

Original Article



Morphological and Molecular Identification of Brown Dog Ticks *Rhipicephalus linnaei* “Tropical Lineage” Isolated From Pet Dogs in Some Small Animal Hospitals Around Hanoi, Vietnam

Nguyen Yen Thi Hoang^{1*} , Tran Thi Huong Giang² , Vu Thi Thu Tra³

1. Department of Parasitology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam.

2. Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam.

3. Department of Public Health, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam.

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ABSTRACT

Background: The brown dog ticks, *Rhipicephalus sanguineus* complex, are distributed worldwide. According to the molecular data and geographical distribution, the *R. sanguineus* complex is divided into two clades: *R. sanguineus* sensu lato “tropical lineage” and *R. sanguineus* sensu stricto “temperate lineage.” However, the recent morphological and molecular identification data re-established a new neotype, *R. linnaei*, that shares similar morphological characteristics to *R. sanguineus*, leading to misidentification.

Objectives: This study aims to identify and confirm that *Rhipicephalus* dog tick species are prevalent in Vietnam.

Methods: Adult ticks were collected from pet dogs in small animal hospitals around Hanoi, Vietnam. They were then identified based on morphological characteristics and molecular analysis, such as PCR and sequencing techniques.

Results: The species of *Rhipicephalus* identified in this study was *R. linnaei* with typical morphological characteristics, including narrowly elongated comma-shaped spiracular in males and broadly U-shape genital pore atrium in females. The phylogenetic analysis indicated that this isolate was closely related to *R. linnaei* from Angola, and the genetic divergences between this isolate in this study with *R. linnaei* Angola strain were 0.24%, 0.5%, and 0.0% for *cox1*, *12S rDNA*, and *16S rDNA* genes, respectively.

Conclusion: This is the first report on the prevalence of *R. linnaei* in dogs in Vietnam and the genetic variation of this species within the referenced *R. sanguineus* complex in Vietnam, providing essential data for tick taxonomy.

Keywords: Pet dogs, *Rhipicephalus linnaei*, *Rhipicephalus sanguineus*, Small animal hospital, Vietnam

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* Corresponding Author:

Nguyen Thi Hoang Yen, PhD.

Address: Department of Parasitology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Trau Quỳ, Gia Lam, Ha Noi, Viet Nam

Phone: + (84) 982595128

E-mail: nthyen@vnu.edu.vn



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Introduction

The *Rhipicephalus sanguineus* complex parasitizing dogs comprises 17 species (Sanches et al., 2016), which have recently been divided into two clades: *R. sanguineus sensu stricto* (s.s), referred to as the “temperate lineage,” which is geographically distributed in temperate regions of Latin America (Argentina, Uruguay, Chile, the United States) and Europe (Spain, Portugal, Italy, France, Germany) and *R. sanguineus sensu lato* (s.l), termed the “tropical lineage,” which is geographically distributed in tropical regions, such as Southeast Asia, China, and India (Chandra et al., 2019; Sanchez-Montes et al., 2021; Pascoe et al., 2022; Grant et al., 2023). However, the identification based on the morphology of these species is difficult because of the similarity of morphological characteristics, leading to potential misidentification. Thus, identification based on morphological characteristics and molecular data has become necessary to distinguish tick species.

The combination of morphological and molecular data has recently been proposed to re-establish the name *R. linnaei* to replace *R. sanguineus* s.l “tropical lineage” species (Šlapeta et al., 2021; Šlapeta et al., 2022; Almazán et al., 2023). Morphologically, *R. linnaei* is characterized by narrowly elongated comma-like spiracles when observed dorsally in males, in contrast to the non-tapered and shorter extension of spiracles in *R. sanguineus* males. Additionally, *R. linnaei* females exhibit a broadly U-shaped genital pore atrium, while *R. sanguineus* females display a narrower U-shape one (Šlapeta et al., 2022). Molecularly, the mitochondrial markers, including *cox1*, *12S rDNA*, and *16S rDNA*, have been extensively utilized for tick identification, phylogenetic analysis, and evaluation of tick population diversity (Moraes-Filho et al., 2011; Chandra et al., 2019; Dantas-Torres et al., 2013). The intra-specific genetic divergence ranges from 1.8% to 8% depending on the target gene (up to 6.6% for *16S rDNA*, 8% for *12S rDNA*, and 3.5% for *cox1*) (Beati et al., 2001; Szabó et al., 2005; Burlini et al., 2010; Dantas-Torres et al., 2013; Almazán et al., 2023). The average level of inter-specific variation was 10.8%, 9.9%, and 13.3% for *16S rDNA*, *12S rDNA*, and *cox1*, respectively (Dantas-Torres et al., 2013).

In Vietnam, *R. sanguineus* s.l tropical lineage has been the sole species identified based on morphological and molecular identification (Nguyen et al., 2018; Huynh et al., 2020; Do et al., 2024). With the redescription of *R. linnaei* as a neotype for *R. sanguineus* s.l tropical lineage, identifying tick species is crucial for epidemiologi-

cal investigations and understanding their role in pathogen transmission to animals and humans. Some workers reported the detection of several pathogens, such as *Anaplasma* spp. *Rickettsia* spp. *Babesia* spp. *Mycoplasma* spp., *Hepatozoon* spp., and *Borrelia* spp., from *R. sanguineus* in Vietnam (Nguyen et al., 2018; Huynh et al., 2020; Do et al., 2024). This study aims to identify and confirm the presence of *R. linnaei* collected from pet dogs in Hanoi, Vietnam, and to assess the molecular diversity of this species in relation to referenced species using mitochondrial markers. By confirming the presence of *R. linnaei* and assessing its molecular diversity in Hanoi, this study seeks to fill the critical knowledge gap regarding this species’ presence and genetic variation within the referenced *R. sanguineus* complex in Vietnam, providing essential data for tick taxonomy.

Materials and Methods

Collection of ticks

Two hundred and eight ticks (predominantly in the adult stage) were collected from 47 naturally infected pet dogs during grooming and health checks at several small animal hospitals near Hanoi. The ticks were preserved in 70% ethanol for morphological examination (Barker, 2023; Barker & Walker, 2014). Images of the ticks were captured using a stereo microscope connected to a camera (Olympus, SZX7, Japan).

Isolation of tick genomic DNA

After morphological examination, male ticks were individually isolated and placed into 1.5 mL Eppendorf tubes. Before DNA extraction, the ticks were dissected to remove their heads. The head was subsequently fragmented using a sterile surgical blade and utilized for DNA extraction. Genomic DNA was extracted using the Genomic DNA Pre Kit for animal tissue (BioFact, Korea) with minor modifications. Specifically, the lysis step was extended to an overnight incubation at 56 °C instead of the recommended 10 minutes. All other steps were performed according to the manufacturer’s instructions. The extracted DNA was stored at -20 °C until further use.

Polymerase chain reaction (PCR) assay was used to amplify mitochondrial cytochrome c oxidase subunit 1 (*cox1*), *12S rDNA*, and *16S rDNA* genes.

The genomic DNA was isolated from individual ticks and subjected to PCR assay. The sequences of primers utilized in this study and the size of genes amplified are listed in Table 1. PCR assays were conducted in-

Table 1. PCR assays to identify tick species

Target Gene	PCR Primer Sequences	Product Size (bp)	Ref.
<i>Cox1</i>	S0725 (F1): 5'-TACTCTACTAATCATAAAGACATTGG-3'	604	Kushimo (2013)
	S0726 (R1): 5'-CTCCTCCTGAAGGGTCAAAAAATGA-3'		
<i>12S rDNA</i>	S0738 (TB1): 5'-AAACTAGGATTAGATACCT-3'	336	Beati & Keiran, (2001)
	S0739 (T2A): 5'-AATGAGAGCGACGGGCGATGT-3'		
	S0726 (R1): 5'-CCTCCTCCTGAAGGGTCAAAAAATGA-3'		
<i>16S rDNA</i>	S0749: 5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3'	276	Black & Piesman, (1994)
	S0750: 5'-TTACGCTGTTATCCCTAGAG-3'		

dependently with each primer pair (Table 1) in a total volume of 30 μ L containing 2 μ L of template DNA, 15 μ L Mastermix 2X (Phusa Biochem LTD. Company, Can Tho, Viet Nam), 0.75 μ L of each primer (10 pmol), and 11.5 μ L PCR water. Amplification conditions were performed as follows: Initial denaturation at 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 10 s, with final extension at 72 °C for 5 min. PCR products were visualized by electrophoresis on a 1.0% agarose gel in TAE 1X at 135V for 25 min. Gels were stained with GelRed® and the bands were observed under ultraviolet light.

Sequencing and phylogenetic tree analysis

Three PCR amplicons were submitted to the 1st BASE Company (Selangon, MY) for sequencing in both forward and reverse strands utilizing the same primers employed in the PCRs. DNA sequences were subsequently aligned using BioEdit software and compared with sequences from the GenBank database via the BLAST search tool. A phylogenetic tree was constructed using the maximum likelihood algorithm with the optimal substitution model in MEGA 6 software. Genetic divergence (%) between groups was calculated using Mega X software.

Results

The morphological identification of rhipicephalus ticks

The morphological characteristics observed in ticks collected in this study were as follows. Male ticks exhibited a pear-shaped body, with the widest region between legs IV and the spiracle plate. Dorsally, the scutum was slightly convex, inornate, and red-brown with unequally sized dots densely distributed anteriorly. The eleven quadrangular festoons possessed a broad caudal ap-

pendage, the eyes were flat and mildly convex, the palps were short and stout, the hypostome was short and blunt, and the basis capituli was distinctly angular hexagonal. Ventrally, the adanal plates were sub-triangular, and the spiracular plate displayed a narrowly elongated comma shape. Coxa I was deeply cleft into two elongated, large, and equal spurs (Figures 1A, 1B, 1C, 1D, and 1F). Females possessed an oval shape; the scutum was inornate, and the alloscutum was gray, distinguishing it from the scutum; the eyes were large, oval, and mildly convex. Ventrally, the festoons were indistinct when engorged, the spiracle plates exhibited a broad comma shape, the genital aperture was broadly U-shaped, and concave, and the anal opening was bean-shaped with a posterior anal groove (Figures 1G, 1H, 1I, 1K, 1L, and 1M).

The molecular identification of hard ticks

PCR assays successfully amplified three bands with lengths of approximately 350, 650, and 300 bp, corresponding to the expected sizes of *12S rDNA*, *cox1*, and *16S rDNA* genes, respectively (Figure 2). Alignment results yielded precise nucleotide counts of 358, 622, and 311 nucleotides. Phylogenetic analysis based on the partial sequence of the *cox1* gene revealed that the isolate collected in this study clustered with *R. sanguineus* reported from South Africa (KC243786), Brazil (KC243873), India (KC243872), and Vietnam (PP389595, PP398596). Notably, it clustered with strains identified as *R. linnaei* from Australia (MW429381), Fiji (MW429382), Laos (MW429383), China (JX416325) (Šlapeta et al. 2021), and the *R. linnaei* strain reported from Angola (MF425595). Furthermore, all strains were classified within the “tropical lineage” group (Figure 3).

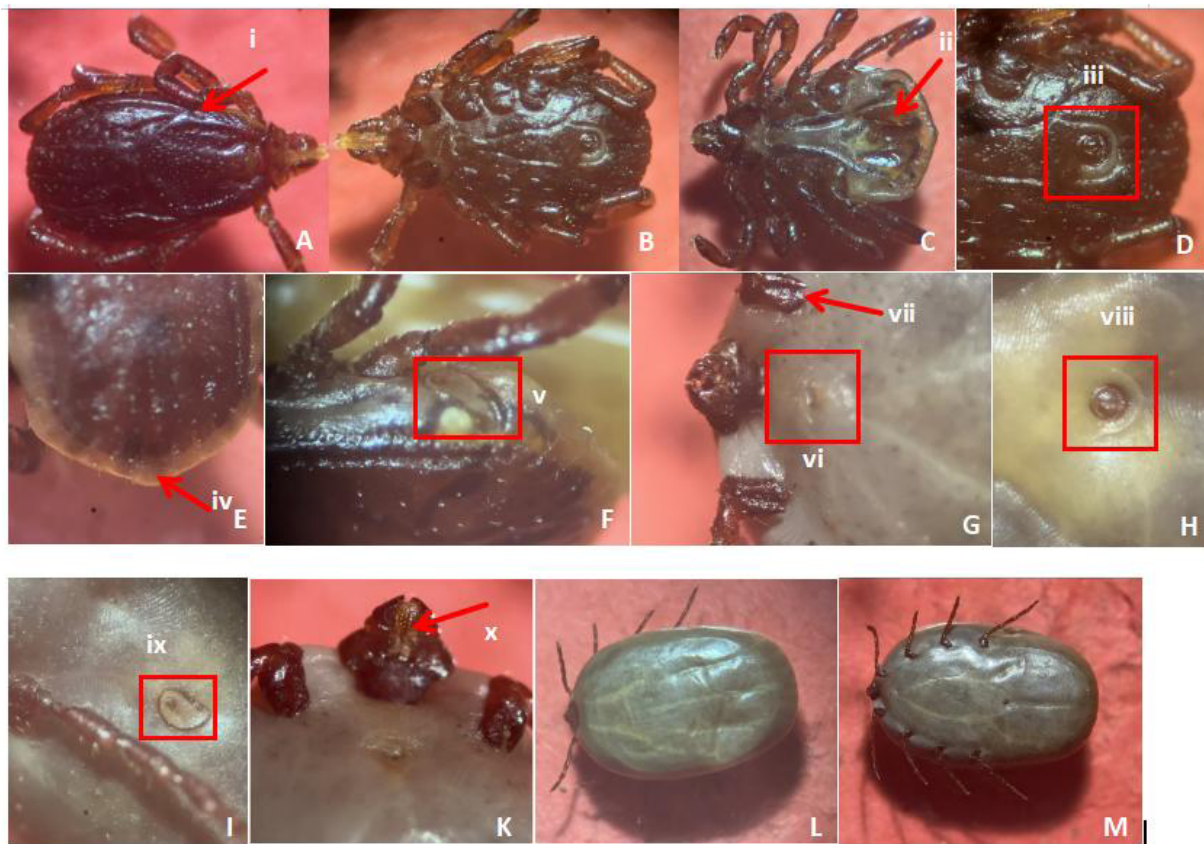


Figure 1. Characteristics of *Rhipicephalus* sp. ticks

Note: A-F, male; G-M, female. Eye (i), ventral plate (ii), anal (iii, viii), festoon (iv), spiracular plate (v, ix), genital aperture (vi), spurs in coxa I (vii), and hypostome (x).

Based on the partial sequence of the *12S rDNA* gene, the isolate in this study clustered with *R. sanguineus* species reported from South Africa (KC243835), China (JQ625664), France (KC243789), Thailand (KC018075), India (OP019271), Cuba (KC018075), Brazil (KC243787, KC018070), and Argentina (JX206969). However, it was also closely related to *R. linnaei* from Angola (MF425971) (Coimbra-Dores et al., 2018) and *R. linnaei* from Portugal (MF425958). In contrast, it was distinct from the *R. linnaei* group (Australia: MW429381, Fiji: MW429382, Laos: MW429383, and China: JX416325) reported by Šlapeta et al. (2021), although they remained within the same “tropical lineage” group (Figure 4).

Additionally, similar observations were made for the partial sequence of the *16S rDNA* gene. Specifically, the isolate collected in this study was closely related to the *R. sanguineus* strains reported from South Africa (KC243835), China (OQ725571), Taiwan (DQ093297), Thailand (JX997387), Colombia (KC243838), and Brazil (JX997391). It was distinct from the cluster contain-

ing the *R. linnaei* group reported by Šlapeta et al. (2021). However, it was closely related to *R. linnaei* reported from Angola (MF425981, MF425978) (Coimbra-Dores et al., 2018). All strains mentioned above were classified within the “tropical lineage” group (Figure 5).

Discussion

Hard ticks (Ixodidae) are among the most prevalent ectoparasites infesting dogs. They comprise numerous genera, such as *Rhipicephalus*, *Dermacentor*, *Haemaphysalis*, etc. In Vietnam, *Rhipicephalus* and *Haemaphysalis* genera were previously reported in dogs (Koloinin, 1995). However, *R. sanguineus* is the sole tick species reported recently in various regions of Vietnam (Nguyen et al., 2018; Huynh et al., 2020; Do et al., 2024). Based on the *cox1*, *12S rDNA*, and *16S rDNA* sequences, we identified the tick species in this study as belonging to the *Rhipicephalus* genus and the “tropical lineage” group. It is also the predominant tick genus in Southeast Asian countries (Colella et al., 2020).

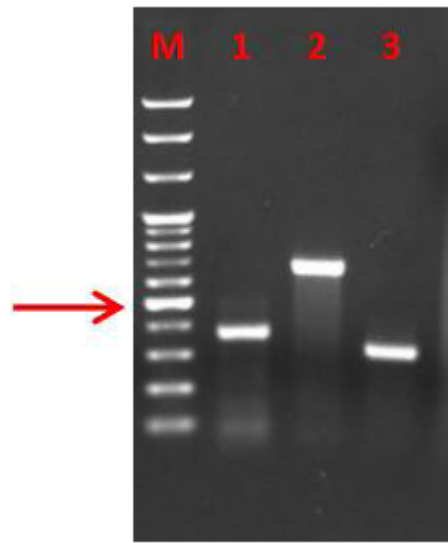


Figure 2. The amplification of three mitochondrial markers of the tick

Note: M: 100 bp ladder; lane 1: For *12S rDNA* gene; lane 2: For *cox1* gene; lane 3: For *16S rDNA* gene. The red arrow shows the position of the 500 bp band.

For morphological identification, the ticks collected in this study were identified as *R. sanguineus* using the established guidelines of Walker and Barker (Walker et al., 2003; Barker & Walker, 2014). However, upon comparison with the morphological characteristics of *R. linnaei* re-described by Šlapeta et al. (2022) and Almazán et al. (2023), these ticks exhibited similar characteristics to *R. linnaei*, such as the spiracular plate in males (narrow elongate comma shape) and the genital aperture in females (broadly U shape), along with other features mentioned above. Notably, based on the molecular identification of the partial sequences of the mitochondrial *12S rDNA* and *16S rDNA* markers, which are more widely used for tick molecular identification, phylogenetic analyses, and the diversity of tick populations, the isolate in this study clustered with *R. sanguineus* “tropical lineage” from most countries mentioned in the phylogenetic trees, but was distinct from the group of *R. linnaei* re-described by Šlapeta et al. (2021), except for its inclusion in the same cluster as *R. linnaei* from Angola (Coimbra-Dores et al., 2018). Conversely, analysis of the partial sequence of the *cox1* gene revealed that the isolate in this study not only clustered with the *R. linnaei* Angola strain but also with *R. linnaei* strains re-described in the research of Šlapeta et al. (2021). Indeed, the *cox1* marker is less frequently utilized in tick diversity research due to observed amplification issues (Lv et al., 2014; Low et al., 2015; Dantas-Torres et al., 2017). However, using the *cox1* gene would be advisable as it is longer than other mitochondrial markers (*12S rDNA* and *16S rDNA*) and translates into protein sequences, thereby reducing alignment ambiguity. To delineate cryptic

species, using mitochondrial markers such as *cox1* is imperative (Hebert et al., 2004; Burger et al., 2004).

Additionally, analysis of the genetic divergence between the isolate in this study and reported *R. linnaei* strains revealed certain differences when using all three markers. Specifically, the low genetic divergences for *cox1* were 0.24% and 0.32% (data not shown), respectively, between this isolate and *R. linnaei* strains from Angola and Šlapeta’s research (2021). However, significant genetic divergence was observed between this isolate and *R. linnaei* from Šlapeta’s study (2021) for *12S rDNA* (17%) and *16S rDNA* (15%). In contrast, these percentages are substantially lower when compared with *R. linnaei* from Angola (0.5 % and 0.0% for *12S rDNA* and *16S rDNA*, respectively) (data not shown). The intra-specific variation was reported up to 1.8% when *12S rDNA* and *16S rDNA* were used in Almazán’s research (Almazán et al., 2023). In Dantas-Torres’s research, these percentages were higher (2.2%, 2.5%, and 3.5% for *16S rDNA*, *12S rDNA*, and *cox1*, respectively) (Dantas-Torres et al., 2016). Some other studies reported higher percentages (up to 2.7% and 6.6% for *16S rDNA* and *12S rDNA*, respectively) (Burlini et al., 2010) and even higher (7.8% to 8% for *12S rDNA*) (Beati et al., 2001; Szabó et al., 2005). The average level of inter-specific variation among *Rhipicephalus* species was 10.8%, 9.9%, and 13.3% for *16S rDNA*, *12S rDNA*, and *cox1* (Dantas-Torres et al., 2013). These data indicated that the isolate in this study is closely related to *R. linnaei* from Angola.

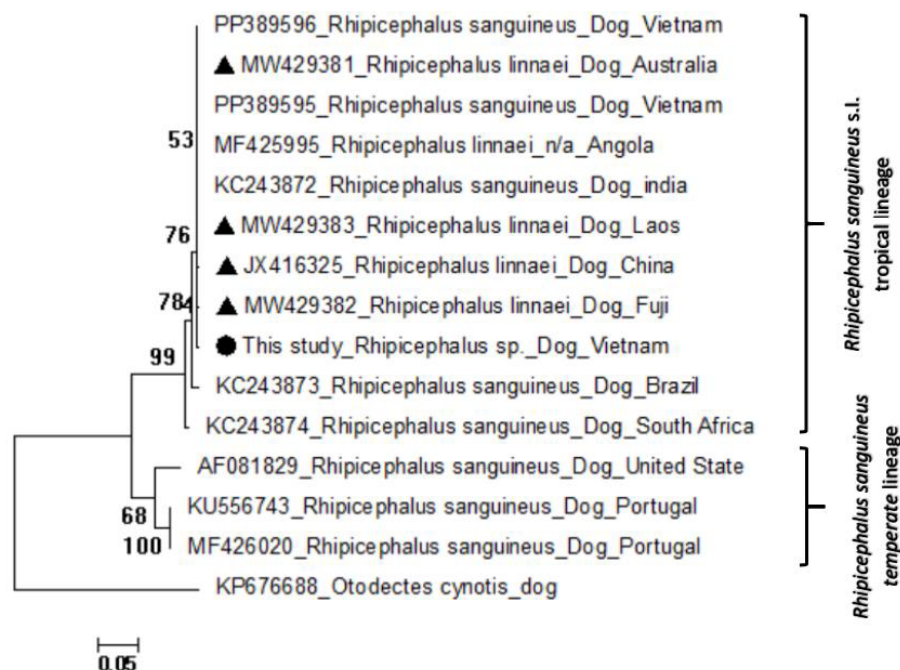


Figure 3. The phylogenetic tree of the partial sequence of the *cox1* Gene of genus *Rhipicephalus*

Note: It was constructed based on the maximum likelihood method with 1000 replicates of bootstrap value by MEGA 6. Accession numbers of reference sequences have been reported from GenBank (NCBI), and host species were included if available. "n/a" implies that no host species information was provided. The black round bullets show the sequence obtained from this study. The black triangle bullets show the species *R. linnaei* identified by Šlapeta et al. (2021).

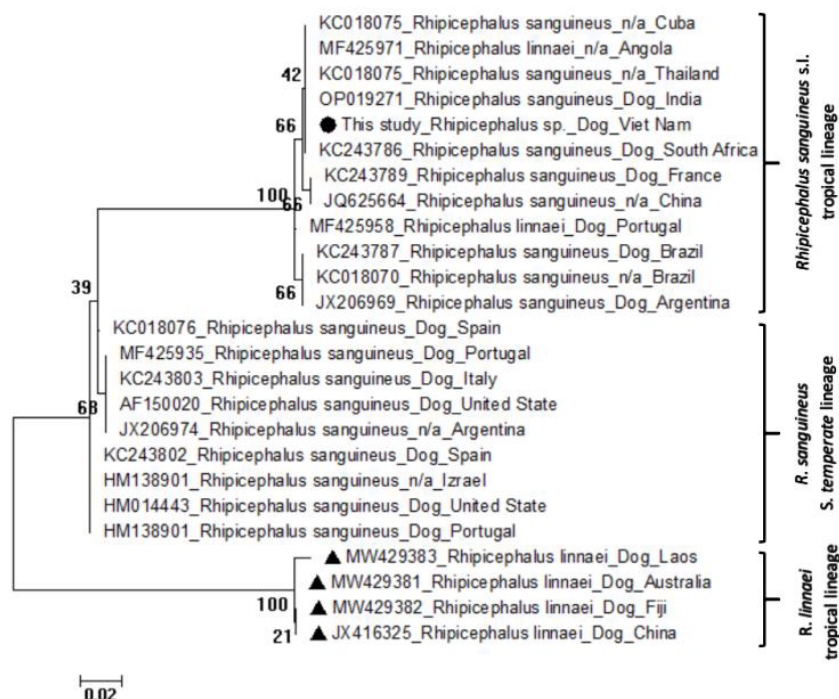


Figure 4. A phylogenetic tree of the partial sequence of the 12S rDNA gene of genus *Rhipicephalus*

Note: It was constructed based on the maximum likelihood method, using 1000 replicates of bootstrap value by MEGA 6. Accession numbers of reference sequences have been reported from GenBank (NCBI), and host species were included if available. "n/a" implies that no host species information was provided. The black round bullets show the sequence obtained from this study. The black triangle bullets show the species *R. linnaei* identified by Šlapeta et al. (2021).

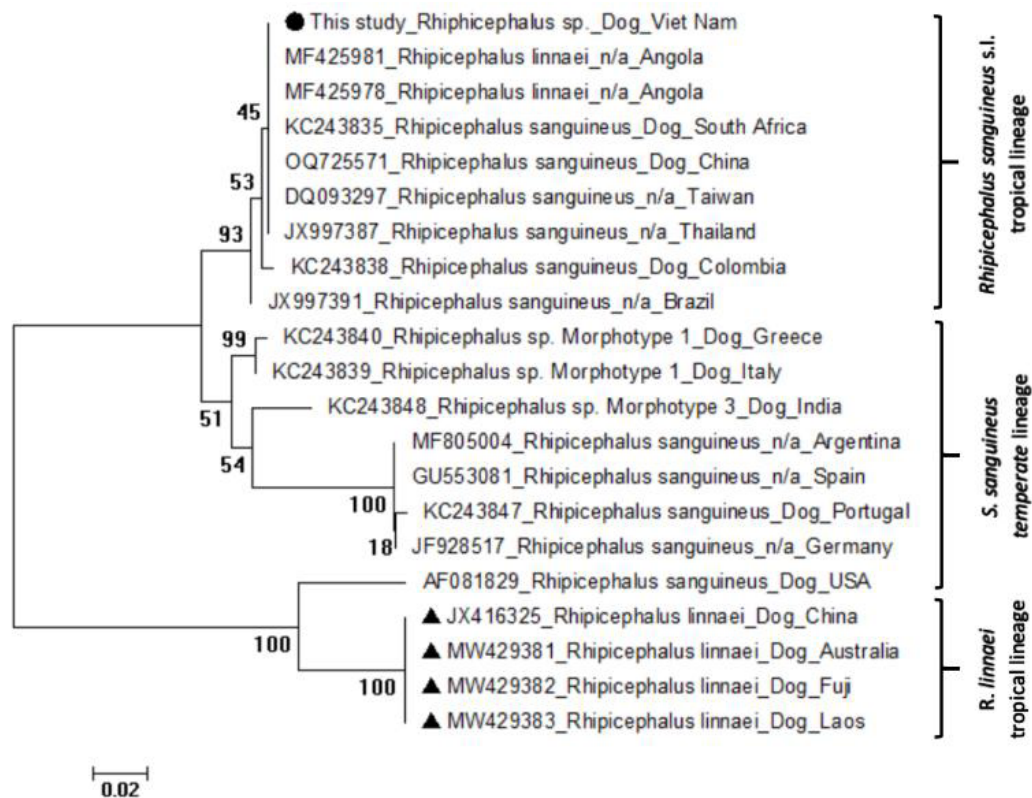


Figure 5. A phylogenetic tree of the partial sequence of the 16S rDNA gene of genus *Rhipicephalus*

Note: It was constructed based on the maximum likelihood method, using 1000 replicates of bootstrap value by MEGA 6. Accession numbers of reference sequences have been reported from GenBank (NCBI), and host species were included if available. "n/a" implies that no host species information was provided. The black round bullets show the sequence obtained from this study. The black triangle bullets show the species *R. linnaei* identified by Šlapeta et al. (2021).

In Vietnam, *R. sanguineus* tropical lineage is the only species reported in dogs using all three aforementioned mitochondrial markers (Nguyen et al., 2018; Huynh et al., 2021; Do et al., 2024). The findings from this study indicated that the isolate was more closely related to *R. linnaei* from Angola. Similar results were observed in China and Laos, where *R. sanguineus* was the sole species documented in dogs (Nguyen et al., 2020; Hu et al., 2022; Wu et al., 2024), except for two strains with accession No. JX416325 (China) and MW429383 (Laos) were re-described as *R. linnaei* (Šlapeta et al., 2021). The findings of this study confirmed the presence of *R. linnaei* in Vietnam, thereby contributing to the understanding of tick taxonomy in canines. Furthermore, *R. linnaei* is capable of surviving and propagating in kennels and residential environments (Teo et al., 2024), serving as a vector for pathogens of medical, veterinary, and zoonotic significance, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Hepatozoon*, and *Rickettsia* (Mumcuoglu et al., 2002; Brown et al., 2006; Greay et al., 2018; Soltani & Dalimi, 2018; Hosseini-Chegeni et al., 2020; Snellgrove et al., 2020; Chaber et al., 2022). Additionally, the detection of *Ehrlichia canis*, *Rickettsia* spp., and *Anaplasma platys* from *R. sanguineus*, which could be re-described as

R. linnaei, in dogs in Vietnam (Nguyen et al., 2018; Huynh et al., 2020; Do et al., 2024) underscores the necessity for implementing control measures at both the individual (pet owner) and community levels.

Conclusion

Based on the results of morphological identification of the adult stage of ticks, and the analysis of the phylogenetic relationship as well as the genetic divergence of three mitochondrial markers (*cox1*, *12S rDNA*, *16S rDNA*) among tick strains, it is provisionally concluded that the *Rhipicephalus* species in this study was identified as *R. linnaei* reported from Angola and also belonged to the "tropical lineage" group. However, due to the limited number of samples sequenced, this study has not evaluated the level of intra-specific variation among *Rhipicephalus* species. Besides, collection sites were limited to small animal hospitals around Hanoi. Further comprehensive research, particularly regarding population dynamics in different regions throughout Vietnam, is necessary to corroborate the findings of this study.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations in this research.

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Authors' contributions

Sample collection, experiments, data analysis, revising the article, and final approval: All authors; Writing the original manuscript: Nguyen Yen Thi Hoang.

Conflict of interest

The authors declared no conflict of interest.

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