



Morphological abnormalities and multiple mitochondrial clades of *Rhipicephalus haemaphysaloides* (Ixodida: Ixodidae)

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Received: 28 April 2022 / Accepted: 25 June 2022 / Published online: 11 July 2022
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Abstract

Rhipicephalus haemaphysaloides is endemic in South and Southeast Asia, and it is known to parasitise domestic animals such as cattle, horses, sheep, and dogs. Despite infestation on multiple hosts, little attention has been paid to its morphological and molecular variation. The present study describes local morphological abnormalities for the first time in *R. haemaphysaloides* from Malaysia, and highlights the presence of three cytochrome c oxidase subunit I (*COI*) operational taxonomic units with 0.46–9.81% distances within the so-called *R. haemaphysaloides* in Malaysia, Thailand, and Pakistan.

Keywords Ticks · Ixodidae · *Rhipicephalus* · Abnormalities · Malaysia

Introduction

Morphological abnormalities in ticks are an extraordinary phenomenon in nature (Shuaib et al. 2020). The occurrence of these monstrosities in ticks could happen naturally (Latif et al. 1988; Dergousoff and Chilton 2007) or by experimental manipulation (Oliver and Delfin 1967; Buczek 2000). Campana-Rouget (1959a, b) classified the morphological abnormalities of ticks into two groups: general and local anomalies. General anomalies are described as malformations affecting the whole tick idiosoma morphology, such as asymmetry, gynandromorphism, duplication, nanism and gigantism (Campana-Rouget 1959a). Local anomalies, on the other hand, include changes or abnormalities of specific parts of the tick idiosoma, such as asymmetry of spiracle and scutum, fusion of adanal plates and malformation of capitulum (Campana-Rouget 1959b). Morphological abnormalities in ticks belonging to various genera and families

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from different parts of the world have been documented (Ren et al. 2016; Chitimia-Dobler and Pfeffer 2017; Chitimia-Dobler et al. 2017; Soghigian et al. 2017; Keskin 2018; Molaei and Little 2018; Muñoz-Leal et al. 2018; Azzi et al. 2019; Balinandi et al. 2019; Chong et al. 2020; Molaei and Little 2020; Molaei et al. 2020; Salceda-Sánchez et al. 2020; Shuaib et al. 2020).

In the present study, we report the morphological abnormalities in a native male *R. haemaphysaloides*, for the first time from Peninsular Malaysia. We also compare the genetic profile of both normal and abnormal specimens, and other reference sequences by using the mitochondria-encoded *COI* gene—a marker that has been proven useful for discerning hidden genetic diversity of *Rhipicephalus* ticks (Low et al. 2015).

Materials and methods

Ticks were collected from cattle farms in Selangor and Pahang from August 2021 to December 2021. The ectoparasites were collected using forceps and preserved in collection tubes filled with 90% ethanol. Collected samples were then transferred to the Parasitology Laboratory in the Institute of Medical Molecular Biology (IMMB), Universiti Teknologi MARA (UiTM) Sungai Buloh Campus for morphological examination under a stereo microscope (Olympus SZX7 Zoom Stereo Microscope, Japan). Ticks were identified at the species level using specialised taxonomic keys (Anastos 1950; Kohls 1957; and Walker et al. 2000).

An abnormal male and a normal male of *R. haemaphysaloides* were subjected to DNA extraction using the Nucleospin DNA Extraction Tissue Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. Due to the difficulty of amplifying the tick *COI* gene fragments using the standard conventional polymerase chain reaction (PCR), a nested PCR of targeting the same gene was conducted instead, following the protocols described in Low et al. (2015). The PCR products were sent to a local company (Apical Scientific, Selangor, Malaysia) for Sanger DNA sequencing.

The *COI* sequences of normal (ON076883) and abnormal ticks (ON076884) generated from this study were deposited in the National Center for Biotechnology Information (NCBI) GenBank DNA sequence database. The phylogenetic tree was constructed based on the reference sequences in Bakkes et al. (2021) and closest reference nucleotide sequences displayed in the Basic Local Sequence Alignment Tool. All sequences were aligned using MAFFT v.7 (Katoh et al. 2019). A maximum likelihood (ML) analysis was performed on an on-line web-based server PhyML (Lefort et al. 2017). Automatic model selection was implemented based on the Akaike information criterion (AIC). The best fit model was the general time-reversible (GTR+G+I) model with a proportion of invariable sites of 0.500 and with a gamma shape parameter of 0.507. An Assemble Species by Automatic Partitioning (ASAP) analysis (Puillandre et al. 2021) was performed to delimit species boundaries among *Rhipicephalus* ticks. Pairwise genetic distances among *Rhipicephalus* ticks were computed using MEGA X (Kumar et al. 2018).

Results and discussion

In total, seven *R. haemaphysaloides* ticks (four males and three females) were morphologically identified. The males were identified by the presence of sickle-shaped adanal plates, comma-shaped spiracles and smooth scutum, whereas females were distinguished

by the narrowly U-shaped genital aperture, less coarse scutum punctuations, and weak and sparsely distributed body setae (Anastos 1950; Kohls 1957; Walker et al. 2000). Of the seven specimens, only one male showed local abnormalities at both sides of coxae (Fig. 1A, C). On the left side of the ventrum, the coxa III and its associated trochanter were presented with atrophy, where the width and length of both appendages were considerably shorter (coxa III width and length: 264 and 356 μm , respectively; trochanter III width and length: 151 and 296 μm , respectively) than those in coxa II (coxa II width and length: 284 and 397 μm , respectively; trochanter II width and length: 193 and 352 μm , respectively) (Fig. 1C). A close-up of the normal size of coxa III and its trochanter is shown in Fig. 1D. On the right side of the tick ventrum, ectomely was seen in coxa II, and the abnormal enlargement of coxa III where the size was similar or slightly larger than coxa IV (Fig. 1C).

This study is the first report of morphological abnormalities in ticks from Peninsular Malaysia and represents the third report of this phenomenon in *R. haemaphysaloides* worldwide (Campana-Rouget 1959a). Additionally, this is also the first case of local abnormalities (both leg atrophy and ectomely) in this species, as the previous two only reported general abnormalities (Campana-Rouget 1959a). In the first report of morphological anomaly, Warburton (1912) described a case of gigantism in *R. haemaphysaloides* females from Asia (number and locality not specified). According to the author, the abnormal females (presumably un-engorged according to the figure in the article) were measured

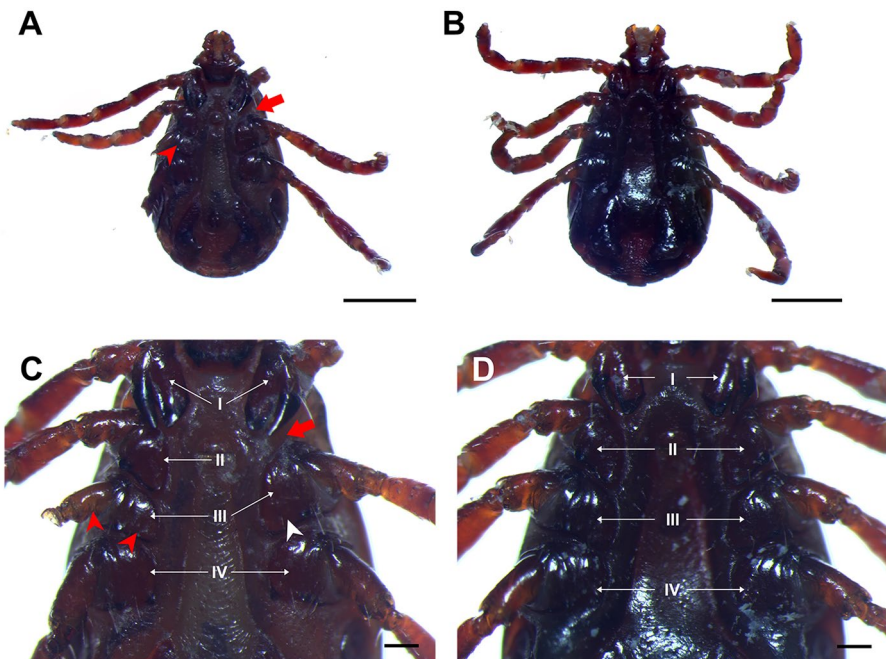


Fig. 1 Morphological comparison of an abnormal (A, C) and normal (B, D) *Rhipicephalus haemaphysaloides* male. Full view of the A abnormal and B normal ventrum. The arrow indicates ectomely of the right second leg and coxa, whereas the red arrowhead indicates atrophy of the left coxa III. Scale bars: 1000 μm . Close-up of the C abnormal and D normal ventrum. The red arrow shows ectomely of the right coxa II, red arrowheads indicate atrophy of the left coxa III and its associated trochanter, and the white arrowhead shows abnormal enlargement of the right coxa III. Coxae numbers are represented by Roman numerals (I–IV). Scale bars: 200 μm . (Color figure online)

1.62× longer than the normal specimens, and all were sampled from the same environmental setting. The difference in size of these ticks was thought to be mainly attributed to the dietary nutrition in the immature stages (Campana-Rouget 1959a). The second report was documented by Sharif (1930) on the case of body asymmetry in a *R. haemaphysaloides* female from India. The tick was collected from an infested calf, and showed curvatures of the body, capitulum and scutum towards the right side (according to the ventral point of view). The author also noted that the fourth leg on the right side was missing. In the latter statement, we cannot consider the missing leg in Sharif's tick as a local abnormality, due to the unknown cause whether it was due to external damage or defect. This is accentuated by the extremely brief description of the missing leg (no explanation on whether the coxa IV was intact or absent) and the use of the silhouette in the figure section, which did not provide answers on the cause of the missing leg.

To date, there are 17 *Rhipicephalus* species reported with morphological abnormalities worldwide (Table 1). Among them, four belonged to the cattle-specific *Boophilus* subgenus: *R. annulatus*, *R. decoloratus*, *R. geigy*, and *R. microplus*. Almost all the species were of Afrotropical origin (Walker et al. 2000; Guglielmone et al. 2014). *Rhipicephalus haemaphysaloides* was the only Oriental *Rhipicephalus* species reported with morphological anomalies, aside from the cosmopolitan brown dog tick, *R. sanguineus* and cattle tick *R. microplus*. This underlines the lack of information on the morphological abnormalities in *Rhipicephalus* ticks from this region, particularly species that were region-exclusive such as *R. pilans*, *R. tetracornus* and *R. ramachandrai* (Walker et al. 2000).

Thus far, the external cause of morphological anomalies in hard ticks is not known, but a study suggested that heavy metal pollutants may cause abnormalities in ticks (Kittler 2011). Furthermore, several authors also considered various biological and non-biological factors as possible causes of tick abnormalities, such as somatic or germinal mutations, exposure to chemical agents, host resistance to tick infestation and environmental stress (Campana-Rouget 1959a, b; Guglielmone et al. 1999; Dergousoff and Chilton 2007).

The *COI* sequences of both normal and abnormal tick sequences generated from the present study had 0.46% difference over 439 bp, suggesting intraspecific variation within this strain. Both specimens also differed from the strains of Thailand by 0.92–1.38% and Pakistan by the extraordinary high distances of 8.75–9.56%. The ML phylogenetic tree revealed three clades of *R. haemaphysaloides* (Fig. 2), implying the presence of different species within the so-called *R. haemaphysaloides*. Ghafar et al. (2020) also suggested the existence of two distinct species within *R. haemaphysaloides* in Pakistan with 0.2–7.6% distances based on *COI* sequences. A recent study by Tantrawatpan et al. (2022) also reported high levels of haplotype and nucleotide diversity in *R. haemaphysaloides* in northeast Thailand. Nevertheless, both studies did not perform species delimitation test to clarify further their species boundaries. To fill this knowledge gap, we included various species of *Rhipicephalus COI* sequences and subjected them in an ASAP analysis. Likewise, the test suggested three OTUs within *R. haemaphysaloides*: specimens from Malaysia and Thailand of Southeast Asia represent a distinct taxon (OTU 1), whereas specimens from Pakistan of South Asia comprised two distinct taxa (OTU 2 and OTU 3). In future study, additional genetic markers such as 16S and 12S ribosomal RNA (rRNA) genes could be tested on these OTUs to establish whether the results are similar.

In view of the distinctiveness of *R. haemaphysaloides* from various geographic regions, future taxonomic studies involving detailed morphological data on the type material from Myanmar of Southeast Asia and the OTUs identified in this study are warranted to provide a better insight on the taxonomic status of this species. Whether the name of *R. haemaphysaloides* should be applied to populations across the entire

Table 1 List of known *Rhipicephalus* species presented with morphological abnormalities

<i>Rhipicephalus</i> species	Morphological abnormalities		References
	General	Local	
<i>R. annulatus</i>	x	x	Nuttall (1914), Campana-Rouget (1959a), Sakla et al. (1980), Keskin (2018)
<i>R. appendiculatus</i>	x		Nuttall (1914), Campana-Rouget (1959a), Mnase et al. (1987), Keskin (2018)
<i>R. bursa</i>	x	x	Campana-Rouget (1959a), Kar et al. (2015), Keskin et al. (2016)
<i>R. compositus</i>		x	Campana-Rouget (1959b)
<i>R. decoloratus</i>	x	x	Campana-Rouget (1959a), Gothe (1967), Balimandi et al. (2019), Shuaib et al. (2020), Keskin (2018)
<i>R. evertsi</i>	x	x	Campana-Rouget (1959a, b), Shuaib et al. (2020)
<i>R. geigy</i>	x		Salceda-Sánchez et al. (2020)
<i>R. haemaphysaloides</i>	x	x*	Campana-Rouget (1959b), this study
<i>R. kochi</i>	x		Campana-Rouget (1959a)
<i>R. longiceps</i>		x	Nuttall (1914), Campana-Rouget (1959b)
<i>R. maculatus</i>	x	x	Campana-Rouget (1959a, b)
<i>R. microplus</i>	x	x	Campana-Rouget (1959b), Guglielmo et al. (1999), Domínguez and Bermúdez (2020), Diyes and Rajakurama (2021)
<i>R. pulchellus</i>	x		Nuttall (1914), Campana-Rouget (1959a), Keskin (2018)
<i>R. sanguineus</i>	x	x	Warburton and Nuttall (1909), Nuttall (1914), Pereira and Castro (1945), Campana-Rouget (1959a, b), Guglielmo et al. (1999), Estrada-Peña (2001), Labruna et al. (2002), Serra-Freire and Borsoi (2009), Keskin (2018), Salceda-Sánchez et al. (2020), Laatamna et al. (2021)
<i>R. simus</i> **	x	x	Campana-Rouget (1959a, b)
<i>R. tricuspis</i>		x	Campana-Rouget (1959b)
<i>R. turanicus</i>	x	x	Campana-Rouget (1959a), Kar et al. (2015), Keskin et al. (2016)

*First report worldwide

**Salceda-Sánchez et al. (2020) speculated that this species may be *Rhipicephalus praetextatus*, not *R. simus*

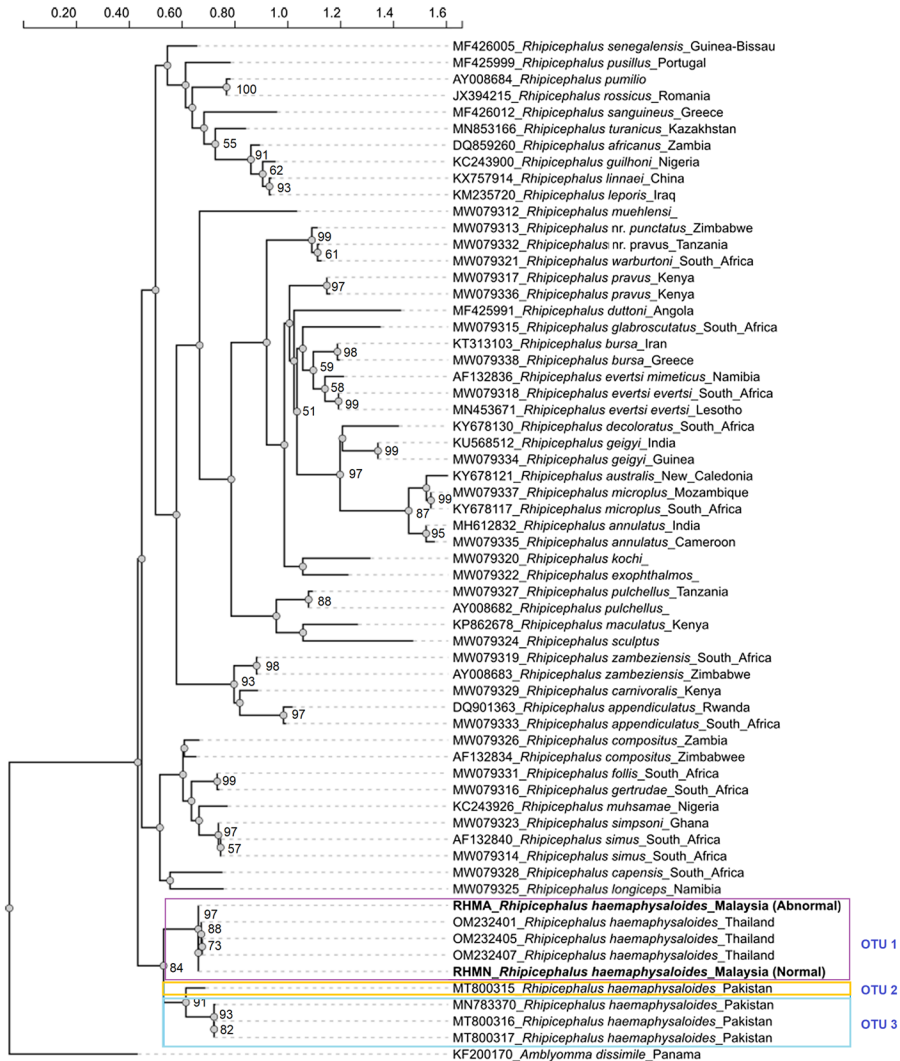


Fig. 2 Maximum likelihood phylogenetic tree of *Rhipicephalus* taxa based on *COI* sequences. Bootstrap values > 50% are shown on the branches. Sequences generated from the present study are in bold

range of Southeast Asia or whether its synonyms should be reinstated for other geographic regions requires further investigation.

Acknowledgements The authors would like to thank the Faculty of Medicine and Institute of Medical Molecular Biotechnology (IMMB), Universiti Teknologi MARA (UiTM), Sungai Buloh campus, Malaysia and the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany for their constant support. This study was supported by the Higher Institution Centre of Excellence (HiCoE) program (MO002-2019) and funded by Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (100-TNCPI/INT 16/6/2 (005/2020)).

Author contributions Abdul Rahman Kazim conducted tick collection, morphological identification, provided figures and tables, and wrote the main manuscript text. Van Lun Low conducted the molecular analyses and contributed to the main manuscript text. Dennis Tappe provided the funds for this project and had also contributed to the main manuscript writing. Jamal Houssaini and Chong Chin Heo contributed to the main manuscript writing. All authors reviewed the manuscript.

Funding Bernhard Nohct Institute for Tropical Medicine, Germany, 100-TNCPI/INT 16/6/2 (005/2020), 100-TNCPI/INT 16/6/2 (005/2020)

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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