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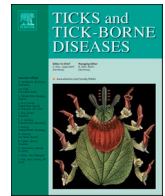
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Short communication

Reevaluating the presence of *Rhipicephalus australis* (Acari: Ixodidae) in Southeast Asia: A phylogenetic approach based on Cambodian tick samples

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ABSTRACT

Morphological variability between *Rhipicephalus australis* and *R. microplus* has led to taxonomic ambiguity, leading to species misidentification. *Rhipicephalus australis* is reported to have a distribution range in Pacific Ocean region extending to several Southeast Asian countries, including Cambodia, although its presence in continental Southeast Asia has not been supported by molecular data. With growing evidence of conflicting morphological characters, this study aimed to evaluate the presence of *R. australis* in Cambodia using both morphological and molecular identification. Tick specimens were collected from cattle across 21 provinces of Cambodia, and a subset of 95 *R. microplus* complex (37 morphologically identified as *R. australis*, 39 *R. microplus*, and 19 nymphs) was selected for molecular analysis. DNA barcoding of the *cox1* gene was performed, and a maximum likelihood phylogenetic tree revealed that all specimens clustered within *R. microplus* clade A. These findings, along with previous observations from other regions, suggest that, in the absence of molecular data, there is no definitive evidence to support the presence of *R. australis* in continental Southeast Asia, particularly in Cambodia.

1. Introduction

The *Rhipicephalus microplus* complex is a group of tick species that commonly infest bovids (Barker and Walker, 2014; Walker et al., 2014). They are of veterinary and public health importance due to their ability to transmit pathogens to both animals, mostly livestock, and humans (Tan et al., 2021; Ybáñez et al., 2013). This species complex is challenging to distinguish morphologically, but analyses of mitochondrial genes as markers determined five phylogenetically distinct clades: *R. australis*, *R. annulatus*, and three *R. microplus* clades (clade A, B, and C) (Burger et al., 2014; Low et al., 2015). The *Rhipicephalus microplus* complex is widely distributed worldwide (Burger et al., 2014). However, a more detailed examination of the five clades provides greater insight into their regional distribution. *Rhipicephalus microplus* clade A has been recorded in various regions, including South America, Southeast Asia, Africa, and North America (Burger et al., 2014; Roy et al., 2018). Clade B has been predominantly identified in China and in India (Burger et al., 2014), while clade C has been documented in South and Southeast Asian countries, such as India, Thailand, Malaysia, Myanmar, Pakistan, and Bangladesh (Low et al., 2015; Roy et al., 2018; Tantrawatpan et al.,

2022). On the other hand, *R. australis* has been described in Australia, New Caledonia, Indonesia, Philippines, New Guinea, Tahiti and Cambodia (Estrada-Peña et al., 2012). Finally, *R. annulatus* has been recorded on various continents, including Southern Europe, West and Central Asia, North and Sub-saharan Africa, and the Americas (Texas-Mexico border) (Burger et al., 2014). In Southeast Asia, *R. annulatus* has only been recorded in Vietnam (Chien et al., 2016; Hornok et al., 2024).

The species status of *R. australis* was reinstated in 2012 by Estrada Peña et al., based on molecular evidence from specimens collected in Australia, New Caledonia and Indonesia (Estrada-Peña et al., 2012). They also provided a set of morphological characters to distinguish them from *R. microplus*. While the genetic data showed clear separation of the two species, the morphological characters to distinguish them are inconsistent and problematic. A study in Malaysia reported that specimens morphologically identified as *R. australis* were actually assigned to *R. microplus* clade A through *cox1* DNA barcoding (Kazim et al., 2022). This discrepancy between morphological and molecular identification has also been reported in other countries, where *R. microplus* clade A and clade C specimens possess characteristics similar to *R. australis* (Low

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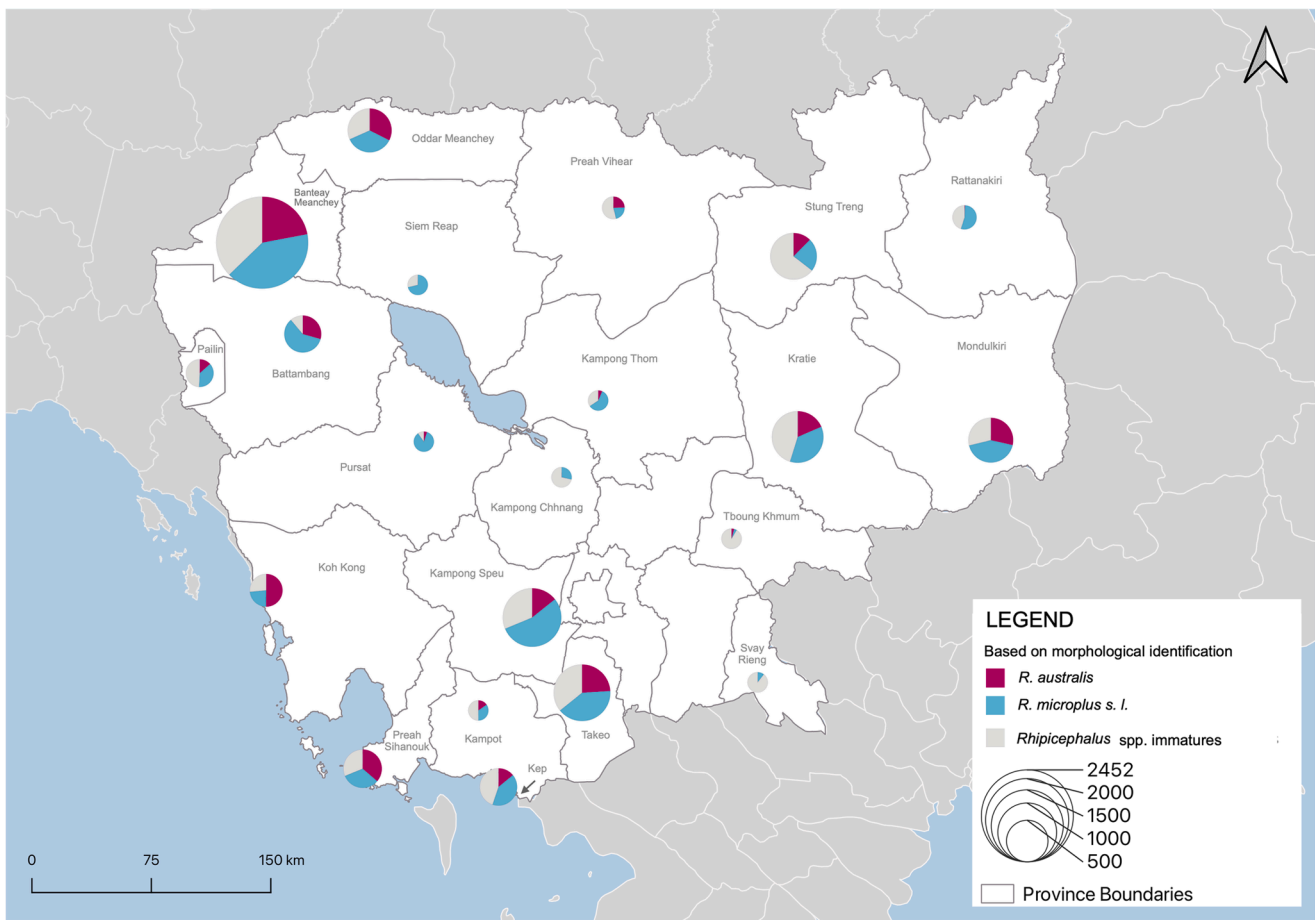


Fig. 1. Distribution of *Rhipicephalus microplus* complex ticks collected in this study based on morphological identification.

et al., 2015; Roy et al., 2018). In the wake of these growing disagreements and the still unresolved distribution of *R. australis* in Southeast Asia, this study aimed to assess its presence in continental Southeast Asia, specifically for Cambodia.

2. Materials and methods

The tick specimens used in the molecular analysis were collected from various locations across 21 provinces of Cambodia, including from cattle farms and household-level cattle rearing in rural and urban settings. Ticks were collected directly from cattle through visual inspection by trained technicians, who examined each animal for a total of 30 min. Ticks found on the cattle were carefully removed using fine-tipped forceps, by grasping the mouthparts and slowly pulling upward to ensure all mouthparts were properly detached from the skin, causing minimal discomfort to the animals. The collected ticks were transferred into 15 ml conical tubes containing 70 % ethanol and labeled according to host, habitat, and locations. In the laboratory, ticks were identified morphologically and sorted by species using species descriptions (Barker and Walker, 2014; Estrada-Peña et al., 2012; Walker et al., 2014; Yamaguti et al., 1971) and identification keys developed in-house by the Medical and Veterinary Entomology Unit at Institut Pasteur du Cambodge (IPC).

A subset of samples was randomly selected by species and location, resulting in a total of 95 individual ticks, morphologically identified as *R. australis* ($n = 37$), *R. microplus* s.l. ($n = 39$), and *Rhipicephalus* spp. nymphs ($n = 19$) and subjected to molecular analysis. DNA from ticks was extracted using a QIAamp DNA mini kit (Qiagen, Germany) with slight modifications to the protocol to incorporate non-destructive methods (Takhampunya et al., 2021). A small incision was made in the abdomen of the tick specimen with a surgical blade before the

addition 180 μ L of buffer ATL and 20 μ L of proteinase K. The ticks were then incubated overnight at 56 $^{\circ}$ C to allow sufficient enzymatic digestion of the tissues. The exoskeleton of the tick was transferred to a new 1.5 ml tube, washed several times in distilled water, and finally kept in 70 % ethanol for taxonomic study, while 200 μ L of the supernatant was used to follow the subsequent steps of the DNA extraction kit, according to the manufacturer's protocol.

A fragment of *cox1* gene was amplified by PCR using a set of primer pairs LCO1490/HCO2198 (Folmer et al., 1994). Each 40 μ L of PCR mix contained 4 μ L of 10X PCR buffer B2 (Solis Biodyne, Estonia), 0.8 μ L of each primer (10 μ M), 0.4 μ L of 20 mM dNTP mix, 5.6 μ L of 25 mM $MgCl_2$, 0.4 μ L of Hot firepol DNA polymerase (5U/ μ L) (Solis Biodyne, Estonia), 23 μ L of nuclease-free water, and 5 μ L of DNA template. PCR assays were conducted using a T100 thermal cycler (Biorad, USA) using protocol as follows: initial denaturation at 95 $^{\circ}$ C for 15 min; followed by 40 cycles of 95 $^{\circ}$ C for 1 min, 50 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 1 min; followed by a final extension of 72 $^{\circ}$ C for 10 min. The resulting amplicons (710 bp) were visualized using gel electrophoresis and were sent to Macrogen (Daejeon, South Korea) for purification and DNA sequencing.

The sequence reads were processed using Unipro UGENE version 50.0 (Okonechnikov et al., 2012) and the consensus sequences (GenBank accession number PQ838663-PQ838765, Table S2) were aligned with reference sequences retrieved from the GenBank database using ClustalW for multiple alignment. A maximum likelihood phylogenetic tree was constructed using the best-fit nucleotide substitution model with 1000 bootstrap replicates in IQ-TREE version 2.3.5 (Minh et al., 2020). The phylogenetic tree was visualized and edited using Interactive Tree of Life (iTOL) version 6.9.1 (Letunic and Bork, 2024).

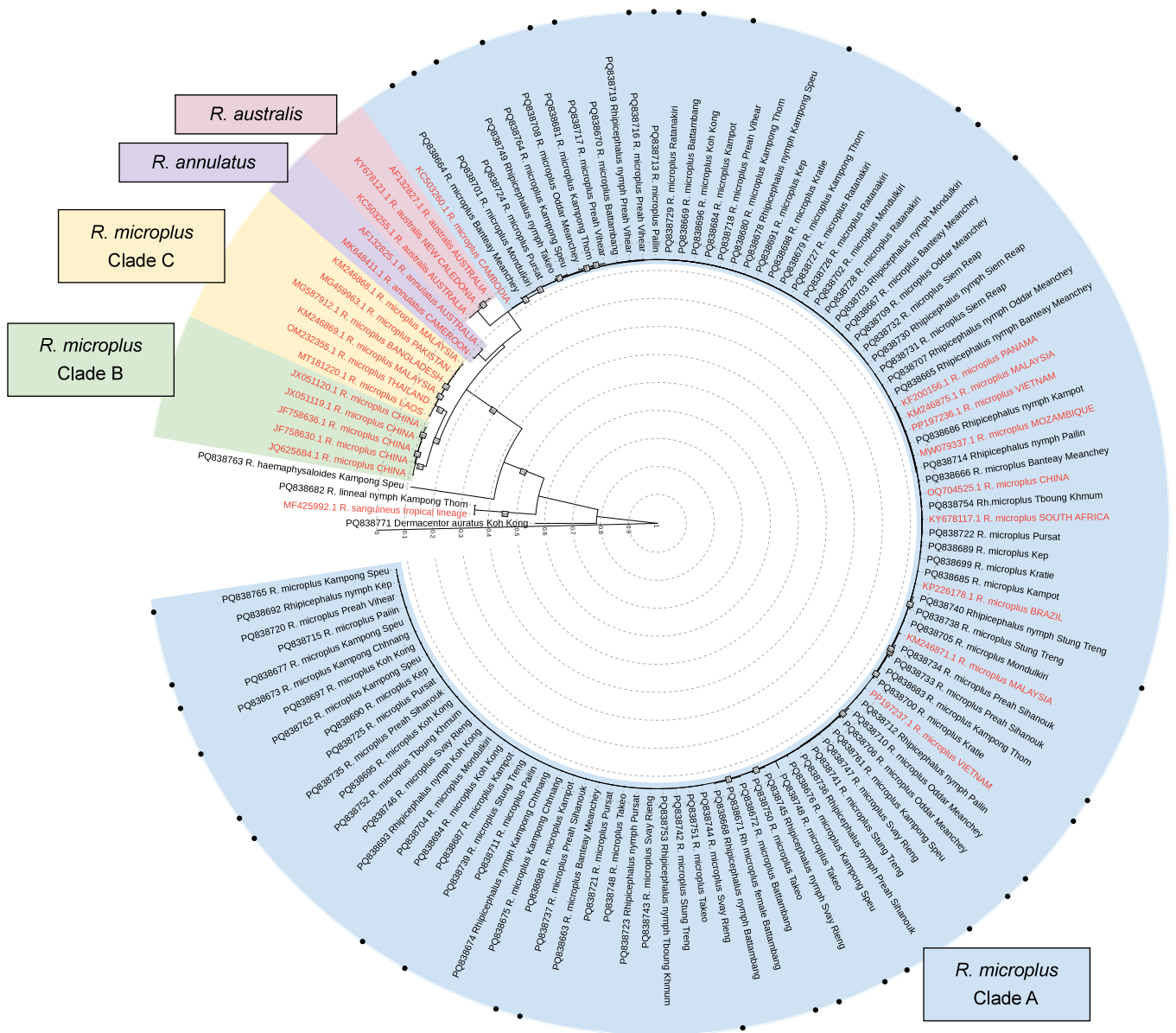


Fig. 2. Maximum likelihood phylogenetic tree of *R. microplus* complex in Cambodia based on a fragment of the *cox1* gene. Text in red indicates sequences obtained from Genbank, while text in black represents sequences from this study. All *R. microplus* complex specimens, including those possessing *R. australis* characters (indicated by black dots), were grouped within *R. microplus* clade A.

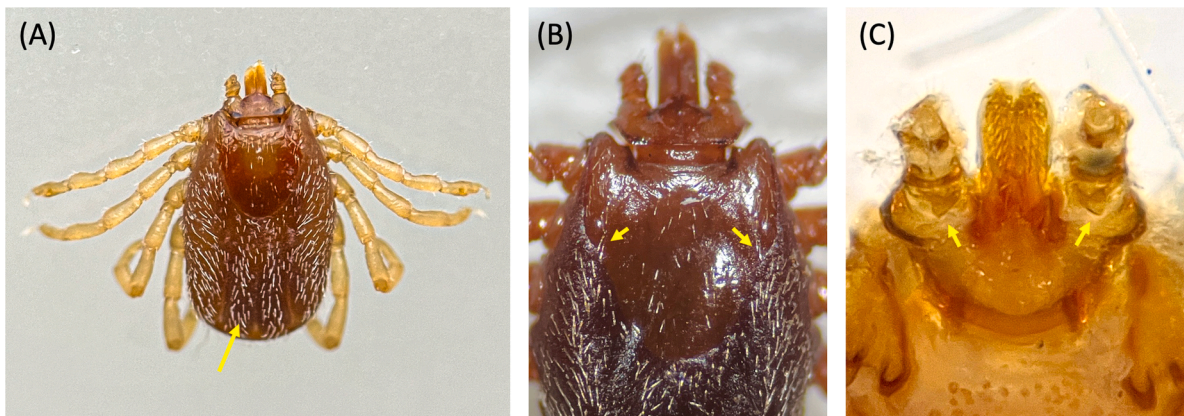


Fig. 3. *Rhipicephalus microplus* clade A from Cambodia exhibit characteristics of *R. australis*, as indicated by yellow arrows. This includes the presence of abundant alloscutal setae (A) and setae posterior to the eyes (B) in female specimens, as well as the retroverted ventral spurs on palpal article 1 (C) in males.

3. Results and discussion

During the nationwide tick sampling, a total of 5304 ticks belonging to the *R. microplus* complex were collected from 97 infested cattle out of 120 inspected. Among them, 1205 were morphologically identified as *R. australis*, 2239 as *R. microplus*, and 1860 immature stages. Both identified species were detected in nearly all sampled provinces, indicating a widespread distribution rather than a geographically clustered pattern (Fig. 1). However, despite these morphological classifications, *cox1* sequence analysis revealed that all ticks belonged to a single species, *R. microplus* (Fig. 2). This result is consistent with studies from Malaysia (Kazim et al., 2022). Despite extensive nationwide sampling, the results indicate that *R. microplus* from Cambodia belongs exclusively to clade A, in contrast to studies from Malaysia and Thailand, where both clades A and C have been reported (Low et al., 2015; Tantrawatpan et al., 2022). However, the limited number of *cox1* sequences analyzed in this study raises the possibility that other clades, if present in very low numbers, may have gone undetected.

Some *R. microplus* from Cambodia possessed morphological characters of *R. australis*, particularly the presence of spurs on the ventral side of palpal article 1 in males, and the abundance of median alloscutal setae that clustered in four to six rows in females (Fig. 3). Same inconsistencies have been reported in Asia, central America and South Africa (Guzmán et al., 2021; Kaewmongkol et al., 2015; Kazim et al., 2022; Londt and Arthur, 1975; Roy et al., 2018). Most male specimens of *R. microplus* examined in a study from Thailand possess spurs on the ventral side of palpal article 1, a characteristic typically observed in *R. australis* males (Kaewmongkol et al., 2015). Similar traits were also noted in specimens from Bangladesh, Myanmar, Pakistan, Cuba and South Africa (Guzmán et al., 2021; Londt and Arthur, 1975; Roy et al., 2018). Additionally, a female specimen of *R. microplus* from Myanmar exhibited abundant dorsal median alloscutal setae arranged in 4–6 rows, and females from Bangladesh, Myanmar, Pakistan displayed setae behind the eyes, both of which are characteristics of *R. australis* (Roy et al., 2018). The high variation in these characters might be associated with the nature of this species complex, which follows a one-host life cycle that may facilitate cohabitation and potential cross-breeding, generating offspring with mixed characters (Ali et al., 2016; Berry, 2017; Kaewmongkol et al., 2015; Kazim et al., 2022).

While the morphological characters and molecular data of *R. australis* appear consistent in specimens from Australia, Indonesia, and New Caledonia, this is not the case for Cambodia and other countries in Southeast Asia, where the presence was never supported by molecular data (Estrada-Peña et al., 2012). The growing evidence of conflicting morphological characters may refute earlier claims of *R. australis* presence in the region and suggest that its actual range may not extend to continental Southeast Asia as previously thought. The findings of this study, along with similar observations elsewhere (Guzmán et al., 2021; Kaewmongkol et al., 2015; Kazim et al., 2022; Londt and Arthur, 1975; Roy et al., 2018), demonstrate that the morphological characters described by Estrada-Peña (Estrada-Peña et al., 2012) cannot be used as the sole reference to distinguish *R. australis* from *R. microplus*. Until validated by molecular data, there is currently no evidence supporting the presence of *R. australis* in continental Southeast Asia.

Ethical statement

The study was authorized and facilitated by the General Directorate of Animal Health and Production (GDAHP) within the Ministry of Agriculture, Forestry, and Fisheries of Cambodia, under permit No. 2981, issued on October 1, 2021.

CRedit authorship contribution statement

Didot Budi Prasetyo: Writing – original draft, Visualization,

Methodology, Formal analysis, Data curation. **Sony Yean:** Writing – original draft, Methodology, Investigation, Data curation. **Sébastien Boyer:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ttbdis.2025.102478](https://doi.org/10.1016/j.ttbdis.2025.102478).

Appendix. Supplementary material

Table S1. *Rhipicephalus microplus cox1* sequences generated in this study

Data availability

All sequence data generated in this study have been deposited in GenBank and are publicly accessible under the accession numbers PQ838663–PQ838754, PQ838761, PQ838762, PQ838764, and PQ838765. Other data will be made available on request.

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