlichia* spp. (*gltA*), *Bartonella* spp. (*gltA*), *Trypanosoma* spp. (*ITS1*) dan *Borrelia* spp. (*flaB*). Pengecaman molekul mengesahkan pengecaman morfologi, dengan semua kutu dikenal pasti sebagai *A. javanense*. Amplifikasi bakteria umum dalam satu kutu menunjukkan dominasi *Staphylococcus* spp., iaitu genus yang merangkumi ahli berpotensi patogen, namun peranannya dalam penularan bawaan kutu masih tidak pasti. Tiada amplifikasi dikesan bagi patogen sasaran. Walaupun tiada patogen bawaan kutu yang diketahui dikesan, kehadiran takson bakteria yang berpotensi relevan menekankan keperluan untuk pemantauan berterusan. Memandangkan risiko limpahan zoonotik melalui perdagangan hidupan liar haram dan interaksi rapat antara manusia dan tenggiling, kajian lanjut dengan saiz sampel yang lebih besar, pengasingan patogen, serta saringan darah perumah adalah penting bagi memahami dengan lebih jelas peranan epidemiologi *A. javanense* dan komuniti mikrob yang berkaitannya.

Kata kunci: *Amblyomma javanense*; tenggiling Sunda; patogen bawaan kutu; zoonosis; pengawasan hidupan liar

INTRODUCTION

The Sunda pangolin (*Manis javanica*) is a critically endangered species of pangolin native to Southeast Asia and southern China (Challender et al. 2019a; Sitam et al. 2023). This arboreal mammal can be found in forest habitats, shrublands, as well as rubber and oil palm plantations (Challender et al. 2019a). They are one of the most highly smuggled mammals in the world, due to their highly-valued scales, bones, and meat, coveted in traditional African and Chinese medicine (Boakye et al. 2015). Previous studies investigating diseases carried by Sunda pangolin found that this scaly toothless mammal harbours various infectious agents, including blood parasites, bacteria, and viruses (Barton et al. 2022; Liu et al. 2019; Wicker et al. 2020). Pangolins are considered omnivores, preying on ants and termites while being preyed on by larger apex predators, thus play important roles in maintaining the stability of the food chain. Despite this, overhunting and increase in illegal smuggling activities have caused pangolin numbers to dwindle in recent years. Additionally, decreases in pangolin numbers may have also been caused by disease, with studies reporting bacterial infections in wild pangolins in Thailand (Parola et al. 2003) and China (Khatri-Chhetri et al. 2016). The COVID-19 pandemic that struck the world in 2020 was an international public health concern that exposed the potential dangers of wildlife trade to public health security, underscoring the importance of pandemic preparedness and proactive pathogen bio-surveillance in wildlife populations. In this context, investigating tick-borne pathogens in Sunda pangolins is particularly significant, as

these animals may act as cryptic reservoirs that facilitate the maintenance and spillover of zoonotic agents between wildlife, domestic animals, and humans.

Ticks are hematophagous parasites that serve as vectors of various zoonotic diseases of medical and veterinary relevance. They are known to parasitise animals, both domestic and wildlife, as well as humans in many regions around the world, including in Southeast Asia (Chong et al. 2020). *Amblyomma javanense* ticks are known to parasitise the Sunda pangolin (Jiang et al. 2021; Li et al. 2024; Zhai et al. 2021), however there is insufficient research on the diversity, prevalence, and molecular characteristics of tick-borne pathogens harboured by these pangolins in Malaysia. Most existing studies in Malaysia have focused on tick-borne pathogens in livestock, companion animals, or more easily accessible wildlife species (Khoo et al. 2017; Watanabe et al. 2015), leaving a significant knowledge gap regarding endangered and trafficked species such as pangolins. This gap is particularly important to address, especially given Malaysia's role as a biodiversity hotspot, and must be given appropriate attention to further our understanding of the pangolin's potential role in regional disease ecology, including cross-border pathogen circulation within Southeast Asia. By doing so, this will not only contribute to conservation biology by improving health assessments of rescued and wild pangolin populations, but will also carry implications for One Health initiatives, strengthening early detection systems for emerging zoonoses and informing risk assessments at the wildlife -human interface. Therefore, this study aimed to perform molecular detection of several tick-borne pathogens, namely *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., *Trypanosoma* spp., and *Borrelia* spp., in ticks collected from captive pangolins in Sungkai Wildlife Conservation Centre, Perak, via polymerase chain reaction (PCR).

MATERIALS AND METHODS

Tick Collection

Ticks were collected from Sunda pangolin hosts that were previously rescued and kept captive in Sungkai Wildlife Conservation Centre, Perak, Malaysia. Pangolins were characterised by life stage (juvenile or adult) and sex. Collection of tick samples was done via observation of undersides of host scales and removal of feeding ticks using forceps. Collected ticks were stored in 1.5 mL tubes and preserved in liquid nitrogen on-site before being transferred to -80°C in the laboratory.

Morphological Identification

All tick specimens were characterised by species, life stage, and sex. Species identification was carried out morphologically referring to established taxonomic keys by Tanskul & Inlao (1989), Voltzit (2002), and Yamaguti et al. (1971) under stereoscopic and compound microscopes.

Genomic DNA Extraction

Genomic DNA extraction began by crushing whole tick samples using pestle and mortar and liquid nitrogen before suspending in phosphate buffer saline (PBS) solution. Genomic DNA was then extracted and purified using QIAGEN DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) following manufacturer protocols. The purified DNA was then stored at -80°C until further use.

Polymerase Chain Reaction (PCR) and DNA Sequencing Analysis

Identification of tick species was molecularly confirmed by carrying out PCR targeting COI gene specific to ticks (n=5). Amplicons were sent to Apical Scientific Sdn. Bhd. to be sequenced. Nucleotide sequences obtained were analysed and edited in Molecular Evolutionary Genetics

Analysis (MEGA) program version 11.0 (Tamura et al. 2021) before being compared to reference sequences deposited in GenBank via BLASTn for confirmation of tick species. Evolutionary analysis was also carried out in MEGA program version 11.0 to construct a maximum likelihood phylogenetic tree using the COI gene. Pathogen detection was carried out via PCR deploying genus-specific primers for the detection of *Rickettsia* spp. citrate synthase (*gltA*), *Ehrlichia* spp. citrate synthase (*gltA*), *Bartonella* spp. (*gltA*), *Anaplasma* spp. 16S (*16S rRNA*), *Trypanosoma* spp. internal transcribed spacer 1 (*ITS1*), and *Borrelia* spp. flagellin B (*flaB*) genes in individual tick samples, alongside general bacterial amplification targeting a fragment of the 16S rRNA gene. Details on the primers deployed and protocols referenced in this study are shown in Table 1. A positive control of pathogen-positive DNA respective to the pathogen targeted and negative control of nuclease-free water was included in each PCR run. The amplification products were subjected to electrophoretic migration in 1.0% agarose gel, except for amplicons for Trypanosoma and Ehrlichia detection, in which 1.5% agarose gel was used, and viewed under blue light.

Table 1. Primers deployed for pathogen detection and their target genes

Organism	Target	Primers	Reference
Ticks	*COI*	LCO1490^{F}	Folmer et al. 1994
		HCO2198^{R}	
General bacteria	*16S rRNA*	27F ^{F}	Lane et al. 1991
		1429R ^{R}	
Borrelia spp.	*flaB*	BflaPAD^{F}	Lau et al. 2023
		BflaPDU^{R}	
		BflaPBU^{F}	
		BflaPCR^{R}	
Anaplasma spp.	*16S rRNA*	EHR16SD^{F}	Parola et al. 2000
		EHR16SR^{R}	
Rickettsia spp.	*gltA*	CS239^{F}	Labruna et al. 2004
		CS1069^{R}	
Trypanosoma spp.	*ITS1*	LeF^{F}	Spanakos et al. 2008
		LeR^{R}	
Bartonella spp.	*gltA*	BhCS.781p^{F}	Norman et al. 1995
		BhCS.1137n^{R}	
Ehrlichia spp.	*gltA*	EgltAf1^{F}	Buysse et al. 2024
		EgltAf2^{F}	
		EgltAr^{R}	

F: Forward primer; R: Reverse primer

RESULTS AND DISCUSSION

Tick Collection and Species Identification

A total of three pangolin females and two pangolin males kept in captivity in Sungkai Wildlife Conservation Centre, Perak, were examined for attached ticks. A total of 122 feeding ticks were collected from the five hosts. Majority of the collected ticks were adult females (81.12%), followed by adult males (16.39%), while only three individuals were identified as nymphs (2.46%). All ticks collected were morphologically identified as *Amblyomma javanense*, and later confirmed as *A. javanense* by sequencing and comparison with reference sequences in GenBank in reference to the COI gene (Table 2). A phylogenetic tree was constructed using

maximum likelihood to investigate the evolutionary history of selected (n=5) *A. javanense* samples (Figure 1). Previously, *A. javanense* has been found on *M. javanica* pangolin (Jiang et al. 2021) and can be found in almost all pangolin species in Asia including from Pakistan, China, Sri Lanka, Myanmar, Philippines, Thailand, Vietnam, Indonesia, Malaysia, and Singapore (Kwak et al. 2018; Voltzit 2002). Hassan et al. (2013) reported that *A. javanense* may occasionally infect humans, although the limitations of their infections are unknown and requires further investigation.

Table 2. BLASTn results of COI gene of selected samples (n = 5)

Tick ID	Organism	Accession number	Identities (%)
T3	*Amblyomma javanense*	PQ048025	419/422 (99.29)
T5	*Amblyomma javanense*	PQ048025	422/425 (99.29)
T6	*Amblyomma javanense*	PQ048025	422/425 (99.29)
T7	*Amblyomma javanense*	PQ048025	423/426 (99.30)
T8	*Amblyomma javanense*	PQ048025	422/425 (99.29)

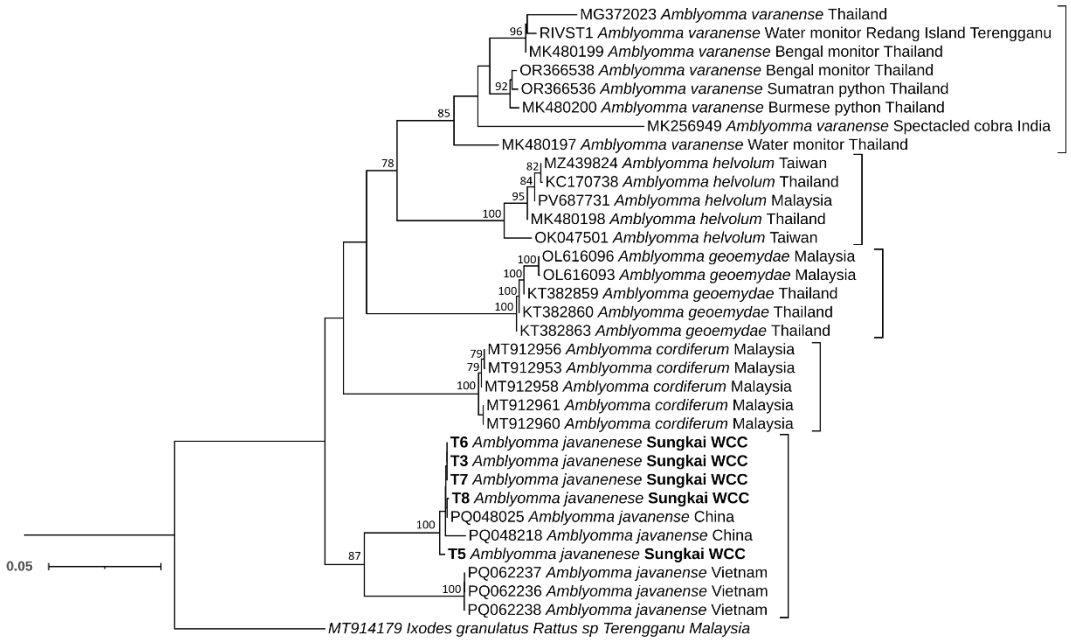

Figure 1. Phylogenetic analysis of *Amblyomma* based on *COI* gene. The phylogenetic tree was constructed by maximum-likelihood method and Tamura-Nei model with 1000 bootstrap replications. GenBank accession numbers with species and country are stated. The sequences obtained in this study are indicated with sample ID and location in bold. Bootstrap values above 75 are indicated on each branch. *Ixodes granulatus* (accession no. MT914179) sequence isolated from *Rattus* sp. from Malaysia is included as outgroup

Molecular Screening for Targeted Pathogens

Whole tick samples were screened for a total of six tick-borne pathogens, namely *Borrelia* spp., *Anaplasma* spp., *Rickettsia* spp., *Trypanosoma* spp., *Bartonella* spp., and *Ehrlichia* spp. via PCR deploying genus-specific primers. PCR results revealed no amplification of targeted pathogens within the sample set. These results could be due to the pangolin hosts being kept

isolated from other wildlife in the forest that may serve as pathogen reservoir, thus preventing transmission of pathogens into the pangolins. Additional studies deploying host blood screening are warranted to further investigate the presence of tick-borne pathogens harboured by pangolins as well as their possible pathogenicity to pangolins themselves, humans and other wildlife. General bacterial screening using samples representative of each host (n=5) revealed dominant presence of *Staphylococcus* spp., though it is not known whether the species present is pathogenic to humans.

Amblyomma ticks have been reported to parasitize several species of reptiles, including water monitors, pythons, and turtles (Kaenkan et al. 2020), and mammals, including wild boars (Khoo et al. 2017; Lim et al. 2020) and domestic cattle (Estrada-Peña & Salman 2013), with occasional reports on humans (Yamauchi et al. 2012). *Amblyomma* are three-host ticks, meaning they latch onto and feed on three different hosts throughout their life cycle, detaching themselves off the host between each life stage (larvae, nymph, and adult) to molt (Nepveu-Traversy et al. 2024). Immature stages of some *Amblyomma* species have been reported to infest birds, which may contribute to the dispersal of these species (Jongejan & Uilenberg 2004). *Amblyomma* spp. are vectors of several pathogenic bacteria including *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Ehrlichia ruminatum*, and several species of *Rickettsia* (Lippi et al. 2021; Steiner et al. 1999). Previous studies have reported the Sunda pangolin to be exclusively parasitized by *A. javanense* (Hassan et al. 2013; Li et al. 2024). A study by Li et al. (2024) detected *Rickettsia* spp., *Ehrlichia* spp., and *Anaplasma* spp. at detection rates of 90.91%, 15.15%, and 6.06%, respectively, on *A. javanense* ticks parasitizing confiscated Sunda pangolins. Another study investigating the ticks and associated pathogens of Formosan pangolins in Southeastern Taiwan identified *Haemaphysalis hystricis*, *Haemaphysalis formosensis*, and *Amblyomma testudinarium* ticks parasitizing the free-roaming pangolins (Khatri-Chhetri et al. 2016). The Formosan pangolin (*Manis pentadactyla pentadactyla*), a subspecies of the Chinese pangolin (*M. pentadactyla*), are critically endangered pangolins distributed across South Asia and Southeast Asia (Challender et al. 2019b), and are closely related to the Sunda pangolin. Khatri-Chhetri et al. (2016) detected presence of *Anaplasma* sp., *Rickettsia* sp., and *Ehrlichia* sp. in the collected ticks via PCR amplification.

In Malaysia, previous studies have reported the presence of tick-borne pathogens in humans and wildlife, including serological evidence of *Ehrlichia* and *Anaplasma* (Koh et al. 2018), as well as molecular evidence of *Borrelia* (Khoo et al. 2018), and *Rickettsia* (Lau et al. 2020), highlighting the diverse circulation of tick-borne bacteria across multiple host taxa. Khoo et al. (2017) previously collected several species of ticks from wild boar carcasses obtained in an Orang Asli settlement in Selangor and found presence of relapsing fever group-associated borreliae in a *H. hystrici*s tick, suggesting that wildlife-associated ticks may serve as important reservoirs for zoonotic pathogens. On the other hand, a study by Ishak et al. (2019) screened a total of 106 ticks collected from entrapped small mammals in Selangor and found no presence of *Rickettsia* sp., indicating possible spatial, temporal, or host-specific variation in pathogen prevalence. To our knowledge, no tick-borne pathogens have been previously reported in ticks collected from Sunda pangolins in Malaysia, revealing a critical gap in current epidemiological understanding of this endangered host species.

CONCLUSION

In conclusion, *Rickettsia* spp., *Ehrlichia* spp., *Bartonella* spp., *Anaplasma* spp., *Trypanosoma* spp., or *Borrelia* spp. were not detected in *A. javanense* ticks collected from captive *M. javanica*

in Sungkai Wildlife Conservation Centre, Perak. However, there still may be presence of pathogens harboured by the pangolins below detectable levels. Further studies incorporating larger sample sizes, pathogen isolation, and host blood screening using more sensitive molecular approaches are recommended to better understand the epidemiological role of *A. javanense* and its associated microbial communities.

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AUTHORS DECLARATIONS

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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.

Ethics Declarations

The study protocol was approved by the University of Malaya Institutional Animal Care and Use Committee (UM IACUC, approval code: T/22052023/13032023-02/R). No human subjects were involved.

Data Availability Statement

Data supporting main conclusions of this study are included in the manuscript. Files containing raw data on sample details can be requested directly from the corresponding author.

Authors' Contributions

Conceptualization: NAH, ZY, AST; Data gathering: NAH, JTAA, AST, MSM; Data visualization: NAH; Original draft preparation: AST, NAH; Review and editing: ZY, NAH; Supervision: ZY, SAB. All authors read and approved the final manuscript.

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