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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

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ABSTRACT

The brown dog tick *Rhipicephalus sanguineus* (*sensu lato*) in the southeastern Mediterranean region and the Middle East is difficult to identify due to the presence of multiple mitochondrial DNA haplogroup lineages. The purpose of this study was to clarify the identity of the "southeastern Europe" lineage of this tick species complex. Our research shows that female ticks of the "southeastern Europe" lineage correspond to the morphology of *R. rutilus* Koch, 1844 as found in type-material at the Museum für Naturkunde Berlin in Germany. We characterised the complete mitogenomes of *R. rutilus*, *R. turanicus* Pomerantsev, 1940 and *Rhipicephalus sanguineus* (Latreille, 1806) in order to improve our understanding of the phylogenetic relationships among species within the *R. sanguineus* (*sensu lato*) complex. The material associated with the morphology of *R. rutilus* was previously labelled as the "southeastern Europe" lineage and found in Israel and Egypt, including Lower Egypt and the Nile Delta, where the original type-material was collected. Based on the morphology, genetic identity, and geographical distribution of the species, we conclude that the name *R. rutilus* is correctly linked to the "southeastern Europe" lineage of *R. sanguineus* (*sensu lato*).

1. Introduction

In 1820 two young naturalists, Dr Wilhelm Hemprich and Dr Christian Ehrenberg, conducted a research expedition to Egypt and neighbouring regions. For an overview of their route and localities, see e.g. Bradley (1968). The expedition was supported by the Prussian government, and brought back an enormous amount of material for the Berlin Museum (Hemprich and Ehrenberg, 1828). Hemprich died from malaria during their travels in 1825. Ehrenberg soon returned to Berlin to become one of the foremost experts on microscopic life and micropaleontology at the time. This expedition was analogous to efforts to rediscover ancient Egyptian culture by Napoleon at the time of his invasion of Egypt in 1798. The French scientific expedition to Egypt was described in major volumes between years 1809 and 1829 in the "Description de l'Egypte" (Audouin, 1826).

Both expeditions brought ticks from the region back to Europe. Napoleon's French expedition gave us *Rhipicephalus linnaei* (Audouin, 1826) – the tropical brown dog tick (Šlapeta et al., 2021). The Hemprich-Ehrenberg expedition yielded *Rhipicephalus* specimens that were subsequently named by the German arachnologist Carl Ludwig Koch as *Rhipicephalus rutilus* Koch, 1844 and *Rhipicephalus limbatus* Koch, 1844, both of which are members of the *Rhipicephalus sanguineus* (*sensu*

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lato) species complex (Camicas et al., 1998). While the type of *R. linnaei* was permanently lost, the types of *R. rutilus* and *R. limbatus* are held in the Museum für Naturkunde Berlin, Germany (Moritz & Fischer, 1981; Šlapeta et al., 2022). The availability of such historical museum material, together with new regional surveys using morphological and genetic tools over the past 10 years, enables the elucidation of their modern identity (Dantas-Torres et al., 2013; Chitimia-Dobler et al., 2017; Hornok et al., 2017; Senbill et al., 2022).

Rhipicephalus rutilus Koch, 1844 was described from a female collected in Egypt on an unknown host. Koch (1847) provided a more detailed description of the same specimen, accompanied by an illustration. Neumann (1897) re-described the species and added a second female specimen from Port Natal (now Durban); however the specimen could not be located in the Museum für Naturkunde Berlin, Germany by us. On the basis of what we now know about the species, the identification of that specimen must be considered as unlikely. Neumann (1911) divided *R. sanguineus* into three subspecies and considered *R. rutilus* to be a synonym of *R. sanguineus sanguineus*. Since that time *R. rutilus* has either been considered as a synonym of *R. sanguineus* (Zumpt, 1950; Floch and Fauran, 1959 (as *rutibus* [*sic*]), Morel and Vassiliades, 1962; Pegram et al., 1987; Keskin, 2009; Walker et al., 2000; Tucker, 2017; Aziz et al., 2018; Ramzan et al., 2020), or as *incertae sedis* (Guglielmone and Nava, 2014).

The aim of the present study was to clarify the identity of the "southeastern Europe" lineage of *R. sanguineus* (*sensu lato*). We show that female ticks that were genetically assigned to the "southeastern Europe" lineage of *R. sanguineus* (*sensu lato*) correspond to the morphology of the *R. rutilus* type-material. We characterised the complete mitochondrial genome (mitogenome) of *R. rutilus*, *R. turanicus*, and *R. sanguineus* (*sensu stricto*) in order to improve our understanding of phylogenetic relationships among species within the *Rhipicephalus sanguineus* (*sensu lato*) species complex.

2. Materials and methods

2.1. Available material and morphological identification

Ticks from several cats, a dog and a hedgehog were collected in Jerusalem, Israel in 2017 (Power et al., 2021). Ticks were collected together with fleas and stored in individual tubes with 70% ethanol. All ticks were observed under a stereo microscope (SMZ-2B, Nikon, Australia) and photographed using a digital microscope (VHX-6000, KEYENCE Inc., Japan) and identified using published keys and guides (Walker et al., 2000). A specimen of *R. sanguineus* (*sensu stricto*) (#208, referred to here as P8/22-208) was collected in 2021 from a dog in Györköny, Hungary. The holotype (ZMB 1093) of *R. rutilus* was observed (JŠ) at the Museum für Naturkunde Berlin, Germany and photographed on the museum's equipment for specimen digitalisation with a DSLR camera.

2.2. DNA isolation and amplification of the partial mitochondrial cox1 gene

DNA was isolated from ticks stored in 70% (v/w) ethanol as previously described by Šlapeta et al. (2022). Briefly, dried ticks with incisions in their idiosoma were subjected to total tick genomic DNA (gDNA) isolation with the Monarch Genomic DNA Purification Kit (New England Biolabs, Australia). Exoskeletons were retained and preserved in 70% (v/w) ethanol. Extracted gDNA was stored at −20 °C.

The ~600 nucleotide (nt) fragment of the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit 1 (*cox*1) gene was amplified using the primer sets S0725/S0726, as described in Chandra et al. (2019) and/or Šlapeta et al. (2022). MyTaq™ Red Mix (Bioline, Australia) was used for DNA amplifications in 30 µl reactions, with 2 µl of template gDNA. All reactions included PCR-grade water (ddH_2O) as a no-template control. The PCR reactions were performed in a T100™ Thermal Cycler (BioRad, Australia) and the PCR products were sequenced at Macrogen Ltd. (South Korea).

2.3. Genome skimming of R. rutilus, R. turanicus and R. sanguineus (sensu stricto) and assembly of mitogenome from next generation sequence data

The gDNA isolated from three adult *Rhipicephalus* spp. ticks was used for genome skimming *via* next-generation sequencing (NGS) using a NEBNext® DNA Library Prep Kit followed by NGS using 150 bp paired-end Illumina sequencing system at a depth of 1 Gb of raw sequence data (Novogene, Singapore). The whole mtDNA (IZ12-T3: JS6028; IZ13-T1: JS6029; P8/22-208: JS6373) was assembled from FastQ data using the MITObim pipeline (Hahn et al., 2013) (https://github.com/chrishah/MITObim) as previously described (Šlapeta et al., 2022). The complete circular mtDNA (mitogenomes) were aligned with all available complete mtDNA sequences of species of the *R. sanguineus* (*sensu lato*) group in CLC Main Workbench 21 (CLC bio, Qiagen, Australia).

2.4. Phylogenetic analysis

Sequence alignments were constructed using the CLC Main Workbench 21 (CLC bio, Qiagen, Australia). Evolutionary analyses including selection of models were conducted in MEGA 11 (Tamura et al., 2021). Whole mitogenome sequence alignments were included for newly obtained mitogenomes as well as all available mitogenomes from within the *R. sanguineus* (*sensu lato*) complex (as of December 20, 2022). The nucleotide mitogenome sequence alignment (15,029 sites) was produced with the aid of amino acid sequence translation. The phylogeny was reconstructed using selected best model, Maximum Likelihood method and General Time Reversible (GTR) model with a discrete Gamma distribution among sites (+G), and the rate variation model was allowed for some sites to be evolutionarily invariable (+I). The percentage of replicate trees in which the associated taxa clustered together was used for a bootstrap test (200 replicates). The *cox*1 gene phylogeny included all newly obtained sequences as well as all *cox*1 sequences belonging to the *R. sanguineus* (*s.l.*) "southeastern Europe" lineage available in GenBank. Sequences from *R. microplus* (Canestrini, 1897) and *R. australis* Fuller, 1899 served as outgroups. The *cox*1 phylogenetic tree based on nucleotide alignment was inferred by using the GTR + G + I for *cox*1 alignment. There was a total of 1001 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together was calculated for the bootstrap test (1000 replicates).

2.5. Voucher material and sequence data deposition

The tick voucher specimens were deposited at the Australian National Insect Collection, CSIRO, Canberra, Australian Capital Territory, Australia, under the following accession numbers: *R. turanicus* (ANIC 48 006 603, ANIC 48 006 604, ANIC 48 006 606, ANIC 48 006 608 to ANIC 48 006 611); *R. rutilus* (ANIC 48 006 605, ANIC 48 006 607); *H. adleri* (ANIC 48 006 612); and *R. sanguineus* (ANIC 48 006 613). Raw FastQ sequence data were deposited at SRA NCBI BioProject: PRJNA917775. The nucleotide sequence data including three assembled mitogenomes generated in this study were deposited in GenBank (NCBI): OQ184022-OQ184024 (complete mtDNA) and OQ180895-OQ180904 (*cox*1). All sequence data, photographic documentation and associated supplementary material and additional data are available at LabArchives (https://dx.doi.org/10.25833/qt1c-z916).

3. Results

Morphological investigation revealed the presence of two female brown dog ticks, *Rhipicephalus sanguineus* (*sensu lato*) (IZ12-T1, IZ12-T3), whose spiracles were similar to those reported by Dantas-Torres et al. (2013) as "*Rhipicephalus* sp. Morphotype 1" with a narrow, short, but distinct dorsal projection (Figs. 1 and 2). The remaining *Rhipicephalus*

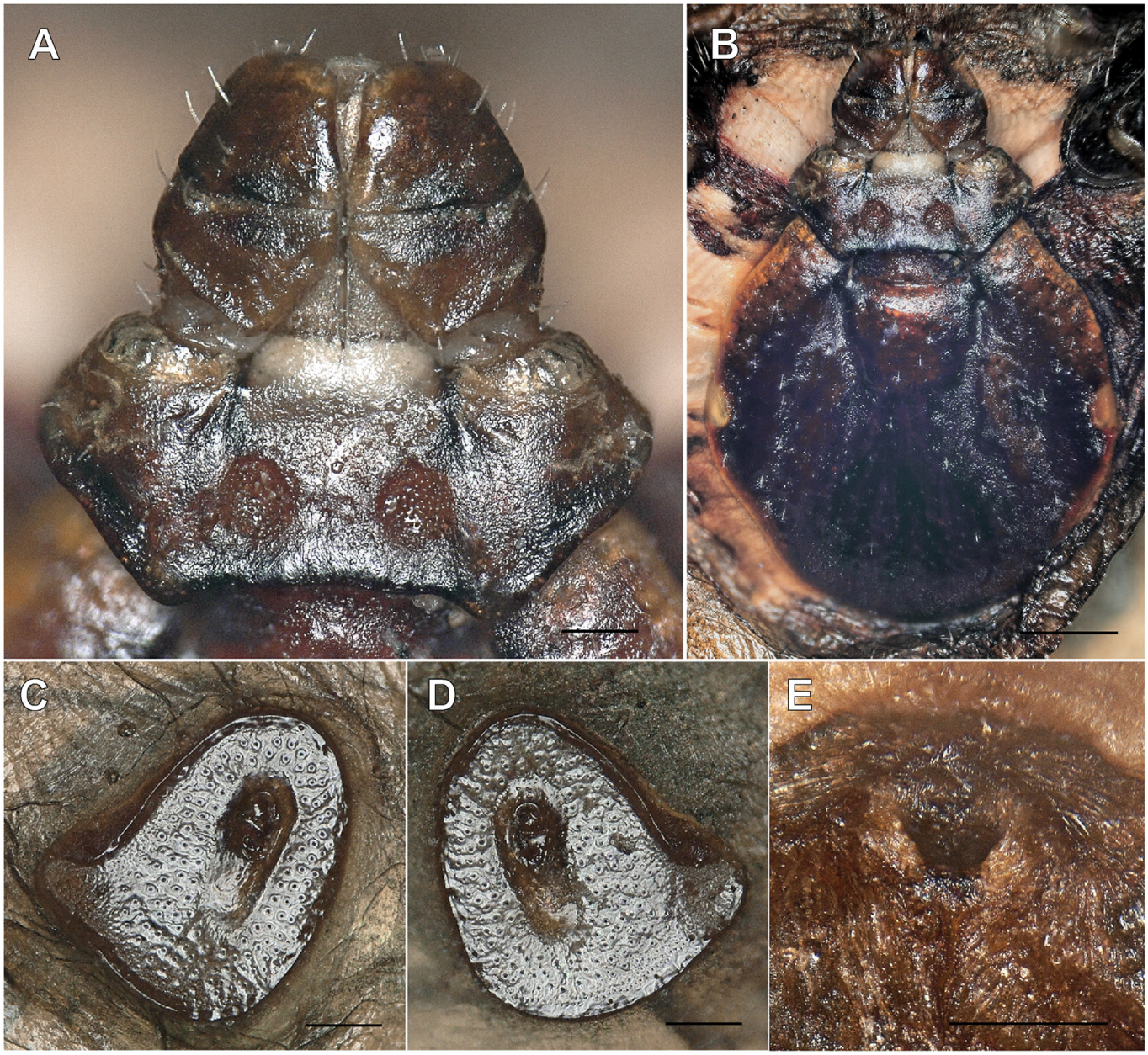

Fig. 1. *Rhipicephalus rutilus* engorged adult female (IZ12-T1, ANIC 48 006 605) collected from a dog from Israel. **A** Basis capitulum with palps. **B** Scutum with eyes. **C**, **D** Spiracles. **E** Genital pore. *Scale-bars*: 100 μm (**A**, **C**, **D**, **E**); 300 μm (**B**).

spp. ticks exhibited spiracles that had a broad dorsal projection and were identified as *R. turanicus.* The recently re-established species *R. secundus* Feldman-Muhsam, 1952 is morphologically similar to *R. turanicus*, and the scutal punctation was examined to differentiate the two species from each other (Fig. 3). The presence of numerous large punctations across the scutum suggested a *R. turanicus* identification (Fig. 3). The additional single tick was identified as *Haemaphysalis adleri* Feldman-Muhsam, 1951 (Table 1).

The presence of a "*Rhipicephalus* sp. Morphotype 1" specimen prompted us to review its identity. "*Rhipicephalus* sp. Morphotype 1" was originally associated with partial mtDNA sequences from Italy and Greece, later identified as an "East Mediterranean" lineage by Hornok et al. (2017) from Romania, Greece, Serbia and Israel, and as the "southeastern Europe" lineage by Chitimia-Dobler et al. (2017) from Egypt (Nava et al., 2018; Bakkes et al., 2020; Šlapeta et al., 2021). This lineage from "southeastern Europe" forms a well-supported phylogenetic clade based on partial *cox*1 (Fig. 4). The general morphology, and specifically the shape of the spiracles (IZ12-T1, IZ12-T3), closely resemble the *R. rutilus* holotype (ZMB 1093) held at the Museum für Naturkunde Berlin, Germany (Fig. 5) (Koch, 1844, 1847). The holotype was collected in Damiette (= Damietta, Egypt) by Ehrenberg.

To verify our morphological investigation, we obtained ten *cox*1 partial sequences from ticks collected from Israel (Table 1). Phylogenetic analysis with related *cox*1 sequences from *Rhipicephalus* species and lineages confirmed the grouping of *R. sanguineus* (*sensu lato*) (IZ12-T1, IZ12-T3) sequences with the "southeastern Europe" lineage that we consider to represent *R. rutilus* (Fig. 4). The *cox*1 sequences from *R. turanicus* were grouped within the haplogroup of other samples of *R. turanicus* (Fig. 4). Using partial *cox*1, the *R. rutilus* clade was sister group to a strongly supported (93%) monophyletic clade consisting of *cox*1 sequences from *R. linnaei*, *R. camicasi*, *R. guilhoni* and *R. afranicus*, but this sister relationship was unsupported (< 50%). Similarly, the placement of the *R. turanicus* partial *cox*1 sequence clade was unsupported (< 50%) within other clades. To improve the phylogenetic resolution, we attempted to

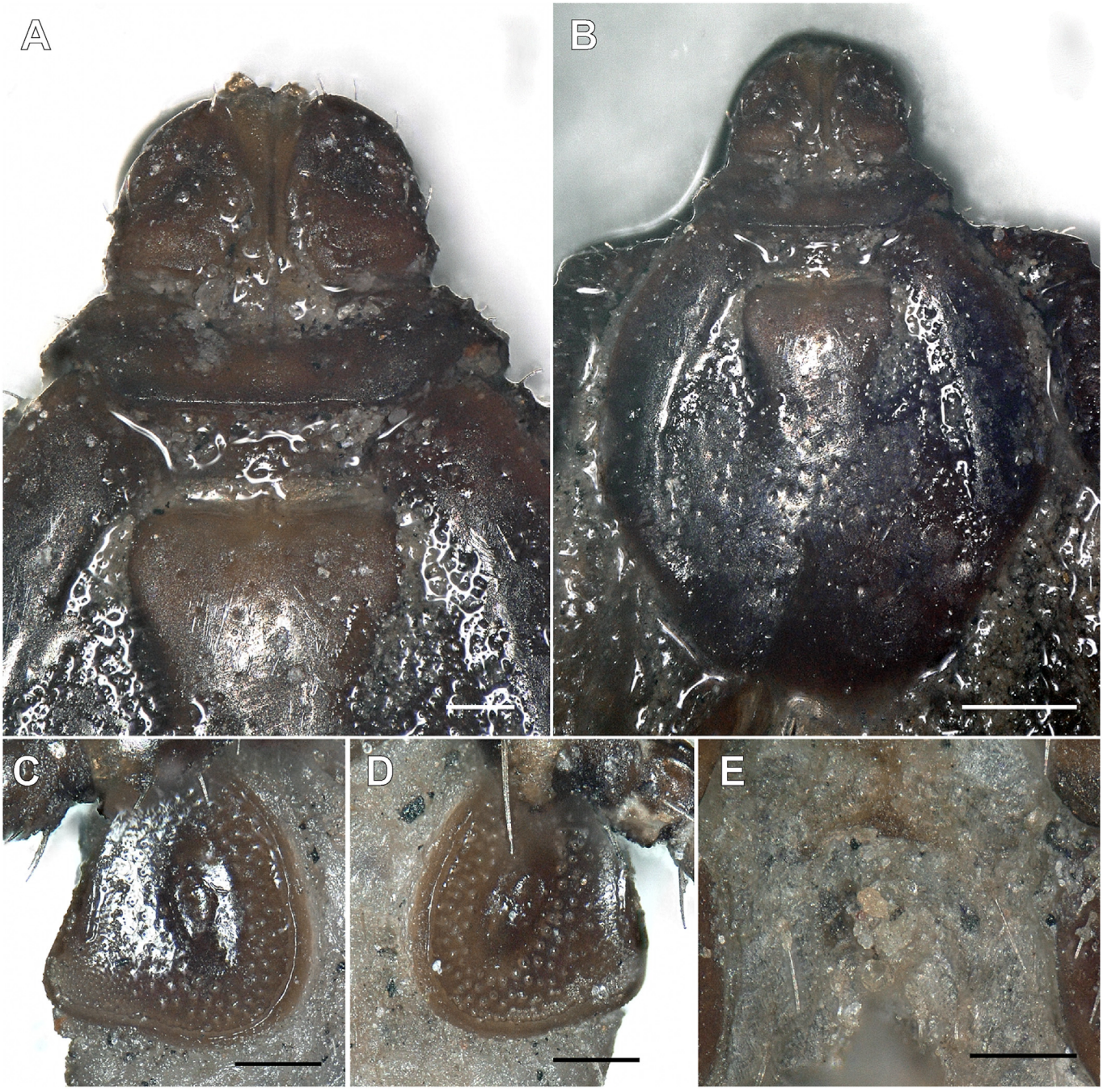

Fig. 2. *Rhipicephalus rutilus* unengorged adult female (IZ12-T3, ANIC 48 006 607) collected from a dog from Israel. **A** Basis capitulum with palps. **B** Scutum with eyes. **C**, **D** Spiracles. **E** Genital pore (obscured by debris). *Scale-bars*: 100 μm (**A**, **C**, **D**, **E**); 500 μm (**B**).

obtain complete mtDNA.

Mitogenomes were assembled from low pass whole genome sequencing (genome skimming) reads for *R. rutilus* (IZ12-T3: JS6028) and *R. turanicus* (IZ13-T1: JS6029) from Israel. To complement the work, we generated whole genome sequence approach and mitogenome assembly for *R. sanguineus* (*sensu stricto*) (P8/22-208: JS6373) from Hungary. Phylogenetic analysis using complete mitogenomes (mtDNA) yielded a robust and highly resolved tree with *R. rutilus* strongly supported (100%) as the sister clade to *R. linnaei* and *R. camicasi* (Fig. 6A). Similarly, *R. turanicus* was also strongly supported as a monophyletic clade (100%) that is sister (100% support) to the mitogenome of *R. secundus* (Fig. 6A). With these three newly obtained mitogenomes, there are currently 24 complete mitogenomes available for six species within the *R. sanguineus* (*sensu lato*) species complex, namely *R. sanguineus*, *R. linnaei*, *R. rutilus*, *R. turanicus*, *R. secundus* and *R. camicasi* (Fig. 6B).

4. Discussion

This study took advantage of ectoparasites that were collected during a study that focused on fleas (*Ctenocephaldes felis*, *Ctenocephalides canis*) and their carriage of *Bartonella* and *Rickettsia* in Israel (Power et al., 2021). Out of the 33 animals (28 cats, 4 dogs and 1 hedgehog) there were six cats (21%), one dog (25%) and one hedgehog (100%) with ticks that remained unprocessed (Power et al., 2021). Initial morphological inspection of the tick specimens indicated that 11 belonged to the genus

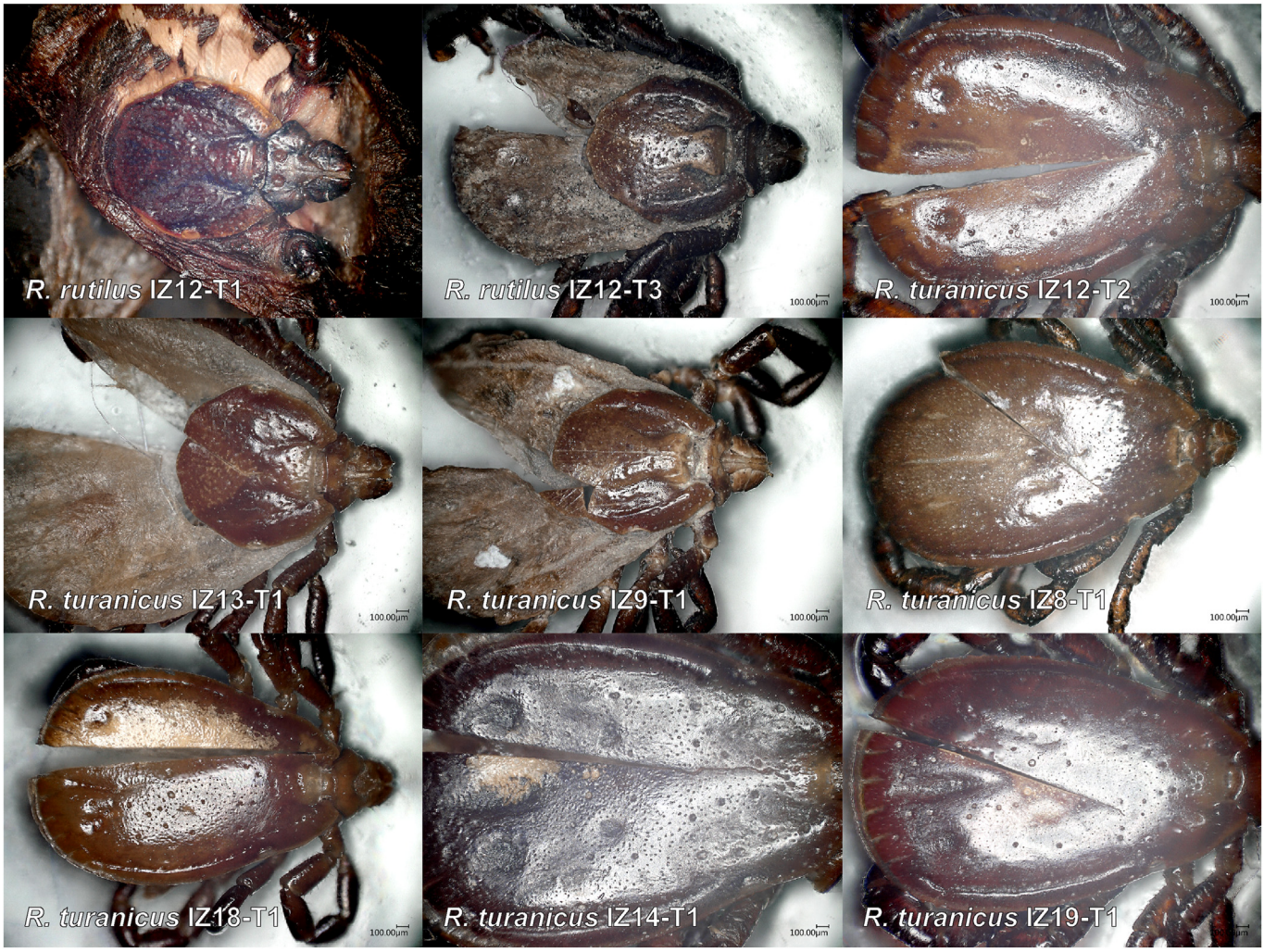

Fig. 3. Scutum of *Rhipicephalus rutilus* and *Rhipicephalus turanicus* from Israel. Note the incision to the scutum and alloscutum is an artefact, as it was part of the process to isolate DNA from these specimens. *Scale-bars*: 100 μm.

Table 1
Summary of ticks and hosts from Jerusalem, Israel.

ID#	ANIC number	Tick species	Tick stage and sex	Host, age	Date	Sequence data
IZ08-T1	ANIC 48 006 603	*Rhipicephalus turanicus*	Unengorged male	Cat, NA	April 3, 2017	OQ180900
IZ09-T1	ANIC 48 006 604	*Rhipicephalus turanicus*	Unengorged female	Cat, NA	April 4, 2017	OQ180899
IZ12-T1	ANIC 48 006 605	*Rhipicephalus rutilus*	Engorged female	Dog, 6 weeks	April 30, 2017	OQ180895
IZ12-T2	ANIC 48 006 606	*Rhipicephalus turanicus*	Unengorged male			OQ180897
IZ12-T3	ANIC 48 006 607	*Rhipicephalus rutilus*[a]	Unengorged female			OQ180896, OQ184022
IZ13-T1	ANIC 48 006 608	*Rhipicephalus turanicus*[a]	Unengorged female	Cat, 1 year	May 4, 2017	OQ180898, OQ184023
IZ14-T1	ANIC 48 006 609	*Rhipicephalus turanicus*	Unengorged male	Hedgehog, NA	April 30, 2017	OQ180902
IZ18-T1	ANIC 48 006 610	*Rhipicephalus turanicus*	Unengorged male	Cat, 1.5 years	May 10, 2017	OQ180901
IZ19-T1	ANIC 48 006 611	*Rhipicephalus turanicus*	Unengorged male	Cat, 7 month	May 11, 2017	OQ180903
IZ20-T1	ANIC 48 006 612	*Haemaphysalis adleri*	Engorged female	Cat, 1 year	May 11, 2017	OQ180904

Notes: ANIC, Australian National Insect Collection; dog, *Canis lupus familiaris*; cat, *Felis catus*; hedgehog, *Erinaceus concolor*; NA, not known.
[a] Mitogenome available.

Rhipicephalus and one to the genus *Haemaphysalis*. The majority of the *Rhipicephalus* ticks exhibited features (i.e. shape of spiracles) that suggested *R. turanicus*, with several specimens from a dog suggesting *R. sanguineus* (*sensu lato*) (Table 1). The tick belonging to the genus *Haemaphysalis* was identified as *H. adleri*. While originally described from the golden jackal (*Canis aureus*) from Israel, the present engorged female was found on a cat and confirms previous findings (Feldman-Muhsam, 1951; Salant et al., 2014). In Israel, recent study into ticks and tick-borne pathogens has demonstrated the common occurrence of both *R. sanguineus* (*sensu lato*) and *R. turanicus* on dogs and cats (Salant et al., 2014; Mumcuoglu et al., 2022a). It was already believed that *R. sanguineus* (*sensu lato*) is the "*Rhipicephalus* sp. Morphotype 1" or "southeastern Europe" lineage because of the scarce sequence records available in public databases (Zemtsova et al., 2016; Hornok et al., 2017). This "southeastern Europe" lineage is now well recognised from neighbouring Egypt, including Lower Egypt and the Nile Delta (Abdullah et al., 2016; Chitimia-Dobler et al., 2017; Senbill et al., 2022).

Carl Ludwig Koch provided the following description of a specimen

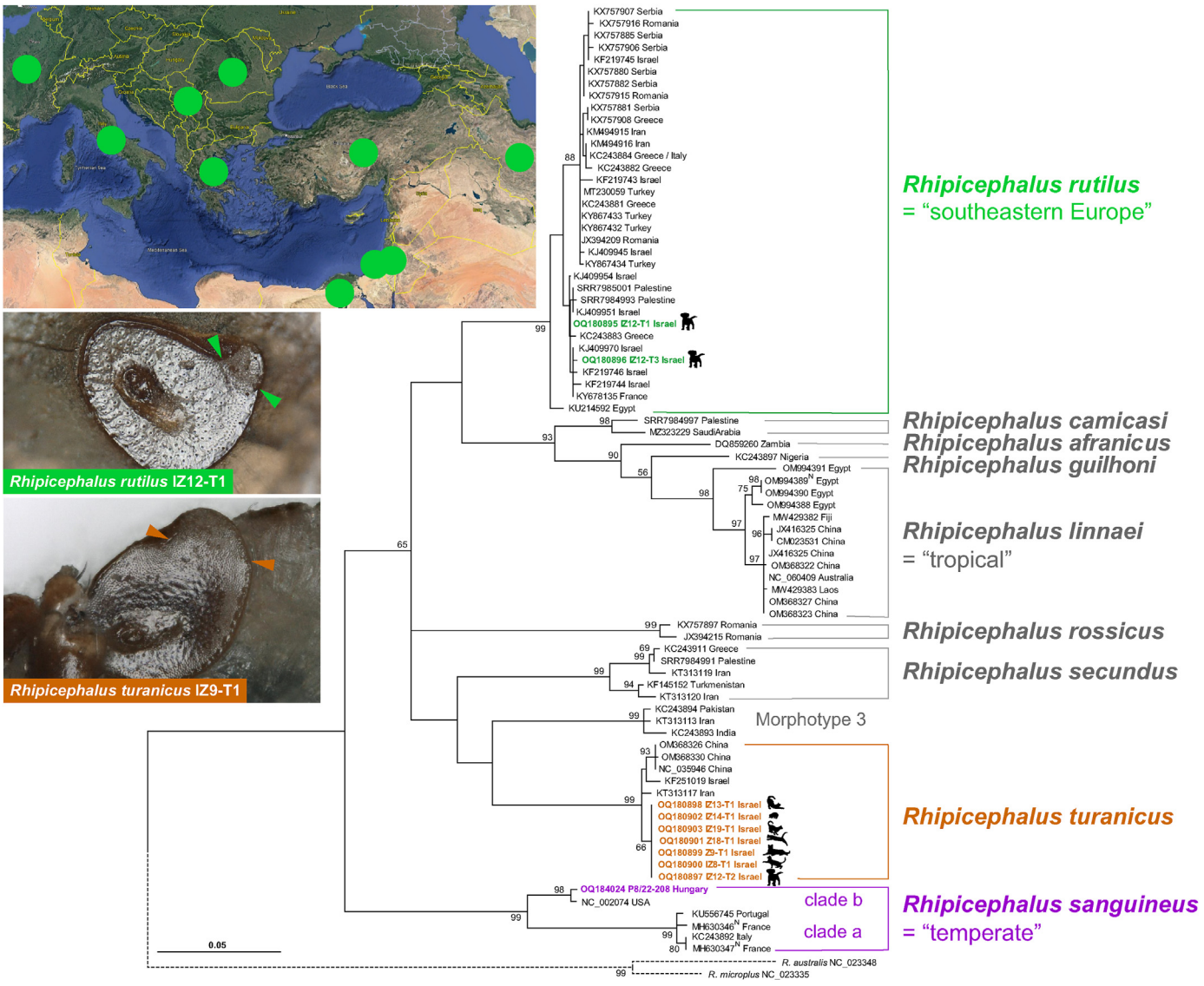

Fig. 4. Phylogenetic analysis of *Rhipicephalus rutilus* based on *cox*1 sequences. The phylogenetic tree was reconstructed using Maximum Likelihood and GTR + G + I model with bootstrap support (> 50%) shown. The newly obtained sequences and the labels are colour-coded. Accession numbers accompanied by (^{N}) indicate sequence from neotype reference material. The taxonomy is indicated on the right of the tree. The dotted line indicates the outgroups (*R. australis*, *R. microplus*). The inset map shows the countries (circles) where *R. rutilus* has been detected based on *cox*1 (map courtesy of Google Earth: SIO, NOAA, U.S. Navy, NSA, GEBCO. Image Landsat/Copernicus). Female spiracle of *R. rutilus* (IZ12-T1, ANIC 48 006 605) and *R. turanicus* (IZ09-T1, ANIC 48 006 604) shown with arrows indicating the start of the dorsal process.

collected during the Hemprich-Ehrenberg expedition to Egypt: "*R. rutilus*. Flach, oval; Thorax sehr fein punktiert, roth; Hinderleib etwas dunkel menningroth. Beine rostroth. Länge 1 1/8‴. Weibchen. Männchen: unbekannt. Vaterland: Aegypten." [*R. rutilus. Flat, oval*; *Thorax very finely dotted, red*; *Abdomen somewhat dark, lead oxide red colour. Rusty legs. Length 1 1/8‴. Female. Male*: *unknown. Origin*: *Egypt*] (Koch, 1844). The length is presented in the historic length unit *Paris line* (ligne, 1‴ = 2.2558 mm) implying that the given length was 2.54 mm. The ZMB 1093 label recorded reveals further details about the locality where the material was collected – Damiette (= Damietta, a port city in the Nile Delta in Lower Egypt) (Moritz and Fischer, 1981). Based on Bradley's (1968) account of the expedition, it was probably collected in the winter or spring of 1823. The morphological identity of the species *R. rutilus* was doubtful and traditionally believed to represent *R. sanguineus*, so the name was treated as a junior synonym of the latter. Re-evaluation of the type-material of *R. rutilus* revealed the key characteristic that links it with the extant material, beyond the general description by Koch (1844) and illustration by Koch (1847). Specifically, the spiracle on female ZMB 1093 held at the Museum für Naturkunde Berlin, Germany (Fig. 5) has the shape and the distinct dorsal process matching the spiracles photographed in our modern material from dogs in Israel and in ticks from Italy and Greece as shown in figure 2f in Dantas-Torres et al. (2013) from dogs. The genital pore of the female holotype could not be observed due to the position of the mounting pin used to display the specimen, which unfortunately pierces the genital region. Older tick specimens in the Berlin Museum were often mounted pinned and dried like insects, and only later was it usual to put the whole (undamaged) specimen in alcohol.

One can consider morphological features of the males of *R. rutilus*. Using the molecular evidence, we can link the features illustrated in figure 1g-i in Dantas-Torres et al. (2013) and figure 7a,b in Hornok et al. (2017) as the current best morphological features of *R. rutilus*, in particular the long, extended dorsal process that tapers towards the narrow opening at the tip as the prominent feature.

Our understanding of the distribution of the "southeastern Europe" lineage and thus *R. rutilus* primarily comes from sequence records generated over the past 10 years. Ticks on Egyptian dogs have been

Fig. 5. Holotype female of *Rhipicephalus rutilus* as illustrated by Koch (1847). On the right are photographs of the holotype held at the Museum für Naturkunde Berlin, Germany (ZMB 1093) in dorsal and lateral view. A close up of the left spiracle is shown in the inset, note the short narrow process. The 1847 illustration is provided courtesy of the Biodiversity Heritage Library (biodiversitylibrary.org). The specimen label and view of the pinned voucher ZMB 1093 is shown as is at the Museum für Naturkunde Berlin, Germany in the lower part.

characterised multiple times. Chitimia-Dobler et al. (2017) characterised ticks from dogs found in Luxor, Upper Egypt, and found both *R. linnaei* and *R. rutilus*. Šlapeta et al. (2022) found only *R. linnaei* and *R. turanicus* on dogs in Esna, a town about 60 km south of Luxor along the River Nile. In Lower Egypt, only *R. rutilus* genetic records exist from ticks on dogs from Cairo and Alexandria (Abdullah et al., 2016; Senbill et al., 2022). The "southeastern Europe" lineage itself was recognised in 2017 based on material from the Balkan region in Europe and the eastern Mediterranean including Türkiye and Israel (Chitimia-Dobler et al., 2017; Hornok et al., 2017). This *cox*1 haplogroup representing *R. rutilus* has been reported from Italy as the "*Rhipicephalus* sp. Morphotype 1" *sensu* Dantas-Torres et al. (2013) under the GenBank accession number KC243884. A more puzzling record that will require verification is a *cox*1 under the GenBank accession number KY678135 obtained from a specimen collected from a dog in France with no further history about the dog (Duron et al., 2017). There are also two *cox*1 records (GenBank: KM494915, KM494915) from ticks collected from dogs in Iran that have not yet been published. A recent study into *Rhipicephalus* spp. on sheep and goats from Iran recorded three distinct *Rhipicephalus* spp. haplogroups, besides *R. bursa* (Hosseini-Chegeni et al., 2019). The authors originally suggested the presence of *R. sanguineus* (*sensu lato*), *R. sanguineus* (*sensu stricto*), and *R. turanicus* based on *cox*1. However, our reanalysis investigating whether the *R. sanguineus* (*sensu lato*) haplogroup could be *R. rutilus* provides new evidence that these three Iranian haplogroups on livestock are *R. turanicus*, *R. secundus* and as yet unknown species that was previously referred to as "*Rhipicephalus* sp. Morphotype 3" *sensu* Dantas-Torres et al. (2013); with none of them being *R. rutilus* or *R. sanguineus*. It is possible that *R. rutilus* will prove to be restricted to dogs in Iran and hence absent from livestock (Hosseini-Chegeni et al., 2019). Dogs are the main host of *R. rutilus*. Taking available *cox*1 sequences as evidence of host association, *R. rutilus* was also found on a cat (KJ409951) and a hedgehog (KJ409970) from Israel.

Using the evidence documented above – the morphology, genetic identity and geographical distribution – we believe we are justified to link *R. rutilus* with what is recognised as the "southeastern Europe" lineage of *R. sanguineus* (*sensu lato*). To our knowledge there is no older available name for this species (Camicas et al., 1998; Guglielmone et al., 2015 and updates). Attempting to extract DNA non-destructively from a 200-year-old specimen is not impossible (Ivanova et al., 2006; Chandra et al., 2021), but in our opinion the risk of permanently damaging the *R. rutilus* holotype is too high and the existing collateral and morphological evidence presented here is sufficient.

To complement our work on mitogenomes, we have made available the first mitogenome of *R. sanguineus* from Europe. Phylogenetic analysis confirmed previously demonstrated divergence of two well-defined clades (a + b) of *R. sanguineus* (Hornok et al., 2017). The complete mitogenome from the Hungarian specimen is almost identical to the reference mitogenome of *R. sanguineus* from the USA, but partly distinct from the neotype material *cox*1 partial sequences (Nava et al., 2018). Hornok et al. (2017) have already reported that these two mtDNA lineages coexist in certain sampling sites such as Piacenza in Italy and Zagreb in Croatia.

The identity of brown dog ticks within the south-eastern Mediterranean regions or/and the Middle East is still far from completely understood. It seems that *R. sanguineus* (*sensu stricto*) is largely absent. Species such as *R. linnaei*, *R. rutilus* and *R. camicasi* need to be considered together with those related to *R. turanicus* and *R. secundus* (Chandra et al., 2022; Mumcuoglu et al., 2022b; Okely et al., 2022; Šlapeta et al., 2022). Morphological differentiation is feasible if large series of locally collected ticks are assembled and compared, but such differentiation is not without its limitations (e.g. Hornok et al., 2017). Establishing good reference material, both morphological and genetic, will be of particular value when comparing studies from different regions since these ticks are vectors of serious disease agents and the role of different species to be

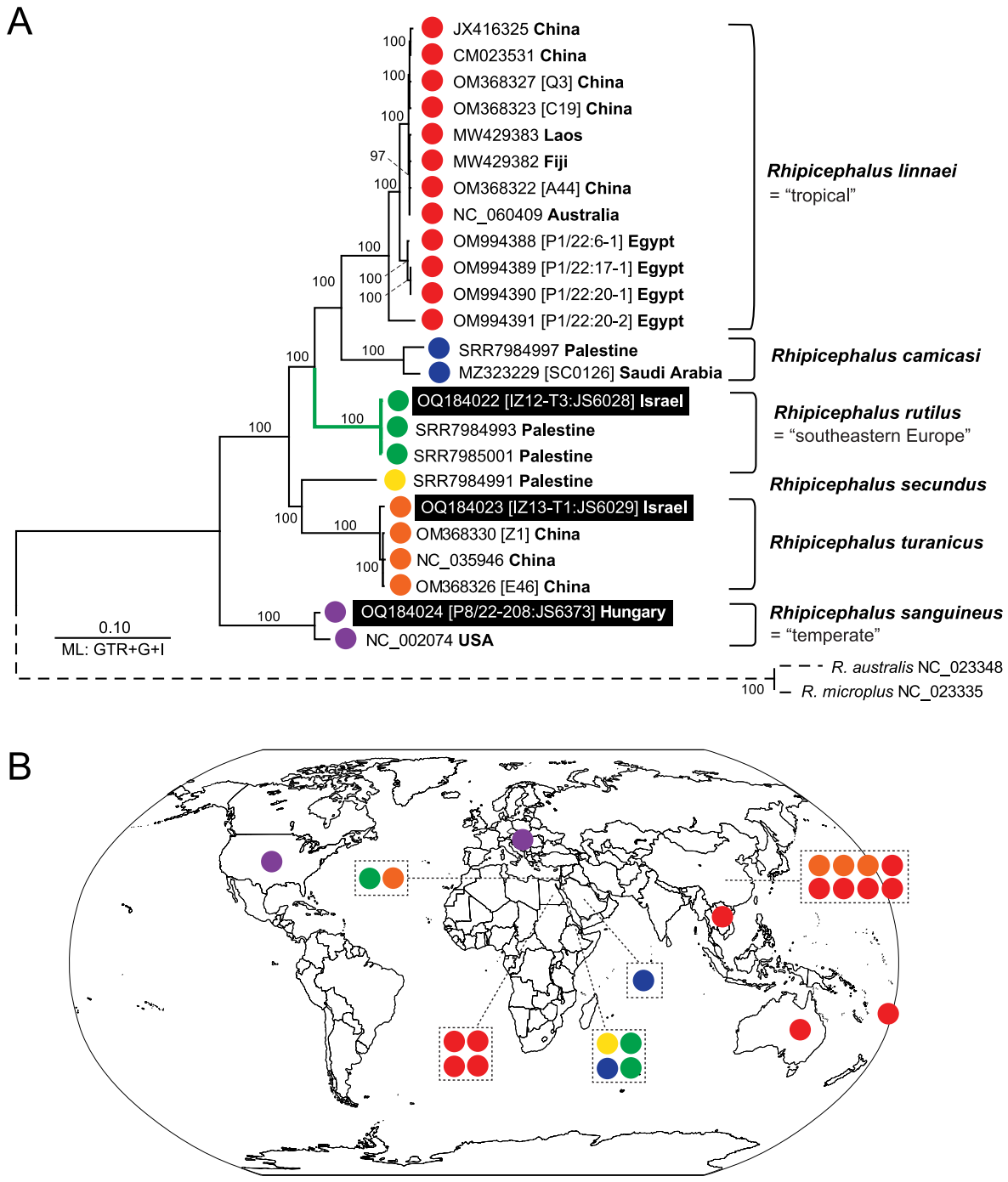

Fig. 6. Mitogenome phylogeny of *Rhipicephalus rutilus* with related species within the *Rhipicephalus sanguineus* (*sensu lato*) species complex. **A** The phylogenetic tree was reconstructed using Maximum Likelihood and GTR + G + I model with bootstrap support (> 50%) shown. The three newly obtained mitogenomes are on a black background. Tree taxonomy is indicated on the right of the tree and is colour-coded. The dotted line indicates the outgroup (*R. australis*, *R. microplus*). **B** World map with country locations for 24 mitogenomes currently available for *Rhipicephalus sanguineus* (*sensu lato*) species complex.

competent vectors is masked by the incomplete record of the species identity. Mitogenomes are easy to obtain and cost-effective, in fact if obtained *via* genome skimming, they enable screening for pathogen DNA as well (Ravi et al., 2019; Jia et al., 2020; Kneubehl et al., 2022; Šlapeta et al., 2022; Kelava et al., 2023). Adoption of the formal available name *R. rutilus*, together with a permanent genomic reference, eliminates the need for informal names such as "Morphotype 1" or "southeastern Europe" in future studies.

5. Conclusions

Based on the morphology, genetic identity, and geographical distribution of the species, we conclude that the name *R. rutilus* is correctly linked to the "southeastern Europe" lineage of *R. sanguineus* (*sensu lato*). We are justified to call material previously labelled as the "southeastern Europe" with a formal name *R. rutilus* for which, we provide molecular reference in form of complete mitogenome.

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Ethical approval

Not applicable.

CRediT authorship contribution statement

Jan Šlapeta: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. Bruce Halliday: Data curation, Investigation, Resources, Writing – review & editing. Jason A. Dunlop: Data curation, Investigation, Resources, Writing – review & editing. Yaarit Nachum-Biala: Investigation, Writing – review & editing. Harold Salant: Investigation, Writing – review & editing. Sajjad Ghodrati: Investigation, Data curation, Writing – review & editing. David Modrý: Resources, Supervision, Writing – review & editing. Shimon Harrus: Resources, Supervision, Writing – review & editing. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The tick voucher specimens were deposited at the Australian National Insect Collection, CSIRO, Canberra, Australian Capital Territory, Australia, under the following accession numbers: *R. turanicus* (ANIC 48 006 603, ANIC 48 006 604, ANIC 48 006 606, ANIC 48 006 608 to ANIC 48 006 611); *R. rutilus* (ANIC 48 006 605, ANIC 48 006 607); *H. adleri* (ANIC 48 006 612); and *R. sanguineus* (ANIC 48 006 613). Raw FastQ sequence data were deposited at SRA NCBI BioProject: PRJNA917775. The nucleotide sequence data including three assembled mitogenomes generated in this study were deposited in GenBank (NCBI): OQ184022-OQ184024 (complete mtDNA) and OQ180895-OQ180904 (*cox*1). All sequence data, photographic documentation and associated supplementary material and additional data are available at LabArchives (https://dx.doi.org/10.25833/qt1c-z916).

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