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Redefining *Rhipicephalus sanguineus* (*sensu lato*) species complex in Greece focusing on the mitogenome of *Rhipicephalus secundus*

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ABSTRACT

Rhipicephalus sanguineus species complex, referred to as *R. sanguineus* (*sensu lato*), is distributed globally with some species distributed in specific regions and others spread globally. In Greece, *R. sanguineus* (*s.l.*) in dogs, and *Rhipicephalus turanicus* and *Rhipicephalus bursa* in livestock, have been repeatedly reported however only based on morphological identification. Recently, there has been a great effort to accurately identify the species belonging to the *R. sanguineus* species complex, using modern molecular tools and describe their spatial distribution. The aim of this study was to expand the studies on the molecular characterisation of species of the *R. sanguineus* (*s.l.*) complex in the Mediterranean region, by molecularly characterising ticks collected from various locations and host species in Greece. Results confirm the presence of *Rhipicephalus rutilus*, *Rhipicephalus secundus*, *R. bursa*, and *R. turanicus*. The complete mitochondrial genome of *R. secundus* (approx. 15 kb) from sheep ($n = 3$) in Greece was sequenced and matched to sequences and morphological data from the type-material from Israel. Additionally, the mitogenome of *R. bursa* from goats ($n = 2$) in Greece was sequenced. This study provides a molecular reference for *R. secundus*, a tick species distributed in the Eastern Mediterranean region.

1. Introduction

Ixodid ticks are highly abundant and widespread ectoparasites, strongly related to the local environmental conditions which are quite favourable in the Mediterranean countries. Their species distribution has been thoroughly studied in many countries, including Greece, where up to date 26 tick species have been identified in domestic animals (cattle, sheep, goats and dogs), wildlife, and humans. *Rhipicephalus sanguineus* (Latreille, 1806) (*sensu lato*) in dogs, and *Rhipicephalus turanicus* (Pomerantzev, 1940) and *Rhipicephalus bursa* (Canestrini and Fanzago, 1878), in livestock, are the most commonly reported (Chaligiannis et al., 2014, 2016, 2018; Latrofa et al., 2017; Saratsis et al., 2022; Ligda et al., 2023). In all above cases ticks were solely morphologically identified.

The taxonomic status of *R. turanicus* has long been unclear, because the species appeared to be represented by two distinct clades using partial mtDNA genes. Bakkes et al. (2020) redescribed *R. turanicus* (*sensu stricto*) assigning genetic sequences from Asia to this name and speculated that clades from Middle East could represent different species, including *Rhipicephalus secundus* Feldman-Muhsam, 1952 considered to be a synonym of *R. turanicus*. Šlapeta et al. (2021) suggested that the assembled complete tick mitogenome from Palestinian territory, published by Ravi et al. (2019) and collected from a sheep, could represent *R. secundus*, as it was distinct from *R. turanicus* (*s.s.*). Finally, Mumcuoglu et al. (2022) re-established the name *R. secundus* Feldman-Muhsam, 1952 and removed it from the list of synonyms of *R. turanicus*. The sequence data from Israel by Mumcuoglu et al. (2022) matched those from Palestinian territory by Šlapeta et al. (2021). The type-locality of *R. secundus* is Israel, and its type-host is the goat. Using available public sequence data, *R. secundus* has been recorded in Turkey, Israel, the Palestinian Territories, Albania, southern Italy, and France (Corsica) (Šlapeta et al., 2021; Mumcuoglu et al., 2022). However, the continuous distribution within the eastern Mediterranean is far from completely understood.

The aim of this study was to characterise *Rhipicephalus* species in the Mediterranean region by molecularly analysing ticks of this genus

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collected from various locations and host species in Greece. Additionally, the study aimed to create a high-quality reference material for *R. secundus*.

2. Materials and methods

2.1. Study design

To address the above objectives, several visits to small ruminant farms were conducted in different locations in Greece, Peloponnese (southern Greece), Epirus and West Macedonia (western Greece), Central Macedonia (northern Greece), Central Greece, Crete and South Aegean islands, between 2020 and 2023. Selected animals per farm were thoroughly checked for the presence of ticks at the head (ears, horns), udder, perianal region, hindlegs, tail, and withers/back. To collect ticks from dogs, local veterinary clinics in the same locations were visited and dogs were thoroughly checked for ticks as well. Collected ticks were placed in numbered and dated tubes containing 70% ethanol immediately after collection and identified according to specific keys (Walker and Keirans, 2000; Estrada-Peña et al., 2004).

From all ticks identified, ticks belonging to *Rhipicephalus* spp. from ruminants and dogs were separated for further characterisation. Overall, 69 *Rhipicephalus* spp. ticks (22 from dogs and 47 from sheep and goats) were selected. To ensure better species diversity each one of the selected ticks was collected from a different animal, thus representing all animals infested with at least one tick of a given species.

Twenty-one ticks collected from dogs were identified as *R. sanguineus* (*s.l.*) and further proceeded to molecular analyses by conventional PCR. The remaining 1 tick was identified as *Ixodes ricinus* and no further action was taken.

Ticks collected from sheep and goats were identified as *R. bursa*, *R. turanicus* and *R. sanguineus* (*s.l.*). Of these, 5 ticks were selected as representative of each species, according to morphological criteria, and shipped to the University of Sydney, Australia, stored in 70% (v/w) ethanol, for further mtDNA analysis by next-generation sequencing techniques.

2.2. DNA isolation and conventional PCR analyses

DNA was extracted from ticks collected from dogs ($n = 21$) using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final elution volume was 100 µl. Disruption and homogenization were performed in the lysis buffer of the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), using the Bead Mill 4 Mini Homogenizer (Fisherbrand™) and 2.4 mm metal beads according to the manufacturer's specifications. Extracted genomic DNA (gDNA) from ticks was stored at −20 °C.

All gDNA samples were processed with touch down PCRs to amplify: a 604 bp fragment of cytochrome *c* oxidase subunit 1 (*cox*1) gene (Kushimo, 2013); a 336 bp fragment of 12S ribosomal RNA (12S rRNA) gene (Beati and Keirans, 2001); and a 276 bp fragment of 16S ribosomal RNA (16S rRNA) gene (Black and Piesman, 1994), with previously described cycling conditions (Meiklejohn et al., 2019). Amplification products were visualized on 1.5% agarose gels stained with ethidium bromide.

Successfully amplified PCR products were sent to a commercial service (CeMIA SA, Larissa, Greece) for purification and sequencing on both strands (Sanger sequencing). The bidirectionally assembled sequences were visually inspected and appended to a reference alignment of verified *cox*1, 12S rRNA and 16S rRNA genes derived from complete mitochondrial genomes from Šlapeta et al. (2023) using CLC Main Workbench 23 (CLC bio, Qiagen, Australia). Based on alignment positional homology and phylogenetic position, the sequences were assigned to their respective taxonomic identity. To corroborate and compare the data of this study, sequences published by Dantas-Torres et al. (2013) were compared to match these data with their "haplotype" identification. Briefly, all sequences from Dantas-Torres et al. (2013) were downloaded, separated based on the gene sequenced and appended sequence data of the present study for pairwise comparison to identify perfect (100%) matches.

2.3. Specific morphological description of selected ticks

Selected ticks were morphologically identified using a stereo microscope (SMZ-2B, Nikon, Rhodes, Australia) with various keys and original descriptions/redescriptions of *Rhipicephalus* spp. and comparison with vouchers from molecularly characterised specimens of *R. sanguineus* (*s.l.*). Morphologically important features were closely examined and photographed using a digital microscope (VHX-6000, KEYENCE Inc., Osaka, Japan). Key characteristics were observed and recorded using the calibrated VHX-6000 digital microscope.

2.4. DNA isolation and genome skimming to assemble mitogenomes from next-generation sequence data

Ticks were cut through their idiosoma and dried prior to the isolation of gDNA using the Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, USA), as previously described by Šlapeta et al. (2022). Exoskeletons were retained and preserved in 70% (v/w) ethanol. Extracted gDNA was stored at −20 °C. The gDNA isolated from five adult *Rhipicephalus* spp. ticks was subjected to next-generation sequencing (NGS) using a NEBNext® DNA Library Prep Kit followed by NGS using 150 bp paired-end Illumina NovaSeq 6000 sequencing system at a depth of 3 Gb or 10 Gb of raw sequence data (Novogene, Biopolis Way, Singapore). The data were analysed on Artemis HPC (Sydney Informatics Hub, The University of Sydney). The whole mitochondrial DNA (mtDNA) was assembled from FastQ data using the GetOrganelle v1.7.5.3 pipeline (Jin et al., 2020) (https://github.com/Kinggerm/GetOrganelle). The complete circular mtDNA sequences were aligned with available complete mtDNA sequences from species of the *R. sanguineus* (*s.l.*) complex using CLC Main Workbench 23 (CLC bio, Qiagen, Australia).

2.5. Phylogenetic analysis using mitogenomes of Rhipicephalus sanguineus (s.l.)

Phylogenetic analysis was performed using the maximum likelihood (ML) and minimum evolution (ME) methods. This analysis involved 42 nucleotide sequences and included the alignment from Almazán et al. (2024). The final dataset comprised 15,743 positions. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). The evolutionary model was selected by fitting 24 different nucleotide substitution models, with the model having the lowest Bayesian information criterion (BIC) score being chosen to describe the substitution pattern. The best model was the general time reversible model (GTR) with gamma distribution (+G) and a proportion of invariable sites (+I), with 67 parameters and BIC score 161243.142 (ln = −80014.736) used for ML analysis. The final tree (highest ln = −80015.45) used a model with evolutionary rate differences among sites (5 categories, +G parameter = 1.0630) and allowed for some sites to be evolutionary invariable (+I, 46.96% of sites). The ME evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004). A bootstrap test was conducted to calculate the percentage of replicate trees in which the associated mitogenomes clustered together, with 1000 replicates for ME and 100 replicates for ML.

3. Results

3.1. Molecular data for ticks collected from dogs

All tested gDNA samples from dogs had a band at the expected size of the target genes (i.e. *cox*1, 12S rRNA, and 16S rRNA genes). The ticks

initially identified as *R. sanguineus* (*s.l.*) belonged to *Rhipicephalus rutilus* (Koch, 1844) (16/21), *R. secundus* (2/21), and *R. turanicus* (1/21), while for 2 samples the sequencing was not successful for any of the three target genes (Table 1). All sequences exhibited > 98.4% sequence identity with reference sequences of either *R. rutilus*, *R. turanicus* or *R. secundus* at their respective gene fragments (LabArchives: https://dx.doi.org/10.25833/98es-qx45). The sequences for all tick species identified were deposited in the GenBank database under the accession numbers PQ001973-PQ001983 (*cox*1 gene), PQ002408-PQ002421 (12S rRNA gene) and PQ002089-PQ002107 (16S rRNA gene) (Table 1).

Comparison with Dantas-Torres et al. (2013) was enabled by aligning our sequences across 400, 270 and 320 nucleotide positions across *cox*1, 16S rRNA and 12S rRNA genes, respectively. In our dataset, there were 7 and 2 *cox*1 sequences matching "haplotype 1" and "haplotype 2" of *R. rutilus* published by Dantas-Torres et al. (2013), respectively. At 16S rRNA gene, 8 and 3 sequences matched "haplotype 3" and "haplotype 1" of *R. rutilus* published by Dantas-Torres et al. (2013), respectively. At 12S rRNA gene, 5, 2 and 1 sequences matched "haplotype 4", "haplotype 1" and "haplotype 3" of *R. rutilus* published by Dantas-Torres et al. (2013), respectively. The remaining *R. rutilus* sequences from Greece did not match previously identified haplotypes of *R. rutilus* from Dantas-Torres et al. (2013). At *cox*1 and 16S rRNA genes, our *R. secundus* sequences were most closely related to *R. turanicus sensu* Dantas-Torres et al. (2013). The 16S rRNA gene sequence (GenBank: PQ002101) from tick R05G23 is 100% identical with sequence of *R. turanicus* "haplotype 10" *sensu* Dantas-Torres et al. (2013) (GenBank: KC243865). No *R. turanicus cox*1 and 16S rRNA gene sequence were identified within the Dantas-Torres et al. (2013) data. At 12S rRNA gene, it was impossible to consistently differentiate *R. turanicus* from *R. secundus* within the sequences of Dantas-Torres et al. (2013).

3.2. Mitogenome analyses of ticks from sheep and goats

Five ticks (three initially identified as *R. sanguineus* (*s.l.*) and two *R. bursa*) underwent DNA isolation with DNA concentration from 1.52 ng/μl to 16.08 ng/μl. Sequencing of DNA from the *R. sanguineus* (*s.l.*) tick vouchers JS6647 (GR4: P10-S1), JS6754 (GR9: P5-S5) and JS6755 (GR10: P6-S2) produced 78,226,994, 28,656,694 and 23,606,828 raw 150 nt paired reads totalling 11.7 Gb, 4.3 Gb and 3.5 Gb of raw data (Q30 = 91.98%, 94.15% and 92.60%; GC = 47.56%, 61.49% and 47.13%).

The assembly yielded 14,714–14,720 bp long contigs that were complete circular mtDNA, mitogenomes. The three mitogenomes were almost identical with each other (uncorrected pairwise identity of 99.5–99.7%). The mitogenomes were closest (99.2–99.3% identity) to the mitogenome of *R. secundus* assembled by Šlapeta et al. (2021) from data provided by Ravi et al. (2019). Using the partial 16S rRNA gene sequences (GenBank: OM030339 and OM030342; 98.9% identical with each other) from the *R. secundus* material re-described by Mumcuoglu et al. (2022), the corresponding 16S rRNA gene sequence from the present mitogenomes were 98.6–99.4% identical across 353 positions in the alignment. The new mitogenomes were 91.6–91.7% identical with the reference *R. turanicus* mitogenome (GenBank: NC_035946), 90.5–90.6% identical with the reference *Rhipicephalus linnaei* (Audouin, 1826) mitogenome (GenBank: NC_060409) and 88.9–89.1% identical with the reference *R. sanguineus* (*s.s.*) mitogenome (GenBank: NC_002074). The circular mtDNA of *R. secundus* from Greece encoded 13 protein-coding genes, two rRNA genes, and 22 tRNAs.

The newly generated mitogenomes were added to the mtDNA alignment of available *R. sanguineus* (*s.l.*) mitogenomes and subsequent phylogenetic inference confirmed highly supported (100% bootstrap in ML and ME) monophyly of all new *R. secundus* mitogenomes (Fig. 1). The clades of *R. turanicus* and *R. secundus* sequences formed strongly (100%) supported sister groups that were monophyletic (Fig. 1).

Additionally, we sequenced two *R. bursa* (Fig. 1) collected from goats. Sequencing of DNA from the *R. bursa* tick vouchers JS6644 (GR1: KZ24 6(g)) and JS6645 (GR2: KZ44 G6A) produced 41,135,556 and 25,234,798 raw 150 nt reads totalling 6.2 Gb and 3.8 Gb of raw data (Q30 = 91.51% and 92.19%; GC = 47.62% and 47.80%). The assembled complete mitogenomes were 14,738 nt [JS6644 (GR1: KZ24 6(g)] and 14,740 nt [JS6645 (GR2: KZ44 G6A)] long circular mitogenomes. The circular mtDNA of *R. bursa* from Greece encoded 13 protein-coding genes, two rRNA genes, and 22 tRNAs.

3.3. Morphological data for the molecular vouchers of R. secundus

Ticks from sheep and goats from Greece were identified based on morphology as *R. sanguineus* (*s.l.*) ($n = 7$; 4 male and 3 female), *R. bursa* ($n = 20$; 8 male and 12 female) and *R. turanicus* ($n = 20$; 9 male and 11 female). The morphology of three molecular vouchers (2 males and 1

Table 1
Summary of ticks collected from dogs initially assigned to *R. sanguineus* (*s.l.*) and GenBank accession numbers for the newly generated sequences used for species identification.

Sample ID	Locality	Region	Tick species ID after sequencing	GenBank ID		
				*cox*1	12S rDNA	16S rDNA
R02G06	Thessaloniki	Central Macedonia	*R. rutilus*	PQ001973	PQ002408	PQ002089
R02G20A	Thessaloniki	Central Macedonia	*R. rutilus*	PQ001974	PQ002409	PQ002090
R02G20B	Thessaloniki	Central Macedonia	*R. rutilus*	na	PQ002410	PQ002091
R04G01	Lamia	Central Greece	*R. rutilus*	PQ001975	PQ002411	PQ002092
R04G10	Lamia	Central Greece	*R. rutilus*	na	na	PQ002093
R04G27A	Lamia	Central Greece	*R. rutilus*	na	na	PQ002094
R04G27B	Lamia	Central Greece	*R. rutilus*	na	PQ002412	PQ002095
R04G36	Lamia	Central Greece	*R. rutilus*	PQ001976	PQ002413	PQ002096
R15G10	Kozani	West Macedonia	na	na	na	na
R05G12	Giannitsa	Central Macedonia	*R. rutilus*	na	na	PQ002097
R05G17	Serres	Central Macedonia	*R. rutilus*	PQ001977	na	PQ002098
R05G19	Giannitsa	Central Macedonia	*R. rutilus*	PQ001978	PQ002414	PQ002099
R05G21A	Kozani	Central Macedonia	na	na	na	na
R05G21B	Katerini	Central Macedonia	*R. rutilus*	PQ001979	PQ002415	PQ002100
R05G23	Katerini	Central Macedonia	*R. secundus*	PQ001982	PQ002420	PQ002101
R05G25A	Katerini	Central Macedonia	*R. rutilus*	na	PQ002416	PQ002102
R05G25B	Katerini	Central Macedonia	*R. rutilus*	PQ001980	PQ002417	PQ002103
R05G39	Kilkis	Central Macedonia	*R. rutilus*	PQ001981	na	PQ002104
R06G87	Crete	Crete	*R. turanicus*	na	PQ002419	PQ002105
R06G193	Crete	Crete	*R. secundus*	PQ001983	PQ002421	PQ002106
R12G38	Santorini	South Aegean	*R. rutilus*	na	PQ002418	PQ002107

Abbreviation: na, not amplified/available.

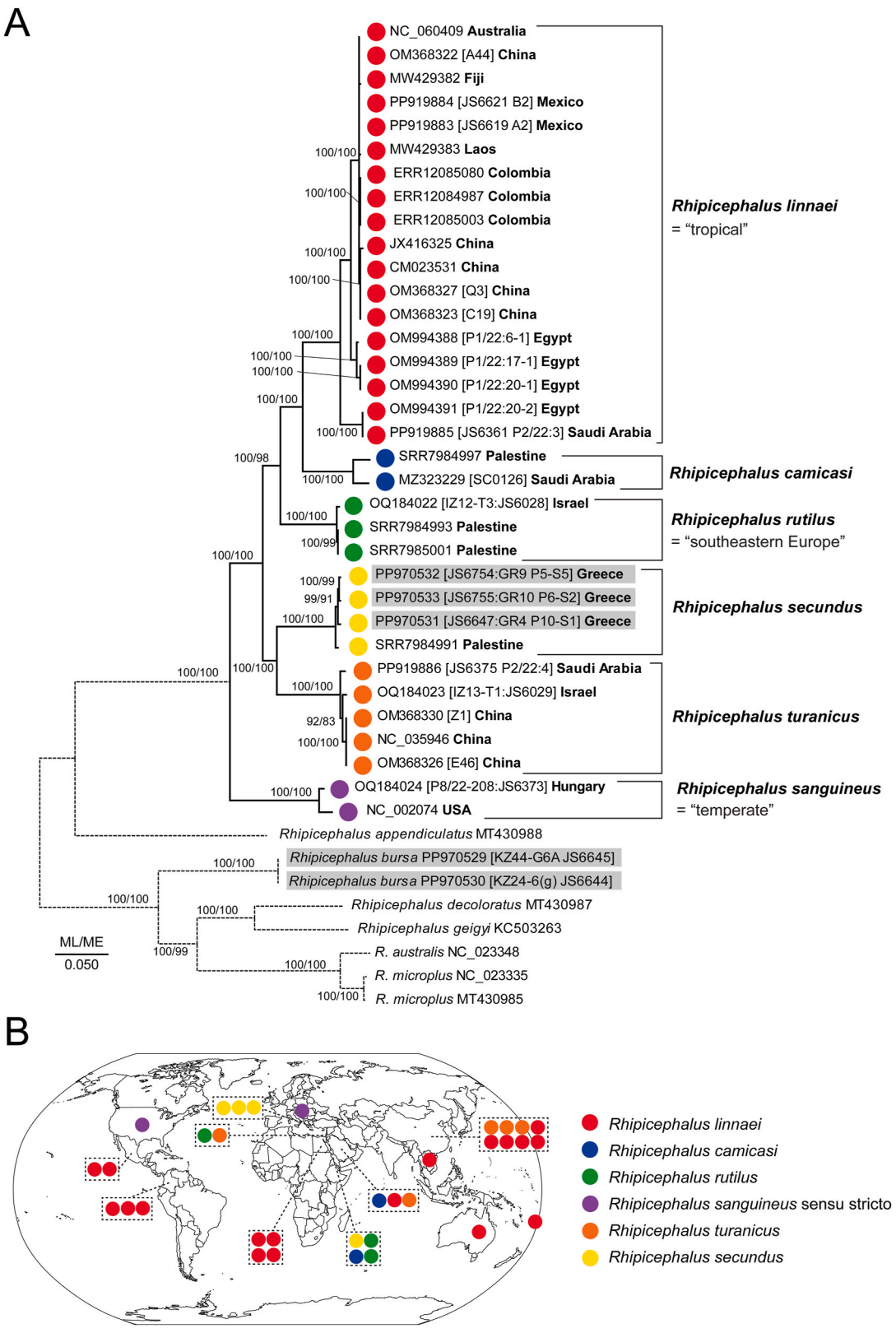

Fig. 1. *Rhipicephalus secundus* complete mitochondrial DNA and new *Rhipicephalus bursa* mitochondrial DNA genomes. **A** Phylogenetic relationships of *Rhipicephalus sanguineus* (*s.l.*) based on complete mitogenomes. Mitogenomes of *R. sanguineus* (*s.l.*) were aligned and the tree was inferred from nucleotide data. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The tree was reconstructed using maximum likelihood method (ML). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates for ML/1000 replicates for minimum evolution method) are shown next to the branches. The species to which the mitogenome belongs is indicated on the right and colour-coded. Each mitogenome is identified by its GenBank accession number and voucher identifier together with the country where it was collected. The new mitogenomes from this study are shaded. **B** Mitogenome country location for the species of the *R. sanguineus* (*s.l.*) group. Each mitogenome is colour-coded based on species identification, each circle represents one mitogenome. *Rhipicephalus sanguineus* (*s.s.*) was previously recognized as the "temperate lineage".

female) for the complete mitogenome sequences of *R. secundus* initially assigned to *R. sanguineus* (*s.l.*) was examined and compared with reference ticks and published descriptions. Brief descriptions of this material follow. A series of photomicrographs of the molecular vouchers of *R. bursa* is available at LabArchives (https://dx.doi.org/10.25833/98es-qx45).

3.3.1. Description of *R. secundus* male vouchers

Host: Sheep, *Ovis aries*.

Locality: Peloponnese, Greece.

Voucher material: GR4: P10-S1 and GR10: P6-S2 (unengorged males); submitted to the Australian National Insect Collection (ANIC), CSIRO, Canberra, Australian Capital Territory, Australia under accession numbers ANIC 48 006 616 and ANIC 48 006 617.

Sequence data: Complete mitogenomes are available from GenBank (PP970533 and PP970531).

Description: *Scutum*: Broadly oval, broadest posteriorly. Punctuations moderate in number and unequal in size. Narrow margins slightly concave. Colouration generally reddish-brown (Fig. 2A and 3A). Patches of yellowish and reddish colouration can be observed across the scutum in an irregular pattern (Fig. 2A). *Adanal plate*: Subtriangular in shape, margins slightly concave, with long elongations anteriorly (Fig. 2B and 3B). *Spiracular plate*: Elongate-subtriangular in shape, narrowly comma-shaped, posterior to leg 4. Dorsal prolongation same size as adjacent festoon. Abundant small goblets surrounding spiracular opening, goblets lacking on the dorsal prolongation (Fig. 2C, D, 3C, D).

3.3.2. Description of *R. secundus* female voucher

Host: Sheep, *Ovis aries*.

Locality: Peloponnese, Greece.

Voucher material: GR9: P5-S5 (engorged female); submitted to the Australian National Insect Collection (ANIC), CSIRO, Canberra, Australian Capital Territory, Australia under accession number ANIC 48 006 618.

Sequence data: Complete mitogenome is available from GenBank (PP970532).

Description: *Scutum*: Broadly oval, broadest posteriorly. Punctuations greater in number and unequal in size. Narrow margins slightly concave. Colouration dark reddish-brown (Fig. 4A). *Genital aperture*: Wide U-shaped (Fig. 4B). Genital aperture was difficult to observe in its natural form due to specimen preservation. *Spiracular plate*: Subtriangular in shape, with slight elongation posteriorly, posterior to leg 4. Dorsal prolongation as wide as adjacent festoon. Dark, reddish-brown, narrow margin. Abundant, brown goblets surrounding spiracular opening, goblets lacking on dorsal prolongation (Fig. 4C and D).

4. Discussion

This study was designed to complement the efforts undertaken recently to characterise the identity of *Rhipicephalus* species in south-eastern Mediterranean region (Hornok et al., 2017; Mumcuoglu et al., 2022; Šlapeta et al., 2023). As indicated in *Section 1*, *R. sanguineus* (*s.l.*), *R. turanicus* and *R. bursa* have been previously recorded in Greece based on morphological identification alone (Chaligiannis et al., 2014, 2016, 2018; Latrofa et al., 2017; Saratsis et al., 2022; Ligda et al., 2023).

Our results confirmed the occurrence of *R. sanguineus*, *R. rutilus* and *R. turanicus* in Greece using molecular tools in agreement with previous studies (Dantas-Torres et al., 2013). Furthermore, we provided the first

Fig. 2. *Rhipicephalus secundus* (GR4: P10-S1; JS6647) unengorged male from Greece. **A** Scutum, dorsal view. **B** Adanal plates. **C** Spiracular plate on left side with festoon (*arrow*), note that the spiracular late projection terminal end (*star*) is as wide as the festoon. **D** Spiracular plate on right side. Scale-bars are in μm. Note the incision to the scutum is an artefact as part of DNA isolation.

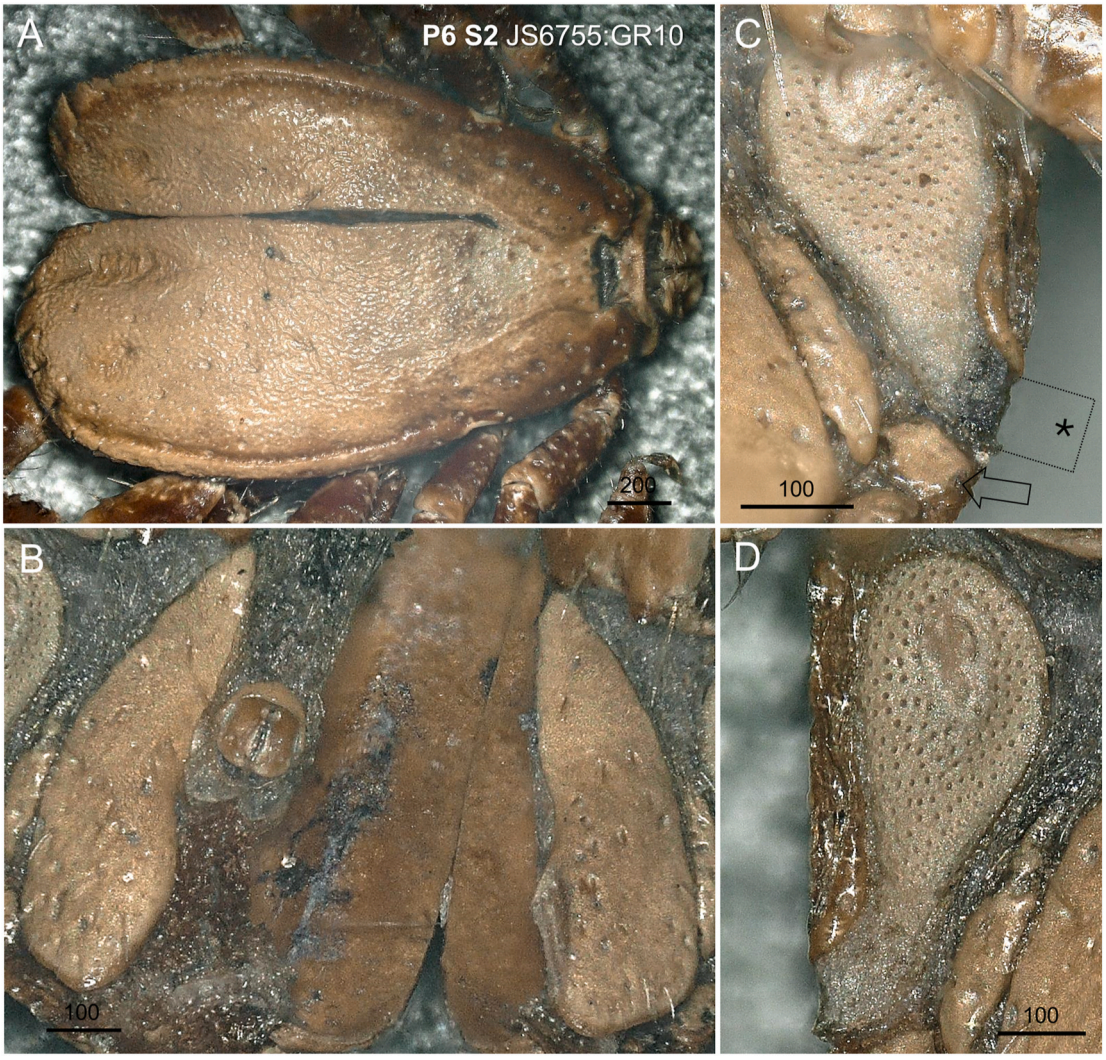

Fig. 3. *Rhipicephalus secundus* (GR10: P6-S2; JS6755) unengorged male from Greece. **A** Scutum, dorsal view. **B** Adanal plates. **C** Spiracular plate on left side with adjacent festoon (*arrow*), note that the spiracular plate projection terminal end (*star*) is as wide as the festoon. **D** Spiracular plate on right side. Scale-bars are in μm. Note the incision to the scutum is an artefact as part of DNA isolation.

molecular evidence for the occurrence of *R. bursa* in Greece, and characterised molecularly and morphologically *R. secundus* based on Greek material. We demonstrate that the material labelled as *R. turanicus* by Dantas-Torres et al. (2013) likely represents *R. secundus* at least at partial *cox*1 and 16S rRNA gene sequences. Finally, our study generated the first mitogenomes of *R. bursa* and three mitogenomes of *R. secundus* based on Greek material.

Dogs from across Greece were primarily parasitized by *R. rutilus*, which in this context is colloquially recognized as *R. sanguineus* (*s.l.*) in the region (Latrofa et al., 2017; Chaligiannis et al., 2018; Ligda et al., 2023). The morphology of *R. rutilus* resembles what was previously considered the "tropical lineage" of *R. sanguineus* (*s.l.*), now identified as a distinct species, *R. linnaei* (Šlapeta et al., 2022). In Dantas-Torres et al. (2013), *R. rutilus* (named *Rhipicehalus* sp. I) was identified on dogs in southern Italy (Puglia) and northeastern Greece (Xanthi) based on molecular data, partial *cox*1, 12S rRNA and 16S rRNA gene sequences. Additional work established distribution of *R. rutilius* in the Eastern Mediterranean region, including Greece, under a vernacular name "southeastern Europe" lineage of *R. sanguineus* (*s.l.*) (Chitimia-Dobler et al., 2017; Hornok et al., 2017). Šlapeta et al. (2023) have shown unequivocally based on morphological and mitogenome data that this lineage represents *R. rutilus*. Dantas-Torres et al. (2013) highlighted key morphological features such as the shape of the spiracular plate and genital opening of *R. rutilus* (as *Rhipicephalus* sp. I). It is unlikely that *R. rutilus* would be mistaken for *R. sanguineus* (*s.s.*), as their spiracular plates are quite distinct (Nava et al., 2018). The only other tick species of the *R. sanguineus* (*s.l.*) species complex sequenced from dogs in the present study were *R. turanicus* and *R. secundus*.

It is interesting that ticks of *R. secundus* morphologically and molecularly characterised here, were collected not only from dogs but also from ruminants in Greece. The distribution of this species is not well known, as it heavily relies on molecular markers such as the traditionally used partial sequences of 12S rRNA, 16S rRNA and *cox*1 genes (Bakkes et al., 2020; Mumcuoglu et al., 2022). To enable better characterisation, we provide complete mitogenome sequences and complemented them with morphological details for vouchers from Greece.

In the redescription of *R. secundus* by Mumcuoglu et al. (2022) only partial sequences of 12S rRNA and 16S rRNA genes were provided. By delivering a morphological description along with the complete mitogenome, we clarified the taxonomic status of this species thus enabling improved phylogenetic resolution and its relationship to related taxa. The morphology and molecular identity confirmed that the voucher material from Greece matched the description of the type-material of Mumcuoglu et al. (2022), justifying the use of our sequence data as a genetic reference. The current distribution of *R. secundus* includes Greece, Turkey, Israel, the Palestinian Territories, Albania, Italy, and

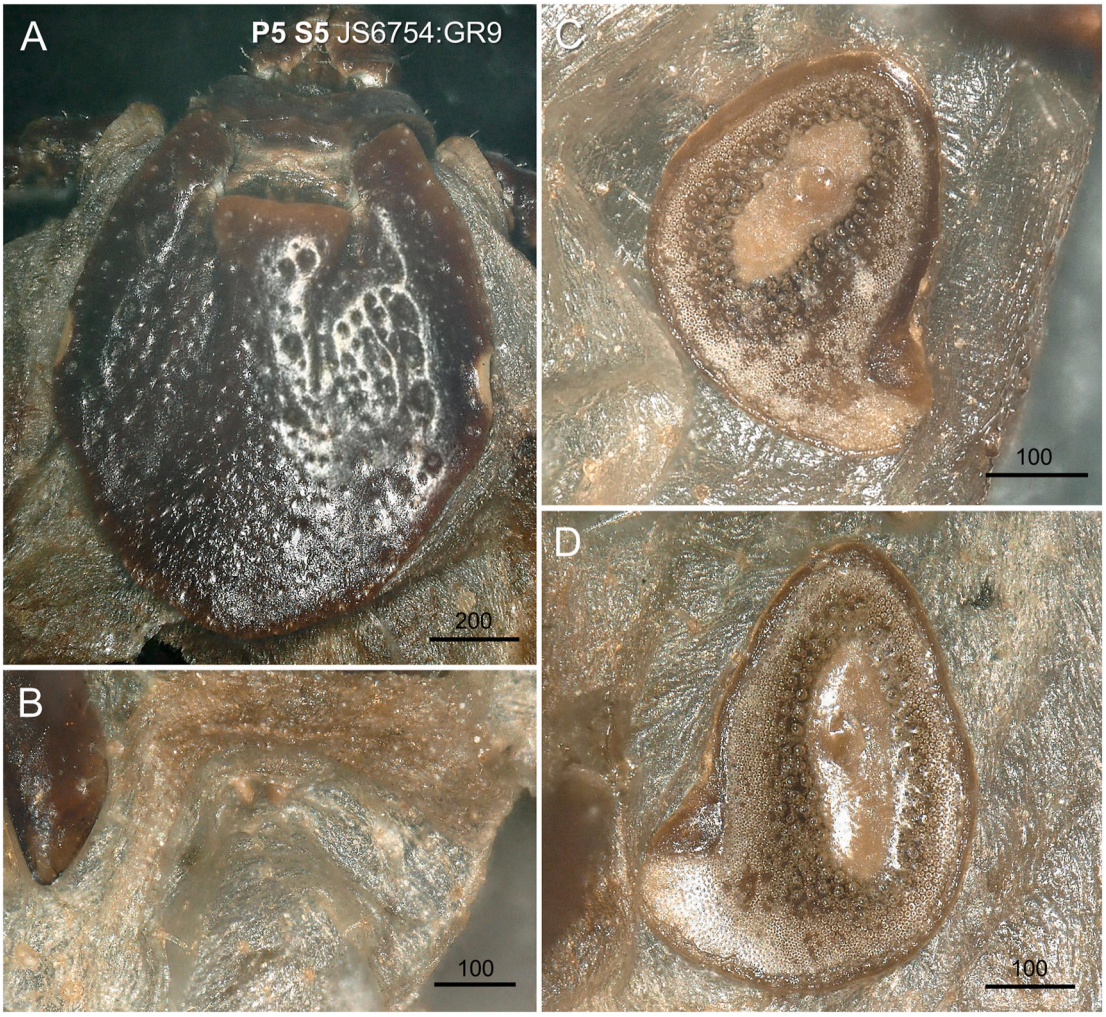

Fig. 4. *Rhipicephalus secundus* (GR9: P5-S5; JS6754) engorged female from Greece. **A** Scutum, dorsal view. **B** Genital aperture. **C** Spiracular plate on left side. **D** Spiracular plate on right side. Scale-bars are in μm.

France; however, it is unlikely that it is complete. Caution is advised when using publicly available sequence data for identifying *R. sanguineus* (*s.l.*), as many sequences may have outdated labels. For example, sequences labelled as *R. turanicus* from Dantas-Torres et al. (2013) likely represent *R. secundus*. It is preferable to use curated sequences sets, ideally continuous sequences from mitogenomes, rather than partial mitochondrial *cox*1, 16S rRNA, or 12S rRNA genes, as references. Further characterisation of *Rhipicephalus* spp. material from ruminants and dogs in both the east and west of Greece is needed to better delineate the distribution of *R. secundus*, as well as *R. turanicus* (Bakkes et al., 2020).

5. Conclusions

In this study, tick species of the *R. sanguineus* (*s.l.*) species complex, i.e. *R. rutilus*, *R. secundus*, and *R. turanicus*, and *R. bursa*, were molecularly characterised based on material from Greece and five mitogenome sequences were generated for *R. secundus* and *R. bursa*. This study created reference material for *R. secundus*, characterised both morphologically and genetically, which can be a useful resource in future studies comparing ticks from different regions and host species.

CRediT authorship contribution statement

Panagiota Ligda: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Jan Šlapeta:** Project administration, Methodology, Data curation, Resources, Supervision, Writing – original draft, Writing – review & editing. **Anastasios Saratsis:** Investigation, Data curation, Writing – review & editing. **Vaia Kantzoura:** Investigation, Data curation, Writing – review & editing. **Jaisy Chong:** Methodology, Investigation, Data curation, Writing – review & editing. **Smaragda Sotiraki:** Project administration, Conceptualization, Investigation, Data curation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Ethical approval

The study was carried out in compliance with the national animal welfare regulations. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentations (Directive 86/609/EC) and may be performed to diagnose animal diseases and improve animal welfare. Ticks were collected by registered veterinarians and caused no suffering. All farm owners were duly informed and consented to the sampling for the study.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the outcomes of this study.

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Data availability

Supplementary material, including sequence data, is available online at LabArchives (https://dx.doi.org/10.25833/98es-qx45). Sequence data reported in this study are available from GenBank SRA (PRJNA1129816). Sequences for partial *cox*1, 12S rRNA gene, and 16S rRNA gene fragments from *Rhipicephalus* spp. were submitted to GenBank under the accession numbers PQ001973-PQ001983, PQ002408-PQ002421, and PQ002089-PQ002107, respectively. Mitogenomes of Greek *Rhipicephalus* spp. were submitted under the accession numbers PP970529-PP970533. The voucher exoskeletons were deposited at the Australian National Insect Collection (ANIC), CSIRO, Canberra, Australian Capital Territory, Australia (ANIC 48 006 614 to 48 006 618).

References

Almazán, C., Torres-Rodríguez, L., Alanazi, A.B., Šlapeta, J., 2024. American mitogenome reference for the tropical brown dog tick, *Rhipicephalus linnaei* (Audouin, 1826). Curr. Res. Parasitol. Vector Borne Dis. 6, 100194. https://doi.org/10.1016/j.crpvbd.2024.100194.

Bakkes, D.K., Chitimia-Dobler, L., Matloa, D., Oosthuysen, M., Mumcuoglu, K.Y., Mans, B.J., Matthee, C.A., 2020. Integrative taxonomy and species delimitation of *Rhipicephalus turanicus* (Acari: Ixodida: Ixodidae). Int. J. Parasitol. 50, 577–594. https://doi.org/10.1016/j.ijpara.2020.04.005.

Beati, L., Keirans, J.E., 2001. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. J. Parasitol. 87, 32–48. https://doi.org/10.1645/0022-3395(2001)087[0032:AOTSRA]2.0.CO;2.

Black, W.C., Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. USA 91, 10034–10038. https://doi.org/10.1073/pnas.91.21.10034.

Chaligiannis, I., Musella, V., Rinaldi, L., Cringoli, G., de la Fuente, J., Papa, A., Sotiraki, S., 2016. Species diversity and spatial distribution of ixodid ticks on small ruminants in Greece. Parasitol. Res. 115, 4673–4680.

Chaligiannis, I., Papa, A., Sotiraki, S., 2014. Ticks feeding on ruminants and humans in Greece. Parasites Vectors 7, O1.

Chaligiannis, I., de Mera, I.G.F., Papa, A., Sotiraki, S., de la Fuente, J., 2018. Molecular identification of tick-borne pathogens in ticks collected from dogs and small ruminants from Greece. Exp. Appl. Acarol. 74, 443–453.

Chitimia-Dobler, L., Langguth, J., Pfeffer, M., Kattner, S., Kupper, T., Friese, D., et al., 2017. Genetic analysis of *Rhipicephalus sanguineus sensu lato* ticks parasites of dogs in Africa north of the Sahara based on mitochondrial DNA sequences. Vet. Parasitol. 239, 1–6. https://doi.org/10.1016/j.vetpar.2017.04.012.

Dantas-Torres, F., Latrofa, M.S., Annoscia, G., Giannelli, A., Parisi, A., Otranto, D., 2013. Morphological and genetic diversity of *Rhipicephalus sanguineus sensu lato* from the New and Old Worlds. Parasites Vectors 6, 213. https://doi.org/10.1186/1756-3305-6-213.

Estrada-Peña, A., Bouattour, A., Camica, J.W.A., 2004. Ticks of domestic animals in the Mediterranean Region: A guide to identification of species. University of Zaragosa, Zaragosa, Spain.

Hornok, S., Sandor, A.D., Tomanovic, S., Beck, R., D'Amico, G., Kontschan, J., et al., 2017. East and west separation of *Rhipicephalus sanguineus* mitochondrial lineages in the Mediterranean Basin. Parasites Vectors 10, 39. https://doi.org/10.1186/s13071-017-1985-z.

Jin, J.J., Yu, W.B., Yang, J.B., Song, Y., dePamphilis, C.W., Yi, T.S., Li, D.Z., 2020. GetOrganelle: A fast and versatile toolkit for accurate *de novo* assembly of organelle genomes. Genome Biol. 21, 241. https://doi.org/10.1186/s13059-020-02154-5.

Kushimo, O., 2013. The tick genus *Amblyomma* in Africa: Phylogeny and multilocus DNA Barcoding. MSc Thesis, Georgia Southern University, USA. https://digitalcommons.georgiasouthern.edu/etd/835/.

Latrofa, M.S., Angelou, A., Giannelli, A., Annoscia, G., Ravagnan, S., Dantas-Torres, F., et al., 2017. Ticks and associated pathogens in dogs from Greece. Parasites Vectors 10, 301.

Ligda, P., Gizzarelli, M., Kostopoulou, D., Foglia Manzillo, V., Saratsis, A., Saratsi, K., et al., 2023. Determination of the effect of collars containing 10% w/w imidacloprid and 4.5% w/w flumethrin (Seresto®) on the incidence of *Leishmania* and other canine vector-borne pathogen infections in Greece. Parasites Vectors 16, 89. https://doi.org/10.1186/s13071-023-05678-4.

Meiklejohn, K.A., Damaso, N., Robertson, J.M., 2019. Assessment of BOLD and GenBank – their accuracy and reliability for the identification of biological materials. PLoS One 14, e0217084. https://doi.org/10.1371/journal.pone.0217084.

Mumcuoglu, K.Y., Estrada-Peña, A., Tarragona, E.L., Sebastian, P.S., Guglielmone, A.A., Nava, S., 2022. Reestablishment of *Rhipicephalus secundus* Feldman-Muhsam, 1952 (Acari: Ixodidae). Ticks Tick Borne Dis. 13, 101897. https://doi.org/10.1016/j.ttbdis.2022.101897.

Nava, S., Beati, L., Venzal, J.M., Labruna, M.B., Szabó, M.P.J., Petney, T., et al., 2018. *Rhipicephalus sanguineus* (Latreille, 1806): Neotype designation, morphological redescription of all parasitic stages and molecular characterization. Ticks Tick Borne Dis. 9, 1573–1585. https://doi.org/10.1016/j.ttbdis.2018.08.001.

Ravi, A., Ereqat, S., Al-Jawabreh, A., Abdeen, Z., Abu Shamma, O., Hall, H., et al., 2019. Metagenomic profiling of ticks: Identification of novel rickettsial genomes and detection of tick-borne canine parvovirus. PLoS Negl. Trop. Dis. 13, e0006805. https://doi.org/10.1371/journal.pntd.0006805.

Saratsis, A., Ligda, P., Aal, F., Jelicic, M., Polgar, J., de Vries, M., et al., 2022. The scenario of ticks and tick-borne pathogens of sheep on a Mediterranean island. Microorganisms 10, 1551. https://doi.org/10.3390/microorganisms10081551.

Šlapeta, J., Chandra, S., Halliday, B., 2021. The "tropical lineage" of the brown dog tick *Rhipicephalus sanguineus sensu lato* identified as *Rhipicephalus linnaei* (Audouin, 1826). Int. J. Parasitol. 51, 431–436. https://doi.org/10.1016/j.ijpara.2021.02.001.

Šlapeta, J., Halliday, B., Chandra, S., Alanazi, A.D., Abdel-Shafy, S., 2022. *Rhipicephalus linnaei* (Audouin, 1826) recognised as the "tropical lineage" of the brown dog tick *Rhipicephalus sanguineus sensu lato*: Neotype designation, redescription, and establishment of morphological and molecular reference. Ticks Tick Borne Dis. 13, 102024. https://doi.org/10.1016/j.ttbdis.2022.102024.

Šlapeta, J., Halliday, B., Dunlop, J.A., Nachum-Biala, Y., Salant, H., Ghodrati, S., et al., 2023. The "southeastern Europe" lineage of the brown dog tick *Rhipicephalus sanguineus* (*sensu lato*) identified as *Rhipicephalus rutilus* Koch, 1844: Comparison with holotype and generation of mitogenome reference from Israel. Curr. Res. Parasitol. Vector Borne Dis. 3, 100118. https://doi.org/10.1016/j.crpvbd.2023.100118.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. USA 101, 11030–11035. https://doi.org/10.1073/pnas.0404206101.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. Mol. Biol. Evol. 38, 3022–3027. https://doi.org/10.1093/molbev/msab120.

Walker, J.B., Keirans, J.E.H.I., 2000. Genus *Rhipicephalus* (Acari, Ixodidae). In: A Guide to the Brown Ticks of the World. Cambridge University Press, Cambridge, UK.