Parasitology Research (2019) 118:1067–1071
https://doi.org/10.1007/s00436-019-06214-z

ARTHROPODS AND MEDICAL ENTOMOLOGY - SHORT COMMUNICATION

Genetic analysis of *Rhipicephalus sanguineus* sensu lato ticks, parasites of dogs in the Canary Islands, Cyprus, and Croatia, based on mitochondrial 16S rRNA gene sequences

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Received: 22 November 2018 / Accepted: 10 January 2019 / Published online: 23 January 2019

Abstract
The aim of this work was to perform an analysis based on mtDNA sequences of the 16S rRNA gene in order to determine the phylogenetic position of ticks belonging to the *Rhipicephalus sanguineus* group from the Canary Islands, Cyprus, and Croatia. All the haplotypes obtained from ticks collected in the Canary Islands and Croatia grouped with *R. sanguineus* sensu stricto from France, Portugal, Italy, Switzerland, Argentina, Chile, Uruguay, and the USA. The sequences of *R. sanguineus* sensu lato from Cyprus formed a clade with *R. sanguineus* s.l. from Egypt, Turkey, and Romania, which belongs to the "*Rhipicephalus* sp. morphotype I" or "southeastern European lineage." Ticks determined as *R. turanicus* s.l. from Cyprus clustered separately from the remaining clades of the *R. sanguineus* group, including *R. turanicus* s.s. The data show that *R. sanguineus* s.s. is present in the Canary Islands and Croatia, while *R. sanguineus* "southeastern lineage" is found in Cyprus.

Keywords *Rhipicephalus sanguineus* s.l. · Canary Islands · Cyprus · Croatia · Genetic analyses

Introduction

The *Rhipicephalus sanguineus* group (Acari: Ixodidae) contains tick species of substantial medical, veterinary, and economic importance because they are common parasites and vectors of several disease agents affecting domestic and wild animals and humans. This species group includes *Rhipicephalus bergeoni* Morel & Balis (1976), *Rhipicephalus camicasi* Morel et al., 1976, *Rhipicephalus guilhoni* Morel & Vassiliades, 1963, *Rhipicephalus leporis* Pomerantzev, 1946, *Rhipicephalus moucheti* Morel, 1965, *Rhipicephalus pumilio* Schulze, 1935, *Rhipicephalus pusillus* Gil Collado, 1936, *Rhipicephalus rossicus* Yakimov & Kol-Yakimova, 1911, *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus schulzei* Olenev, 1929, *Rhipicephalus sulcatus* Neumann, 1908, and *Rhipicephalus turanicus* Pomerantzev, 1940 (Pegram et al. 1987). Of these taxa, *R. sanguineus* (hereafter *R. sanguineus* sensu stricto) stands out because it is a parasite of dogs worldwide, a vector of human and animal pathogens, and a target of the commercial pest control market. The presence of different lineages within *R. sanguineus* s.s. showing strong biological and genetic divergence has been demonstrated in several studies (Szabó et al. 2005; Burlini et al. 2010; Eremeeva et al. 2011; Moraes-Filho et al. 2011; Levin et al. 2012; Nava et al. 2012; Dantas-Torres et al. 2013; Liu et al. 2013; Hekimoglu et al. 2016; Sanches et al. 2016; Zemtsova et al. 2016; Chitimia-Dobler et al. 2017; Dantas-Torres et al. 2017; Jones et al. 2017; Labruna et al. 2017). As a result, *R. sanguineus* s.s. is now recognized as a species complex rather than a single species (Dantas-Torres and Otranto 2015; Nava et al. 2015). Recently, *R. sanguineus* s.s. was redefined, with morphological redescriptions of all parasitic stages and phylogenetic analyses based on different molecular markers; a neotype, replacing Latreille's lost type material, was also designated (Nava et al. 2018). However, there remains the task of defining the taxonomic status of some

Handling Editor: Julia Walochnik

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lineages of *R. sanguineus* sensu lato whose classification at the specific level is still uncertain (Nava et al. 2018).

Considering the body of knowledge generated in recent years on the taxonomic status of *R. sanguineus* s.l., much of which was obtained from analyses of mitochondrial DNA (mtDNA) sequences, an analysis based on mtDNA sequences of the 16S rRNA gene was performed in order to determine the phylogenetic position of *R. sanguineus* s.l. ticks occurring in the Canary Islands, Cyprus, and Croatia.

Material and methods

Tick collection

Ticks were collected during 2010, 2012, and 2013 from dogs in 21 animal shelters on the five Canary Islands below and in one case from a pedestrian precinct and urban houses in Costa Teguise, Lanzarote. Collections were made on Lanzarote (N 29° 4′ 14.878″, W 13° 38′ 30.939″; N 29° 0′ 0.417″, W 13° 33′9.714″; N 29° 7′ 45.728″, W 13° 28′ 59.176″), Fuerteventura (N 28° 43′ 43.972″, W 13° 55′ 0.553″), Gran Canaria (N 28° 8′ 30.097″, W 15° 30′ 14.654″), Tenerife (N 28° 30′ 0.596″, W 16° 24′ 35.429″; N 28° 5′ 12.3″, W 16° 32′ 34.809″), and La Palma (N 28° 39′ 28.652″, W 17° 52′ 7.286″). Additionally, 18 ticks were collected from pet and guardian dogs in Cyprus: one *R. sanguineus* s.l. female from Famagusta (Gazimagusa) city (N 35° 08′ 19.05″, E 33° 54′ 54.67) and 13 *R. sanguineus* s.l. (nine females and four males) and four *R. turanicus* s.l. (two females and two males) from Kato Deryneia (N 35° 04′ 32.79″, E 33° 57′ 32.78). One ti0ck was also collected from Kornič, Krk Island, Croatia (N 45° 03′ 60.00″, E 14° 35′ 59.99″). All ticks were preserved in 70% ethanol.

Tick identification and DNA extraction

Ticks were identified using the keys and morphological descriptions in Walker et al. (2000) and Estrada-Peña et al. (2004). DNA was extracted from individual ticks using the MagNA Pure LC RNA/DNA Kit (Roche, Mannheim, Germany) in a MagNA Pure LC instrument (Roche) according to the manufacturer's instructions. A fragment of the 16S rRNA gene was amplified using the polymerase chain reaction (PCR) protocol described by Mangold et al. (1998). The extracted total nucleic acid was stored at − 80 °C until use.

Sequencing and data analysis

The obtained DNA amplicons were identified by size in gel electrophoresis and sequenced by Sanger sequencing (GATC Biotech, Konstanz, Germany). The sequences were edited using BioEdit Sequence Alignment Editor (Hall 1999) with manual edition whenever necessary and aligned using the Clustal W program (Larkin et al. 2007). Phylogenetic analyses were performed with the maximum likelihood (ML) method. To construct the ML tree, the best-fitting substitution model was determined with the Akaike information criterion using the ML model test implemented in MEGA 5 (Tamura et al. 2011). A general time-reversible model with gamma distribution was selected to generate the phylogenetic tree with partial 16S rRNA gene sequences. Support for topologies was tested by bootstrapping over 1000 replications and gaps were excluded by pairwise comparison. *Rhipicephalus microplus* (Canestrini, 1888), *Rhipicephalus annulatus* (Say, 1821), and *Rhipicephalus decoloratus* Koch, 1844 were used as outgroups. All ambiguous positions were removed for each sequence pair.

Results

Of 3021 ticks from the Canary Islands, 2538 were determined as *R. sanguineus* s.l. and 322 as *R. turanicus* s.l. Fourteen of 18 ticks from Cyprus were found to be *R. sanguineus* s.l., while the remaining four were *R. turanicus* s.l., and the single specimen from Croatia was identified as *R. sanguineus* s.l. *Rhipicephalus sanguineus* s.l. was the most common species on all of the Canary Islands, followed by *R. turanicus* s.l., which was collected only on Lanzarote and Fuerteventura. Two additional tick species were collected on the Canary Islands: 10 *R. pusillus* on Lanzarote, Fuerteventura, and La Palma; and three *Hyalomma marginatum* Koch, 1844, on Lanzarote, Fuerteventura, and Tenerife (one from each island). Due to engorgement or damage, 148 ticks could only be identified to genus, but all were clearly *Rhipicephalus*. After morphological identification, 85 tick specimens from the Canary Islands were selected for genetic analyses: 20 from Lanzarote, of which ten were *R. sanguineus* s.l. (five females and five males), six were *R. turanicus* s.l. (two males and four females), and four were *Rhipicephalus* sp. (one male and three females); 21 from Fuerteventura, of which ten were *R. sanguineus* s.l. (five females and five males) and 11 were *R. turanicus* s.l. (one female, five males, and five nymphs); 13 from La Palma, all *R. sanguineus* s.l. (eight females and five males); 11 from Gran Canaria, all *R. sanguineus* s.l. (six females, three males, and two nymphs); and 20 *R. sanguineus* s.l. from Tenerife, (nine females, nine males, and two nymphs). The 85 ticks from the Canary Islands (see above) included in the phylogenic analysis were as follows: 64 specimens morphologically identified as *R. sanguineus* s.l. from Lanzarote, Fuerteventura, Gran Canaria, Tenerife, and La Palma, and 17 identified as *R. turanicus* from Lanzarote and Fuerteventura. Four specimens could not be identified to the species level and were labeled *Rhipicephalus* sp. Only one sequence representing each haplotype was included in the phylogenetic analysis.

The phylogenetic topology obtained with the sequences of the mitochondrial 16S rRNA gene is shown in Fig. 1. A 370-bp fragment was unambiguously aligned. All haplotypes obtained

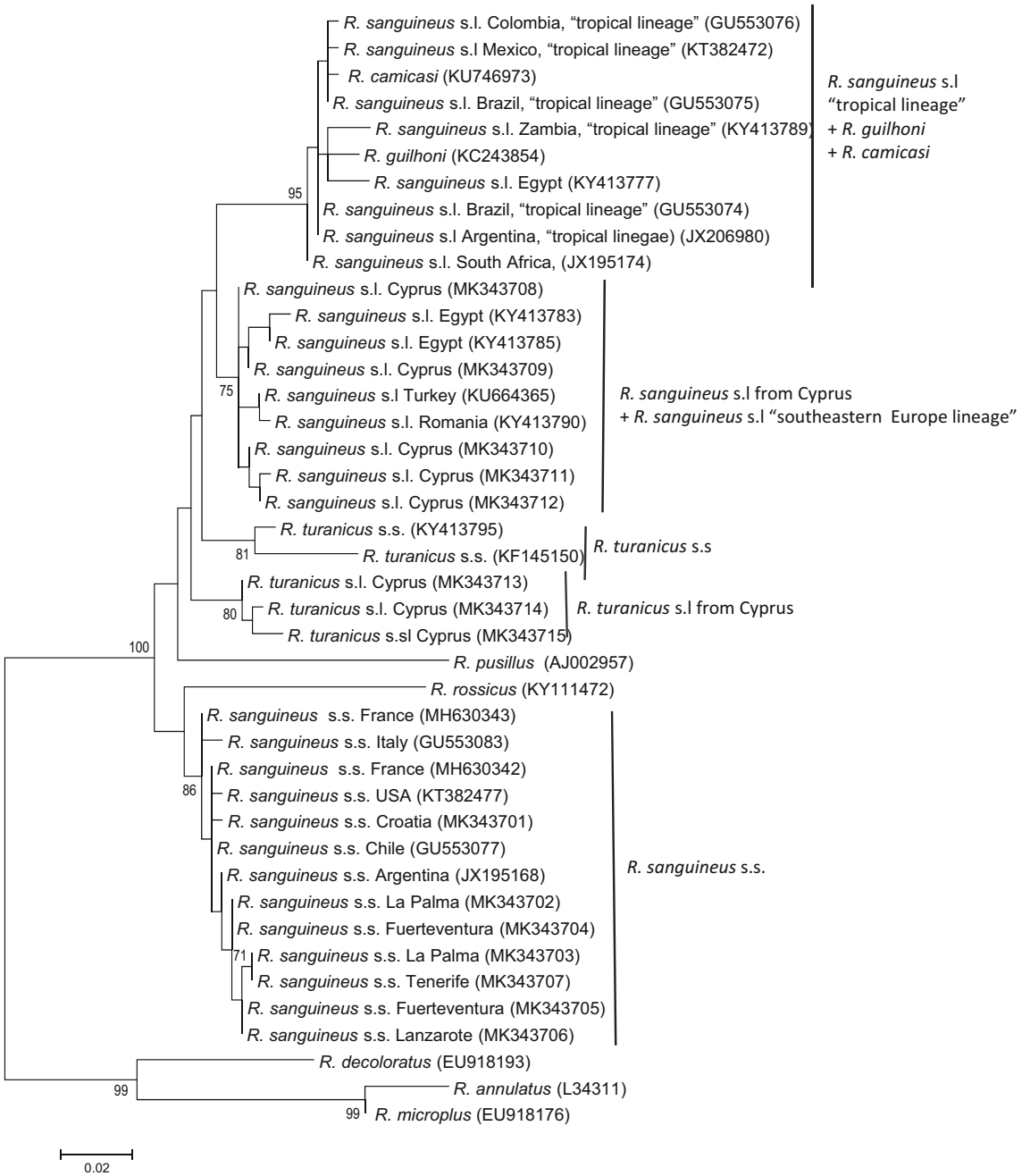

Fig. 1 Phylogenetic tree based on partial 16S rDNA sequences

from ticks collected in the Canary Islands grouped with *R. sanguineus* s.s. from France, Portugal, Italy, Switzerland, Argentina, Chile, Uruguay, and the USA (Fig. 1), despite their earlier morphological determination as *R. sanguineus* s.l. or *R. turanicus* s.l. Sequences obtained from Croatia also corresponded to *R. sanguineus* s.s. (Fig. 1). The 16S sequences of *R. sanguineus* s.l. from Cyprus formed a clade with *R. sanguineus* s.l. from Egypt, Turkey, and Romania belonging to the "*Rhipicephalus* sp. morphotype I" sensu Dantas-Torres et al. (2013) or "southeastern European lineage" sensu Chitimia-Dobler et al. (2017). Ticks determined as *R. turanicus* s.l. from Cyprus clustered separately from the remaining clades of the *R. sanguineus* group, including *R. turanicus* s.s. (Fig. 1). Pairwise genetic divergence among the clades indicated in Fig. 1 was always higher than 3%, while the differences within each clade ranged from 0.1 to 2.5%.

Discussion

In their recent redefinition of *R. sanguineus* s.s., Nava et al. (2018) highlighted the need for studies to determine the exact

geographic range of this taxon. The known distribution of *R. sanguineus* s.s. after its taxonomic redefinition encompasses France, Italy, Spain, Switzerland, Portugal, Argentina, Brazil, Chile, Uruguay, and the USA (Nava et al. 2018), but it is evident that this geographic range is incomplete. Our analysis of 16S rDNA sequences (a molecular marker widely used at the species level within the *R. sanguineus* group) expands the distribution of *R. sanguineus* s.s. to the Canary Islands and Croatia's Krk Island. At this last locality, only one specimen of *R. sanguineus* s.s. was collected. Therefore, further studies are needed to determine whether this record is accidental or represents an established tick population. The clustering of Canary Island ticks morphologically determined as *R. turanicus* s.l. with *R. sanguineus* s.s. is in agreement with previous research, where ticks morphologically determined as *R. turanicus* from western Europe were genetically identical to *R. sanguineus* s.s. (Nava et al. 2018). As discussed by these authors, additional studies are needed to clarify this issue.

The 16S sequences obtained from the ticks collected in Cyprus constituted two phylogenetic groups. The first, comprising ticks morphologically determined as *R. sanguineus* s.l. according to Walker et al. (2000), clustered with sequences of *R. sanguineus* s.l. from Egypt, Turkey, and Romania, all belonging to the "southeastern European lineage." The taxonomic status of this taxon is still unresolved. Dantas-Torres et al. (2018) have found that two lineages of *R. sanguineus* s.l. from southern Europe, namely "southeastern European lineage" (referred to as "*Rhipicephalus* sp. I") from Italy and *R. sanguineus* s.s. (referred to as "*Rhipicephalus* sp. II") from Portugal sensu Dantas-Torres et al. (2013), are biologically compatible, suggesting conspecificity. However, further exhaustive morphological analyses, biological experiments involving populations from different geographic areas, and a formal taxonomic approach following the rules of the International Code of Zoological Nomenclature (ICZN 1999) are needed to definitively resolve the taxonomic status of the "southeastern European lineage" and its relationship with *R. sanguineus* s.s. The second group comprised ticks morphologically determined as *R. turanicus* according to Walker et al. (2000). However, these specimens clustered in a clade unrelated to that of *R. turanicus* s.s. (see Fig. 1). As in the case of the *R. sanguineus* s.s. ticks from Cyprus, additional evidence to resolve the taxonomic status of Cyprus ticks here determined as *R. turanicus* s.l. is needed.

In previous studies, ticks determined as both *R. sanguineus* s.l. and *R. turanicus* s.l. were found parasitizing dogs and other domestic animals in Cyprus (Le Fiche et al. 1974; Chochlakis et al. 2012; Attipa et al. 2017). However, in view of the evolutionary complexity existing within the *R. sanguineus* group revealed by several molecular phylogenetic analyses (Szabó et al. 2005; Burlini et al. 2010; Eremeeva et al. 2011; Moraes-Filho et al. 2011; Levin et al. 2012; Nava et al. 2012; Dantas-Torres et al. 2013; Zemtsova et al. 2016; Chitimia-Dobler et al. 2017; Nava et al. 2018), it is evident that a detailed and integrative analysis of the morphological and molecular diversity of *R. sanguineus* group ticks occurring on Cyprus is necessary, as confirmed by our results.

Acknowledgements Dr. Richard G. Robbins, Walter Reed Biosystematics Unit, Department of Entomology, Smithsonian Institution, Washington, DC, helpfully reviewed and commented on an earlier version of our manuscript.

Funding information Financial support to SN was provided by INTA, Asociación Cooperadora INTA Rafaela, and CONICET.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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