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Research paper

Genetic analysis of *Rhipicephalus sanguineus* sensu lato ticks parasites of dogs in Africa north of the Sahara based on mitochondrial DNA sequences

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ABSTRACT

The aim of this work was to determine the evolutionary relationship among tick populations of *Rhipicephalus sanguineus* sensu lato distributed in Africa north of the Sahara and different lineages of *R. sanguineus* s.l. distributed in different regions of Sub-Saharan Africa, America and Europe through the analysis of DNA sequences of two mitochondrial genes. One hundred and thirty six 16S rRNA gene sequences and twenty-seven 12S rRNA gene sequences of *R. sanguineus* s.l. were analyzed. Phylogenetic analyses were performed including different lineages of *R. sanguineus* s.l. from America, Europe and Africa, and species belonging to the *R. sanguineus* group as *Rhipicephalus camicasi, Rhipicephalus guilhoni, Rhipicephalus sulcatus, Rhipicephalus rossicus, Rhipicephalus pusillus, Rhipicephalus turanicus* and *Rhipicephalus leporis*. At least two different lineages of *R. sanguineus* s.l. are living in sympatry in Africa north of the Sahara. One of these mitochondrial lineages belongs to the same evolutionary entity that *R. sanguineus* s.l. from tropical areas of America, *R. sanguineus* s.l. from Sub-Saharan Africa, *R. camicasi* and *R. guilhoni*. The other mitochondrial lineage of *R. sanguineus* s.l. present in Africa north of the Sahara is phylogenetically associated to *R. sanguineus* s.l. ticks from southeastern Europe (Romania, Turkey and Greece). Both evolutionary entities are clearly different to the evolutionary entity formed by *R. sanguineus* s.l. from western Europe and temperate areas of America. Thus, the name *R. sanguineus* s.s. cannot be assigned to any of the two evolutionary entities present in Africa north of the Sahara. The taxonomic status of these taxa will remain unresolved until new lines of evidence become available to complement the current results based on mitochondrial DNA.

1. Introduction

The *Rhipicephalus sanguineus* group (Acari: Ixodidae) contains tick species with substantial medical, veterinary and economic importance because they are common parasites of domestic mammals and vectors of different disease agents affecting domestic and wildlife animals and humans. This species group is formed by *Rhipicephalus sanguineus* (Latreille, 1806), *Rhipicephalus sulcatus* Neumann, 1908, *Rhipicephalus rossicus* Yakimov and Kohl-Yakimov, 1911, *Rhipicephalus schulzei* Olenev, 1929, *Rhipicephalus pumilio* Schulze, 1935, *Rhipicephalus pusillus* Gil Collado, 1936, *Rhipicephalus turanicus* Pomerantzev, 1940, *Rhipicephalus leporis* Pomerantzev, 1946, *Rhipicephalus guilhoni* Morel and Vassiliades, 1963; *Rhipicephalus moucheti* Morel, 1965, *Rhipicephalus bergeoni* Morel and Balis, 1976 and *Rhipicephalus camicasi* Morel, Mouchet and Rodhain, 1976 (Pegram et al., 1987).[1] Among all these taxa, *R. sanguineus* (Latreille, 1806) (from now on *R. sanguineus* sensu stricto) stands out because it is a parasite of dogs worldwide, a vector of humans and animals pathogens, and a relevant target of the anti-parasitic market. The presence of different lineages within the taxon so-called *R. sanguineus* s.s. with strong biological and genetic divergences has been demonstrated in several studies around the world (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; Labruna et al., 2016;

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[1] The authorities and years of publication of *R. turanicus*, *R. rossicus* and *R. moucheti* were modified from those published in Pegram et al. (1987) and Walker et al. (2000) following Guglielmone and Nava (2014).

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Sanches et al., 2016; Zemtsova et al., 2016; Dantas-Torres et al., 2017; Jones et al., 2017). In fact, *R. sanguineus* s.s. is currently recognized as a species complex rather than a single species (Dantas-Torres and Otranto, 2015; Nava et al., 2015). However and in contrast to this view, other studies have found that ticks previously determined as *R. sanguineus* and *R. turanicus* were genetically indistinguishable (Zahler et al., 1997; Beati and Keirans, 2001). All the above mentioned studies and the formal nomenclatural problems related to the original description of *R. sanguineus* s.s. (see a detailed explanation in Nava et al., 2015) have shown that at the current state of knowledge it is not possible to assign the name *R. sanguineus* s.s. to any tick population worldwide nowadays, and the name *R. sanguineus* sensu lato should be used instead of *R. sanguineus* s.s. until a neotype is properly described.

Africa is the paradigmatic continent for *Rhipicephalus* because 75% of the total number of species belonging to this genus are endemic to the Afrotropical Region (Walker et al., 2000; Guglielmone et al., 2014). In view of the lack of an unambiguous morphological definition of *R. sanguineus* s.l. (Nava et al., 2015), which includes populations from Africa, there is an apparent need for the molecular characterization of *R. sanguineus* s.l. from different regions of this continent. Particularly in areas of the African continent north of the Sahara, there is a lack of genetic studies of *R. sanguineus* s.l. to clarify its taxonomic status. In this context, Egypt becomes relevant not only for its location in the continent, but also due to its proximity to certain areas of southeastern Europe and southwestern Asia where species of the *R. sanguineus* group also coexist. Therefore, this study based on mitochondrial DNA sequences was performed to determine the evolutionary relationship among *R. sanguineus* s.l. from Egypt (considered as a geographical point representative of an African area north of the Sahara) and different lineages of *R. sanguineus* s.l. distributed in different regions of Sub-Saharan Africa, America and Europe, with the main objective of adding information that helps to understand the alfa and beta taxonomy of this group of ticks.

2. Materials and methods

Rhipicephalus sanguineus s.l. adult ticks were collected from dogs in Luxor (25°41′N; 32°65′E), Egypt, during May 2013 and June 2013. Ticks from Egypt were determined as *R. sanguineus* s.l. when they were morphologically compatible with the description of "*R. sanguineus*" presented in Walker et al. (2000). Ticks were stored at −80 °C until further processing. 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 instructions of the manufacturer. A fragment of the 16S rRNA and 12S rRNA genes were amplified using the polymerase chain reaction (PCR) protocols described by Mangold et al. (1998a,b) and Beati and Keirans (2001), respectively.

Sequences of different lineages of *R. sanguineus* s.l. from Sub-Saharan Africa (Mozambique, South Africa), America (temperate and tropical areas) and Europe, as well as sequences of species belonging to the *R. sanguineus* group such as *R. camicasi, R. guilhoni, R. sulcatus, R. rossicus, R. pusillus R. turanicus* and *R. leporis*, were obtained from GenBank and added to the sequence analyses. Additionally, ticks determined as *R. sanguineus* s.l. collected from dogs in Zambia (Localities: Chikowa (13°26′S, 32°52′E), Jumbe (13°17′S, 32°5′E)) and Romania (Localities: Calarasi (44°12N, 27°20′E), Timisoara (45°44′N, 21°13′E), Vrancea (44°32′N, 22°35′E), Craiova (44°19′N, 23°48′E), Giurgiu (43°53′N, 25°58′E)) were also processed for DNA extraction and amplification of the respective 16S rRNA and 12S rRNA gene fragments as described above. The sequences obtained from the ticks from Zambia and Romania were by location of their collection considered to be representatives of those *R. sanguineus* s.l. populations distributed in Sub-Saharan Africa and southeastern Europe.

The sequences were edited using BioEdit Sequence Alignment Editor (Hall, 1999) with manual edition whenever it was necessary and aligned with the program Clustal W (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 make the phylogenetic tree with partial 16S rRNA gene sequences. The tree based on partial 12S rRNA gene sequences was generated with the general time reversible model by using gamma distribution with invariant sites. Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded in the pairwise comparison. *Rhipicephalus microplus* (Canestrini, 1888)*, Rhipicephalus annulatus* (Say, 1821)*, Rhipicephalus decoloratus* Koch, 1844; *Rhipicephalus bursa* Canestrini and Fanzago, 1878*, Rhipicephalus australis* Fuller, 1899 and *Rhipicephalus kochi* Dönitz, 1905 were considered as outgroup.

Finally, the number of variable nucleotide positions between haplotypes was used to calculate pairwise estimates of percent sequence divergence for both 16S rRNA and 12S rRNA genes. These analyses were conducted with the program MEGA 5 (Tamura et al., 2011) by using the Kimura 2-parameter model. All ambiguous positions were removed for each sequence pair.

3. Results

One hundred and thirty six 16S rDNA sequences of *R. sanguineus* s.l. were obtained: 97 from Egypt, 16 from Zambia and 23 from Romania. Sequences from Egypt were grouped in nine different haplotypes (Egypt haplotypes I–IX), those from Zambia in four different haplotypes (Zambia haplotypes I–IV), and the Romanian sequences were grouped in five different haplotypes (Romania haplotypes I–V). Twenty-seven 12S rRNA gene sequences of *R. sanguineus* s.l. were also obtained and included in the analysis: 22 sequences from Egypt grouped in three different haplotypes (haplotypes Egypt I–III), four sequences from Zambia belonging to the same haplotype, and one sequence from Romania. The length of the sequences alignments of the 16S rRNA and 12S rRNA genes were ca. 330 bp.

The ML tree constructed with 16S rRNA gene sequences showed that the species of the *R. sanguineus* group considered in this study are grouped in six different clades formed by the following taxa: clade I) *R. sanguineus* s.l. "tropical lineage" (tropical areas of America, Sub-Saharan Africa), *R. camicasi* and *R. guilhoni*; clade II) *R. turanicus*; III) *R. sanguineus* s.l. lineage form the "southeastern Europe"; IV) *R. pusillus;* V) *R. rossicus*; VI) *R. sanguineus* "temperate lineage" (temperate and cold areas of South America and North America, western Europe). The haplotypes I–VI of *R. sanguineus* s.l. from Egypt (Genbank accession numbers: KY413777-KY413782) were associated to the phylogenetic clade I which is formed by sequences of the "tropical lineage" of *R. sanguineus* s.l., *R. camicasi* and *R. gulhoni* (Fig. 1). The four 16S haplotypes from Zambia (Genbank accession numbers: KY413786-KY413789) were also grouped to this clade (Fig. 1). The remaining three16S haplotypes of *R. sanguineus* s.l. from Egypt (VII–IX; Genbank accession numbers: KY413783-KY413785) formed the clade III (named here colloquially "southeastern Europe") with 16S rRNA gene sequences of *R. sanguineus* s.l. from Turkey and the five 16S haplotypes from Romania (Genbank accession numbers: KY413790-KY413794) obtained in this work (Fig. 1).

In the phylogenetic analysis performed with 12S rRNA gene sequences (Fig. 2), the species belonging to the *R. sanguineus* species group were mostly clustered in the same clades observed in the tree constructed with 16S rRNA gene sequences. The clade I was formed by *R. sanguineus* s.l. "tropical lineage", *R. camicasi, R. guilhoni,* two haplotypes of *R. sanguineus* s.l. from Egypt (haplotypes I–II; Genbank accession numbers: KY413800-KY413801) and one haplotype of *R. sanguineus* s.l. from Zambia (Genbank accession number: KY413803). The clade III was formed by one haplotype of *R. sanguineus* s.l. from Egypt (haplotype III; Genbank accession number: KY413802) and two

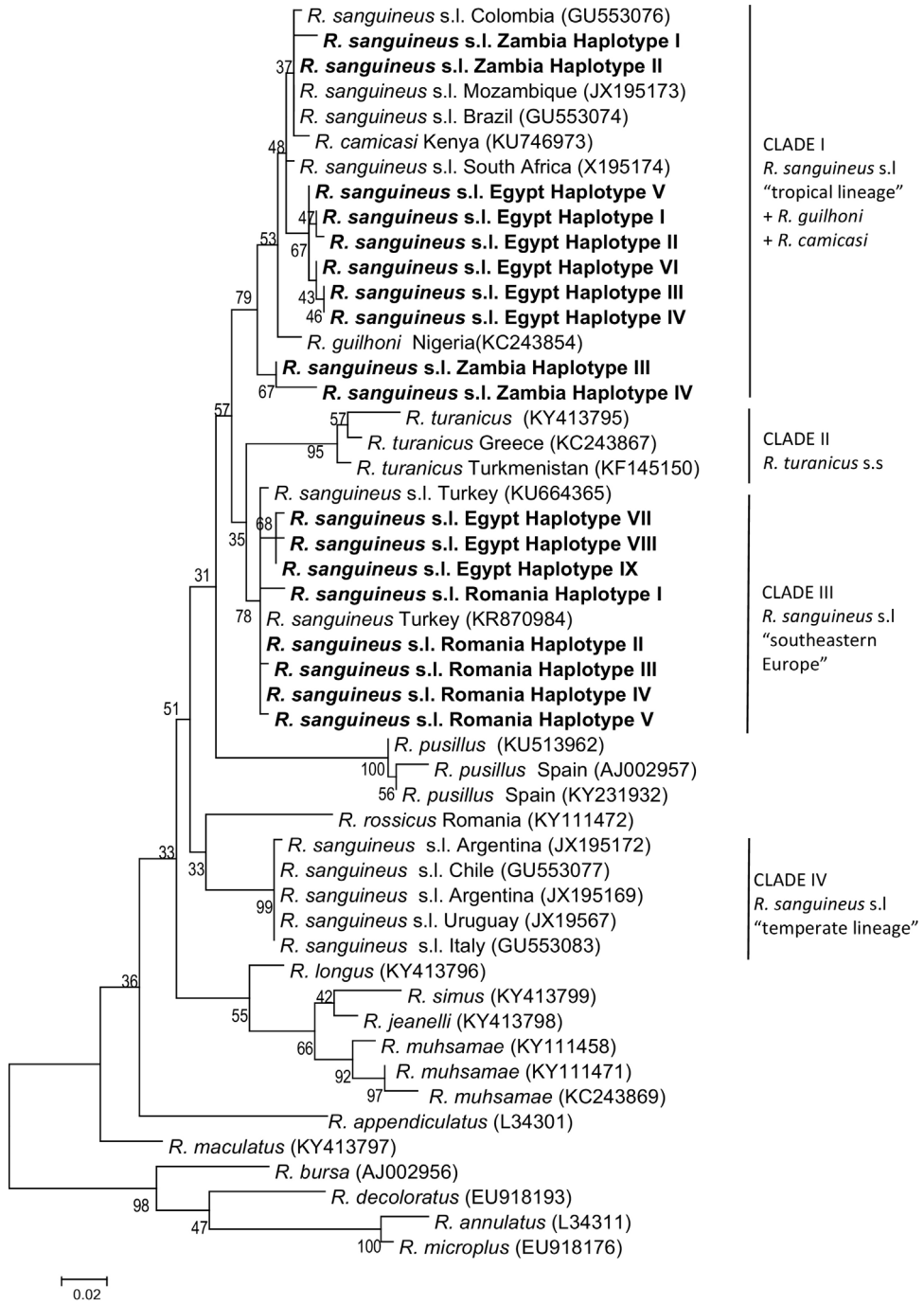

Fig. 1. Maximum-likelihood tree based on partial 16S rRNA gene sequences of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets.

haplotypes from southeastern Europe, one belonging to *R. sanguineus* s.l. from Romania (haplotype I, obtained in this study, Genbank accession number: KY413804) and the other one of *R. sanguineus* s.l. from Greece (morphotype I sensu Dantas-Torres et al., 2013). The remaining three clades of the *R. sanguineus* species group comprise *R. turanicus*, *R. sanguineus* s.l. "temperate lineage" + *R. turanicus* from France,[2] and *R. rossicus* (Fig. 2).

The nucleotide divergence among the 16S rRNA and 12S rRNA gene sequences of *R. sanguineus* s.l. are presented in Tables 1 and 2, respectively. In the case of 16S rRNA gene sequences, the pairwise

[2] It is important to mention that unlike what occurs with *R. sanguineus* s.l. the taxon *R. turanicus* is formally well defined (see Filippova, 1997) and phylogenetically represents an independent lineage (see Figs. 1 and 2 of this work and Dantas-Torres et al., 2013). Currently, from a morphological point of view, it is not possible to assign the specific epithet *turanicus* to any population apart from those morphologically defined in Filippova (1997). Walker et al. (2000) consider *bona fide* data those on *R. turanicus* in Pegram et al. (1987), but under the morphological indefiniteness of most populations of *R. turanicus* that decision is unsound (Guglielmone and Nava, 2014). The study by Pegram et al. (1987) comparing Palearctic and Afrotropical populations did not clarify the status of *R. turanicus* because there is no guarantee that these investigators were working with *bona fide R. turanicus* (Guglielmone and Nava, 2014). The same applies to the work of Estrada-Peña and Sanchez (1988) for specimen from western Europe. In conclusion, the taxonomic status of the ticks determined as *R. turanicus* in western Europe and Africa should be carefully evaluated.

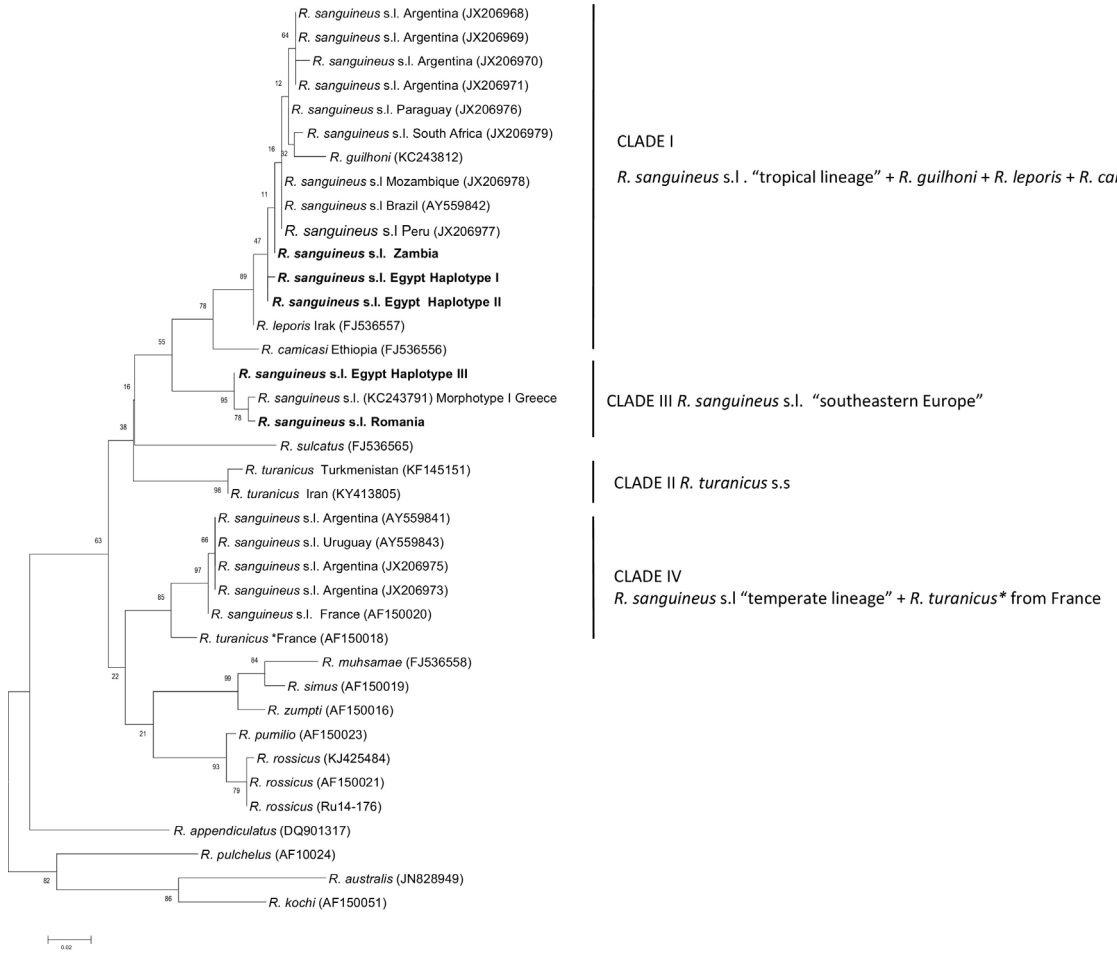

Fig. 2. Maximum-likelihood tree based on partial 12S rRNA gene sequences of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets. * See footnote number 2 in the section "results".

differences within the clade I ranged from 0.1% to 2.8%, and in clade III from 0.1% to 1.8% (Table 1). The differences among all the clades of the *R. sanguineus* species group included in the phylogenetic analysis (Fig. 1) were always higher than 3.1%. Regarding 12S rRNA gene sequences, the nucleotide divergence within clade I formed by *R. sanguineus* s.l. "tropical lineage", *R. guilhoni*, *R. leporis*, *R. camicasi, R. sanguineus* s.l. from Zambia and *R. sanguineus* s.l. from Egypt (haplotypes I–II) ranged from 0% to 2.3%, although the sequence of *R. camicasi* from Ethiopia differed with the remaining sequences of this clade in values ranging from 3.2% to 3.6% (Table 2). The nucleotide divergence in 12S rRNA gene sequences within the clade II (*R. sanguineus* s.l. from southeastern Europe) was from 0.6% to 1.2% (Table 2). The nucleotide divergence in 12S rRNA gene sequences among the clades identified in the phylogenetic tree constructed with 12S rRNA gene sequences (Fig. 2) was always higher than 5.1%.

4. Discussion

The results based on mitochondrial DNA sequences obtained during this work have been shown that at least two different lineages of *R. sanguineus* s.l. (clades I and III in our analyses) are living in sympatry in Africa north of the Sahara. One of these mitochondrial lineages including six different haplotypes belongs to the same evolutionary entity (i.e. the same taxon) as *R. sanguineus* s.l. from tropical areas of America, *R. sanguineus* s.l. from Sub-Saharan Africa (populations from Zambia, Mozambique and South Africa are being considered herein), *R. camicasi* and *R. guilhoni*. In the phylogenetic analysis constructed with 12S rRNA gene sequences, *R. leporis* from Iraq also clustered with this clade. The close relationship within the members of this evolutionary entity suggests that they could belong to the same species which is clearly different to the evolutionary entity formed by *R. sanguineus* s.l. from western Europe and temperate areas of America. As it is widely recognized, these two evolutionary entities are not only genetically different but also biologically incompatible (Szabó et al., 2005; Burlini et al., 2010; Moraes-Filho 2011; Levin et al., 2012; Nava et al., 2012; Dantas-Torres et al., 2013; Sanches et al., 2016). The taxon formed by *R. sanguineus* s.l. ticks from western Europe and temperate areas of America probably represents *R. sanguineus* s.s. (see Nava et al., 2015 for the roots of this taxonomic decision). Consequently, the name *R. sanguineus* s.s. (or *R. sanguineus* (Latreille, 1806)) should neither be assigned to the evolutionary entity formed by *R. sanguineus* s.l. from tropical areas of America, *R. sanguineus* s.l. from Sub-Saharan Africa, *R. camicasi, R. guilhoni* nor *R. sanguineus* s.l. from Africa north of the Sahara (*R. sanguineus* s.l. from Egypt, haplotypes I–VI of 16S and haplotypes I–II of 12S obtained in this study). According to the principle of priority established by the International Code of Zoological Nomenclature (ICZN, 1999), the ticks belonging to this evolutionary entity (clade I of this study) should be called *R. guilhoni* because it was described by Morel and Vassiliades in 1963, while *R. camicasi* was described later by Morel et al. (1976). However, neither Morel and Vassiliades (1963) nor Morel et al. (1976) have compared the specimens that they used for the descriptions of both *R. guilhoni* and *R. camicasi* with the type specimens of some species from central and northern Africa that were considered as synonyms of *R. sanguineus* s.s. in Camicas et al. (1998) and Walker et al. (2000), namely *Rhipicephalus brevicollis* Neumann 1897 (type locality: Mombasa, Kenya), *Rhipicepha-*

Table 1
Matrix of sequence divergence (% nucleotide difference), showing the pair-wise comparisons of the 16S rDNA sequences among the haplotypes of *Rhipicephalus sanguineus* sensu lato.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	–																									
2	0.3																									
3	0.6	0.3																								
4	0.9	0.6	0.1																							
5	0.3	0.6	0.6	0.6																						
6	0.6	0.9	0.3	0.3	0.3																					
7	1.8	2.1	2.1	2.1	1.5	1.8																				
8	1.5	1.8	1.8	1.8	1.2	1.5	0.3																			
9	1.5	1.8	1.8	1.8	1.2	1.5	0.3	0.1																		
10	1.5	1.2	1.2	1.2	1.2	1.5	0.9	0.6	0.6																	
11	1.5	1.8	1.2	1.2	1.2	0.9	0.9	0.6	0.6	1.2																
12	2.4	2.5	2.6	2.3	2.1	2.3	1.8	1.5	1.5	1.5	2.1															
13	2.1	2.3	1.5	2.3	2.1	2.3	1.2	0.9	0.9	1.5	1.5	2.3														
14	1.5	1.8	1.8	1.8	1.2	1.5	0.3	0.1	0.1	0.6	0.6	1.5	0.9													
15	2.6	2.7	2.7	2.6	2.5	2.6	2.5	2.4	2.5	2.5	2.6	2.6	2.5	2.5												
16	2.8	2.8	2.8	2.7	2.8	2.8	2.6	2.6	2.7	2.7	2.8	2.8	2.6	2.7	1.1											
17	3.4	3.4	3.7	4.0	3.7	4.0	4.3	4.0	4.0	3.7	4.0	3.7	4.7	4.0	3.2	4.4										
18	3.7	3.7	3.1	3.1	3.4	3.1	4.4	4.0	4.0	3.7	3.7	3.7	5.0	4.0	3.1	4.7	0.6									
19	3.7	3.7	3.7	3.7	3.4	3.7	4.0	3.7	3.7	3.4	3.7	3.4	4.7	3.7	3.1	4.4	0.3	0.3								
20	4.7	4.7	4.7	4.7	4.3	4.7	4.0	3.7	3.7	3.7	4.3	3.7	4.7	3.7	3.7	5.3	1.8	1.8	1.5							
21	3.7	3.7	3.7	3.7	3.4	3.7	3.4	3.1	3.1	2.9	3.7	2.9	4.0	3.1	3.2	4.4	0.9	0.9	0.6	0.9						
22	4.0	4.0	4.0	4.0	3.7	4.0	3.7	3.4	3.4	3.1	4.0	3.1	4.3	3.4	3.2	4.7	1.2	1.2	0.9	1.2	0.3					
23	3.7	3.7	3.7	3.7	3.4	3.7	3.4	3.1	3.1	2.9	3.7	2.9	4.0	3.4	3.1	4.4	0.9	0.9	0.6	0.9	0.1	0.3				
24	4.0	4.0	4.0	4.0	3.7	4.0	3.7	3.4	3.4	3.1	4.0	3.1	4.3	3.1	3.1	4.7	1.2	1.2	0.9	1.2	0.3	0.6	0.1			
25	3.7	3.7	3.7	3.7	3.4	3.7	3.4	3.1	3.1	2.9	3.7	2.9	4.0	3.1	3.1	4.4	0.9	0.9	0.6	0.9	0.1	0.3	0.1	0.3		
26	4.0	4.0	3.4	3.4	3.7	3.4	3.7	3.4	3.4	3.1	3.4	3.1	4.3	3.4	3.2	4.7	1.2	0.6	0.9	1.2	0.3	0.6	0.3	0.6	0.3	–

H: haplotype. 1. *R. sanguineus* s.l Egypt HI; 2. *R. sanguineus* s.l Egypt HII; 3. *R. sanguineus* s.l Egypt HIII; 4. *R. sanguineus* s.l Egypt HIV; 5. *R. sanguineus* s.l Egypt HV; 6. *R. sanguineus* s.l Egypt HVI; 7. *R. sanguineus* s.l Colombia; 8. *R. sanguineus* s.l Brazil; 9. *R. sanguineus* s.l South Africa; 10. *R. sanguineus* s.l Mozambique; 11. *R camicasi* Kenya; 12. *R. guilhoni* Nigeria; 13. *R. sanguineus* s.l Zambia HI; 14. *R. sanguineus* s.l Zambia HII;15. *R. sanguineus* s.l Zambia HIII; 16. *R. sanguineus* s.l Zambia HIV; 17. *R. sanguineus* s.l Egypt HVII; 18: *R. sanguineus* s.l Egypt HVIII; 19. *R. sanguineus* s.l Egypt HIX; 20. *R. sanguineus* s.l Romania HI; 21. *R. sanguineus* s.l Romania HII: 22. *R. sanguineus* s.l Romania HIII; 23. *R. sanguineus* s.l Romania HIV; 24. *R. sanguineus* s.l Romania HV; 25. *R. sanguineus* Turkey I; 26. *R. sanguineus* Turkey II.

Table 2
Matrix of sequence divergence (% nucleotide difference), showing the pair-wise comparisons of the 12S rDNA sequences among the haplotypes of *Rhipicephalus sanguineus* sensu lato.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–														
2	0.3														
3	0.6	0.3													
4	2.3	2.0	2.3												
5	3.6	3.5	3.6	3.3											
6	0.9	0.6	0.9	2.0	3.2										
7	0.6	0.3	0.6	1.8	3.2	0.9									
8	0.9	0.6	0.3	2.1	3.6	1.2	0.3								
9	0.9	0.6	0.3	2.1	3.6	1.2	0.3	0.0							
10	1.2	0.9	1.2	1.8	3.6	1.5	0.6	0.9	0.9						
11	0.9	0.6	0.3	2.1	3.6	1.2	0.3	0.0	0.0	0.9					
12	0.9	0.6	0.9	2.1	3.6	1.2	0.3	0.6	0.6	0.9	0.6				
13	6.0	5.7	6.0	6.0	5.1	5.4	6.0	6.3	6.3	6.0	6.3	6.3			
14	6.7	6.4	6.7	6.7	5.7	6.0	6.7	7.0	7.0	6.7	7.0	7.0	1.2		
15	6.6	6.3	6.6	6.0	5.1	6.0	6.0	6.3	6.3	6.0	6.3	6.3	1.2	0.6	–

H: haplotype. 1. *R. sanguineus* s.l Egypt HI; 2. *R. sanguineus* s.l Egypt HII; 3. *R. sanguineus* s.l Zambia HI; 4. *R. guilhoni*; 5. *R camicasi* Ethiopia; 6. *R. leporis* Irak; 7. *R. sanguineus* s.l Paraguay; 8. *R. sanguineus* s.l Brazil; 9. *R. sanguineus* s.l Peru; 10. *R. sanguineus* s.l South Africa; 11. *R. sanguineus* s.l Mozambique; 12. *R. sanguineus* s.l Argentina (tropical lineage); 13. *R. sanguineus* s.l Egypt HIII; 14. *R. sanguineus* s.l Romania; 15. *R. sanguineus* s.l Morphotype I Greece.

lus limbatus Koch, 1844 (type locality: Egypt), *Rhipicephalus punctatissimus* Gerstäcker 1873 (type locality: Mombasa, Kenya), *Rhipicephalus rutilus* Koch, 1844 (type locality: Egypt), and *Rhipicephalus stigmaticus* Gerstäcker 1873 (type locality: Mombasa, Kenya). The types of these taxa should be examined to determine whether the name of some of them should be assigned to the evolutionary entity composed by *R. sanguineus* s.l. from Sub-Saharan Africa, *R. camicasi, R. guilhoni* and *R. sanguineus* s.l. from Africa north of the Sahara, because they would have priority over *R. camicasi* and *R. guilhoni*. All the species above described by Koch (1844), Gerstäcker (1873) and Neumann (1897) are treated as names *incertae sedis* in Guglielmone and Nava (2014) but the type specimens for all of them are held in the Berlin Museum (Moritz and Fischer, 1981) therefore available for scrutiny. Also, future studies should include specimens of *R. camicasi* and *R. guilhoni* from areas near their type localities in the genetic and morphological comparisons in order to determine whether these species effectively belong to a same specific entity composed by *R. camicasi, R. guilhoni* and *R. sanguineus* s.l. from Africa north of the Sahara, *R. sanguineus* s.l. from tropical areas of America and *R. sanguineus* s.l. from Sub-Saharan Africa.

The other mitochondrial lineage of *R. sanguineus* s.l. from Egypt found during this study is phylogenetically associated to *R. sanguineus* s.l. ticks from Romania (sequences obtained in this work), Turkey (sequences obtained by Hekimoglu et al., 2016) and Greece (morphotype I sensu Dantas-Torres et al., 2013). The genetic divergence between the two mitochondrial lineages of *R. sanguineus* s.l. detected in Africa north of the Sahara range from 3.1% to 4% in 16S rRNA gene

sequences and from 6% to 6.7% in 12S rRNA gene sequences. The biological significance of these genetic differences in mitochondrial DNA must be evaluated with additional lines of evidence (i.e, cross mating, ecological and morphological comparisons) as it was done for the temperate and tropical lineages of *R. sanguineus* s.l. (Oliveira et al., 2005; Szabó et al., 2005; Levin et al., 2012; Sanches et al., 2016; Labruna et al., 2017). This is important in view of the high probability of frequent inbreeding events between these two mitochondrial lineages of *R. sanguineus* s.l. detected during this study in Africa north of the Sahara. Also, the analyses based on relatively short sequences of mitochondrial genes have some limitations, as the smaller effective population size may lead to coalescence of haplotype lineages in populations that are only temporarily isolated producing an over-resolution of mtDNA trees (Sites and Crandall, 1997; Wiens and Penkrot, 2002). Therefore, further research is needed to establish accurately the taxonomic relationships between the two mitochondrial lineages of *R. sanguineus* s.l. (clades I and III of this work) present in Africa north of the Sahara.

The name *R. sanguineus* s.s. cannot be assigned to any tick population in the world at the current state of knowledge, due to the lack of an accurate morphological definition and because of the existence of different mitochondrial lineages, some of them reproductively incompatible among each other (Nava et al., 2015). The presence of two sympatric lineages in Africa north of the Sahara was demonstrated in this work, one of them closely related to *R. camicasi, R. guilhoni* and *R. sanguineus* s.l. populations from sub-Saharan Africa and tropical areas of America, and the other one closely related to populations of *R. sanguineus* s.l. distributed in southeastern Europe. But the taxonomic status of these taxa will remain unresolved until a neotype of *R. sanguineus* s.s. becomes established and new lines of evidence complement the current results based on mitochondrial DNA.

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