



Original article

Rhipicephalus sanguineus (Latreille, 1806): Neotype designation, morphological re-description of all parasitic stages and molecular characterization

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ABSTRACT

The aims of this work were to re-describe all parasitic stages of *Rhipicephalus sanguineus* sensu stricto, to select and deposit a neotype, and to characterize some of its diagnostic molecular traits. A male of *R. sanguineus* s.s. collected in Montpellier, France, was designated as neotype. The diagnostic characters unique to the male of *R. sanguineus* s.s. are: *spiracular plate* elongated and subtriangular in shape with a dorsal prolongation narrow and usually visible dorsally, with the dorsal prolongation narrower than the width of the adjacent festoon; punctations of the *scutum* moderate in number and unequal in size; marginal groove conspicuous, deep and punctate; posteromedian groove distinct and elongated, and posterolateral grooves often sub-circular, shorter than posteromedian groove; adanal plates long, wide, and subtriangular in shape, with a clear concavity in its inner margin and posterior margin broadly rounded or truncated; accessory adanal plates with the posterior end pointed, narrower than the width of adjacent festoon. The female of *R. sanguineus* s.s. can be diagnosed by a combination of broadly U-shaped genital aperture, *spiracular plate* with a narrow dorsal prolongation visible dorsally, *basis capituli* hexagonal with broad lateral angles, and *scutum* barely longer than broad with posterior margin sinuous and punctations moderate in number and unequal in size, larger and more numerous along cervical fields. The nymph has a *basis capituli* sub-triangular dorsally with lateral angles slightly curved and presence of ventral processes, *scutum* approximately as long as broad with lateral margins nearly straight, posterior margin broadly rounded, and cervical grooves short and sigmoid in shape extending posteriorly to the level of the eyes. The larva is characterized by *basis capituli* broader than long with lateral angles short and slightly curved and with posterior margin slightly convex, cervical grooves short, shallow and subparallel, and *scutum* almost twice broader than long. The phylogenetic analysis of DNA sequences support *R. sanguineus* s.s. as a well-defined taxon when compared with other species of the *R. sanguineus* group: *R. turanicus* s.s., *R. camicasi*, *R. guilhoni*, *R. sulcatus*, *R. pusillus*, *R. rossicus* and *R. leporis*. Molecularly *R. sanguineus* s.s. also encompasses the so-called “temperate lineage” from the New World (Argentina, southern Brazil, Chile, Uruguay, and USA). The evidence currently available supports the presence of *R. sanguineus* s.s. in Europe (France, Italy, Spain, Switzerland and Portugal) and America (Argentina, Brazil, Chile, Uruguay and U.S.A.), but further studies are needed to determine the exact geographic range of this taxon.

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1. Introduction

The tick *Rhipicephalus sanguineus* (Latreille, 1806) (from now on, *R. sanguineus* sensu stricto) is, from a public health and economic perspective, the most important species of the *Rhipicephalus sanguineus* group, which includes *R. sanguineus* s.s., *Rhipicephalus sulcatus* Neumann, 1908, *Rhipicephalus rossicus* Yakimov and Kohl-Yakimova, 1911, *Rhipicephalus schulzei* Olenov, 1929, *Rhipicephalus pumilio* Schulze, 1935, *Rhipicephalus pusillus* Gil Collado, 1936, *Rhipicephalus turanicus* Pomerantzev, 1940, *Rhipicephalus leporis* Pomerantzev, 1946, *Rhipicephalus guilhoni* Morel and Vassiliades (1962), *Rhipicephalus moucheti* Morel, 1965, and *Rhipicephalus camiciasi* Morel, Mouchet and Rodhain, 1976 (Pegram et al., 1987a,b; Walker et al., 2000)¹. In spite of the veterinary, medical and economic relevance of *R. sanguineus* s.s., its name has often been applied to any population of *Rhipicephalus* ticks of the *R. sanguineus* group associated worldwide with dogs. This was often done without following any strict formal, biological, morphological or molecular criteria (Nava et al., 2015). The taxon *R. sanguineus* s.s. is not properly defined because its original description is not informative and the type specimen has been lost. Furthermore, many morphological descriptions of *R. sanguineus* s.s. are based on ticks originating from different populations², showing, in some cases, biological incompatibility and significant genetic divergence (Oliveira et al., 2005; Szabó et al., 2005; Burlini et al., 2010; Moraes-Filho et al., 2011; Levin et al., 2012; Nava et al., 2012; Dantas-Torres et al., 2013; Liu et al., 2013; Nava et al., 2015; Sanches et al., 2016; Zemtsova et al., 2016; Chitimia-Dobler et al., 2017; Dantas-Torres et al., 2017; Labruna et al., 2017; Coimbra-Dores et al., 2018; Díaz et al., 2018). In addition, ticks from Western Europe (Spain, Portugal and France) and southern Switzerland identified morphologically as *R. sanguineus* s.s. and *R. turanicus*³, were shown to be almost identical in terms of their DNA sequences (Mangold et al., 1998; Beati and Keirans, 2001; Bernasconi et al., 2002; Moraes-Filho et al., 2011; Santos-Silva et al., 2011; Almeida et al., 2017; Dantas-Torres et al., 2017).

Nava et al. (2015) explained the reasons why *R. sanguineus* s.s. should be relegated to a *nomen nudum*. However, they also mentioned advantages of keeping the name *R. sanguineus* s.s. as valid: its widespread usage, and the public health and economic importance of the tick populations it represents. These authors also indicated that selecting a neotype would be an important step in clarifying the taxonomic and biological problems involving *R. sanguineus* s.s. According to the rules issued by the International Code of Zoological Nomenclature (ICZN, 1999), the principal criteria and procedures for designating a neotype are as follows: 1) if no name-bearing type is believed to be extant a neotype may be fixed; 2) a neotype is the name-bearing type of a nominal species-group taxon designated under conditions specified in this article (number 75) when no name-bearing type specimen (i.e. holotype, lectotype, syntype or prior neotype) is believed to be extant and an author considers that a name-bearing type is necessary to define the nominal taxon objectively; 3) a statement is required that it is designated with the express purpose of clarifying the taxonomic status or the type locality of a nominal taxon; 4) a statement is required of the characters that the author regards as differentiating from other taxa the nominal species-group taxon for which the neotype is designated, or a bibliographic reference to such a statement; 5) data and a description

sufficient to ensure recognition of the specimen designated are required; 6) the author's reasons for believing the name-bearing type specimen(s) (i.e. holotype, or lectotype, or all syntypes, or prior neotype) to be lost or destroyed, and the steps that had been taken to trace it or them are required; 7) evidence is required that the neotype came as nearly as practicable from the original type locality. The main goals of this study are to clarify the issues surrounding the name "*R. sanguineus* s.s.", to re-describe per current standards every active stage of the species, to select and deposit a neotype, and to characterize some of its molecular traits. This will provide an accurate definition of this taxon as a biological entity against which the taxonomic and ecological diversity represented by other taxa currently assigned to this name can be compared.

2. Materials and methods

2.1. Type locality

Latreille (1806) described *R. sanguineus* s.s. (as *Ixodes sanguineus*) from ticks collected in "Gallia". It was later transferred to the genus *Rhipicephalus* by Koch (1844). The "Gallia" of Latreille (1806) corresponds roughly to the present-day territory of France (Bequaert, 1945; Hoogstraal, 1956; Walker et al., 2000; Nava et al., 2015), but there is no indication of the exact site where the specimen described by Latreille (1806) was collected. Therefore, the choice of the French type locality of the neotype has been arbitrary.

The male neotype of *R. sanguineus* s.s. was collected on the walls of a private kennel in Montpellier (43.33°N, 3.50°E), France. The International Code of Zoological Nomenclature recommends a designated neotype to preferably be of the same sex as the original type (article 75.3.5). In our case, not only could the description of Latreille have applied to different tick species (Nava et al., 2015), but it could also have applied to either sex. The mention of three linear depressions in the posterior part of the body could refer to both, the posterolateral and posteromedian grooves of the male, or the alloscutal grooves of the female. The mention of an unadorned anterior part of the body could refer to a female *scutum*. There is really no way for us to unequivocally assign a sex to the poorly described type. If we opted for a male neotype, it is because male provide slightly more diagnostic morphological characters than females, and because some of the female characters partially disappear after engorgement.

2.2. Ticks

The descriptions of all parasitic stages of *R. sanguineus* s.s. were made from specimens from a colony established from adult ticks collected with the male neotype in Montpellier, France. The morphology of the ticks collected in France was compatible in general terms with that described as characteristic of *R. sanguineus* s.s. in Morel and Vassiliades (1962), Filippova (1997); Walker et al. (2000), and Estrada-Peña et al. (2004, 2017).

Wild caught males and females from the neotype locality were fed on laboratory rabbits by using feeding chambers attached on the dorsum of the hosts. One of the fully engorged female was the origin of the colony we used for morphological descriptions. The adult offspring (F1) was bred again on rabbits to generate F2 and F3 adults and immature ticks. The engorged ticks were kept at 25 °C and 83–86 % relative humidity, with a daily photoperiod of 12 h light–12 h dark. This colony with different generations was established in order to prevent the presence of hybrids among the ticks from which the colony originated, because different lineages or species of the *R. sanguineus* group could occur sympatrically and produce viable hybrids that are sterile. The colony was shown to be highly fertile throughout three generations, and it was maintained with the agreement of the Commission for Animal Ethics of the Faculty of Veterinary Medicine, Zaragoza (2015-06656 A), adhering to the European protocols for animal welfare.

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

² See the sequence of the morphological descriptions of *R. sanguineus* s.s. from its original description in 1806 to the beginning of the XXth century in Nava et al. (2015).

³ The taxonomic status of the ticks determined as *R. turanicus* in Western Europe, southern Switzerland and Africa should be carefully evaluated because the available evidence does not support they belong to the taxon *R. turanicus* s.s. (see Guglielmo and Nava (2014) and the discussion of this work).

For the description of morphological discrete characters, we examined unfed F1-F3 adults and F2-F3 immatures (F1 immatures had all been used for colony maintenance). For measurements of morphological continuous traits, however, in order to prevent as much as possible biases due to repeated feeding on rabbits (not the natural host of *R. sanguineus*), we used only F1 adults and F2 immatures. Representative specimens (males, females, nymphs and larvae) are deposited in the Tick Collection of Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Argentina (INTA 2381). Ticks were cleaned for both light and scanning electron microscopy with ultrasound (20 kHz) using distilled water and commercial detergent in a proportion of 9:1. Unfed adults (ten males and ten females) and nymphs (ten specimens) were measured and photographed using a Nikon Alphaphot-2 YS2 optical microscope. Ten unfed larvae were mounted in Hoyer's medium to make semi-permanent slides and examined and photographed by light microscopy using a Nikon Eclipse

E200 optical microscope. Measurements of adults and nymphs are given in millimetres and those of larvae in micrometres, with the mean followed by the standard deviation, and range in parentheses. Scanning electron photomicrographs were taken at the Servicio de Microscopía Electrónica, Museo de La Plata, Universidad Nacional de La Plata, Argentina, using a JEOL/JSM 6360 LV® Digital Scanning Microscope. The terminology employed in the morphological descriptions follows that of Walker et al. (2000), with the exceptions of marginal groove and cervical groove (see Nava et al., 2017 and Horak et al., 2018) instead of marginal line and internal cervical margin, respectively. The ventral process of the nymph is defined in Nava et al. (2017), and the larval chaetotactic terminology adheres to Clifford and Anastos (1960).

2.3. Molecular analysis

DNA was extracted from *R. sanguineus* ticks (F1, F2 and F3 adults)

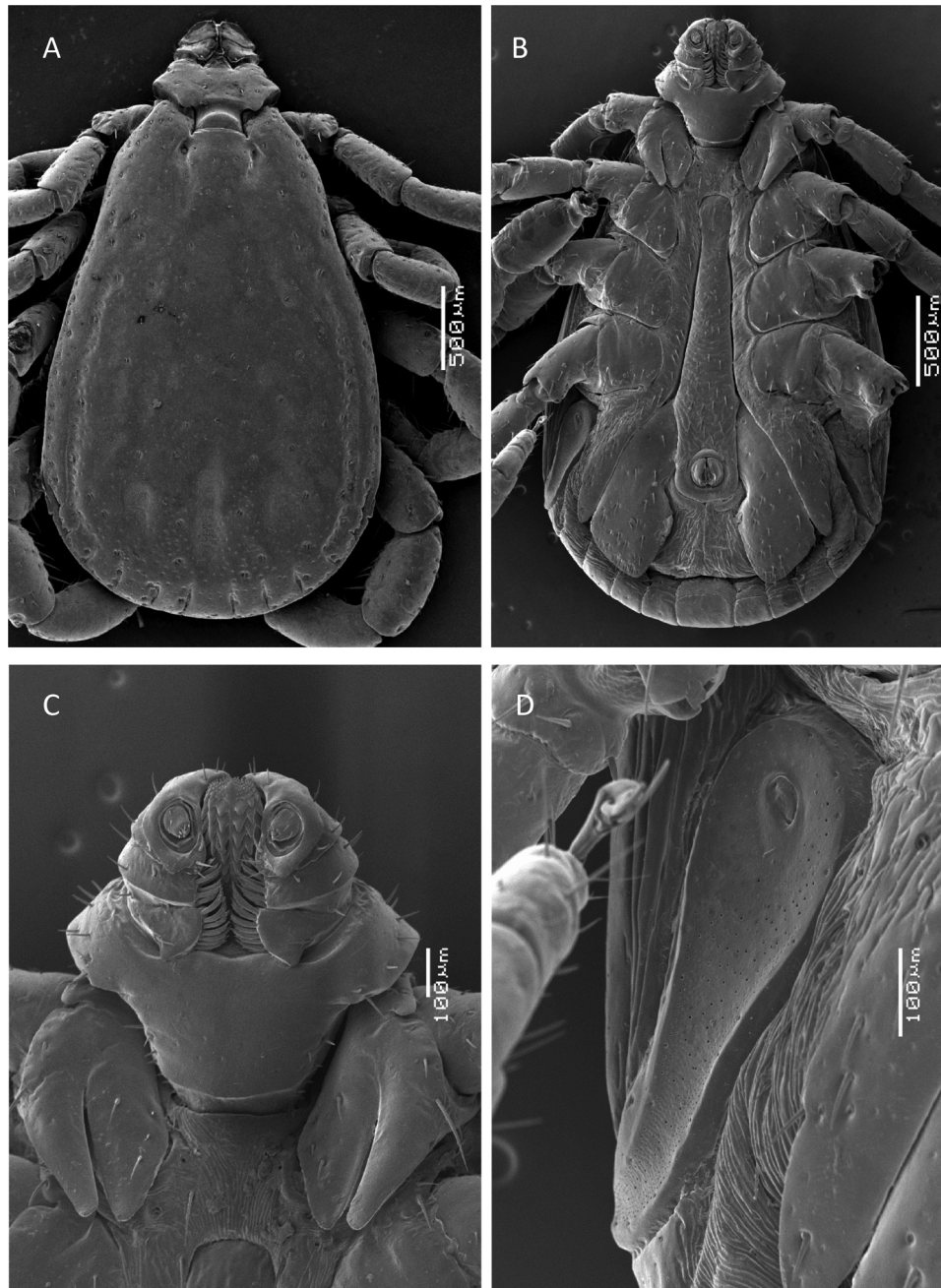


Fig. 1. *Rhipicephalus sanguineus* sensu stricto. Male. A. Body, dorsal view. B. Body, ventral view. C. Capitulum, ventral view. D. Spiracular plate.

by using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA). A 405 base pair (bp) fragment of the mitochondrial 16S rRNA gene was amplified using the primers and the polymerase chain reaction (PCR) conditions designed by Mangold et al. (1998). For amplification of a 375 bp fragment of the mitochondrial 12S rRNA gene, primers and PCR profile were as described in Szabó et al. (2005). Sequences of the mitochondrial *cox1* gene (cytochrome c oxidase subunit 1) were obtained with the primers and protocols described in Folmer et al. (1994) and Herbert et al. (2003). Finally, the amplification of the nuclear ribosomal internal transcribed spacer 2 (ITS2) was conducted by using the primer (1) 5_CCATCGATGTGAAYTGCAGGACA3_in the 5.8S rDNA gene (Zahler et al., 1995) and the primer (2) 5_GTGAATTCTATGCTTAAATTCAG GGGGT3_in the 28S rDNA gene (McLain et al., 1995). The conditions of the PCR reaction were those presented in Labruna et al. (2002).

The amplified DNA was purified using Wizard SV Gel and PCR Clean-Up (Promega, Madison, WI, USA) according to the manufacturer's protocol. Sequencing reactions were performed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and an Applied Biosystem 373 A gene sequencer at the Unidad de Genómica, Instituto de Biotecnología, INTA Castelar. DNA sequences of *R. sanguineus* s.l. available in GenBank were also used for comparative purposes. 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 by Maximum-likelihood (ML) method, after the best-fitting substitution model was selected with the Akaike Information Criterion (AIC) using the ML model test implemented in MEGA 5 (Tamura et al., 2011). Support for the topologies was tested by bootstrapping over 1000 replicates with gaps excluded in the pairwise comparison. Sequences of *Rhipicephalus microplus* (Canestrini, 1888), *Rhipicephalus annulatus* (Say, 1821), *Rhipicephalus decoloratus* Koch, 1844, *Rhipicephalus australis* Fuller, 1899 and *Rhipicephalus kochi* Dönitz, 1905 were used as outgroups. Pairwise distances were calculated after selecting the best-fit model of nucleotide substitution with AIC in MEGA 5 (Tamura et al., 2011).

3. Results

3.1. Morphological re-descriptions

3.1.1. Male

Body: outline broadly oval, broadest at level of legs IV, slightly narrower anteriorly, with a small concave margin at level of eyes; total length 3.50 ± 0.09 (3.15–3.90), length from apices of scapulae to posterior body margin 2.96 ± 0.07 (2.65–3.20), maximum width 1.95 ± 0.09 (1.60–2.10) (Fig. 1A)⁴. **Scutum:** inornate, yellowish- or reddish-brown coloration; punctations moderate in number, unequal in size, larger and more densely distributed in anterior and posterior fields; marginal groove deep and punctate, delimiting the first two festoons, extending anteriorly to level of legs III; cervical grooves short, deep, comma-shaped; posteromedian groove distinct, elongate; posterolateral grooves sub-circular, shorter than posteromedian groove; eyes flat, scapulae rounded (Fig. 1A). **Festoons:** quadrangular in shape, almost as wide as long (except the central festoon which is wider than long), with small punctations and very few setiferous punctations (Fig. 1A). **Ventral plates:** adanal plates long, almost three times as long as wide, subtriangular in shape, fairly broad, posterior margin broadly rounded or truncated, inner margin distinctly concave to posterior level of anus; accessory adanal plates conspicuous, pointed, shorter than adanal plates, posterior end narrower than the width of adjacent festoon (Fig. 1B). **Spiracular plate:** elongate, subtriangular in shape, dorsal prolongation narrow and usually visible dorsally, dorsal prolongation

narrower than the width of adjacent festoon (Fig. 1A, D). **Capitulum:** *basis capituli* dorsally hexagonal, wider than long, width 0.71 ± 0.01 (0.67–0.73), length 0.22 ± 0.01 (0.20–0.25) (dorsal view); length from palpal apices to cornua apices 0.56 ± 0.02 (0.48–0.64), cornua broad and triangular; palps short and rounded apically; hypostome short, blunt, dental formula 3/3 in 6–7 rows, apex with corona of fine denticles; palps total length 0.28 ± 0.001 (0.23–0.33), segment I length 0.04 ± 0.002 (0.03–0.05), segment II length 0.11 ± 0.004 (0.10–0.13), segment III length 0.12 ± 0.006 (0.10–0.13) (Fig. 1A, C). **Legs:** coxa I with two long triangular spurs, the external narrower than the internal, both spurs almost parallel; coxae II–III each with a single short external spur; coxa IV with two short spurs; coxae increasing in size from I to IV (Fig. 1B). Genital aperture at level of coxae II (Fig. 1B).

3.1.2. Female

Body: outline broadly oval, broadest at level of insertion of legs IV, slightly narrower anteriorly, total length 3.70 ± 0.16 (3.20–4.60), length from apices of scapulae to posterior body margin 3.13 ± 0.12 (2.70–3.80), maximum width 2.00 ± 0.07 (1.75–2.40) (Fig. 2A). **Scutum:** inornate, yellowish- or reddish-brown coloration, barely longer than broad, length 1.48 ± 0.08 (1.20–1.86), maximum width 1.41 ± 0.08 (1.15–1.80), length to width ratio 1.05 ± 0.01 (1.03–1.09), posterior margin sinuous; cervical grooves broad, shallow, diverging posteriorly; cervical fields depressed; punctations moderate in number, unequal in size, larger and more densely distributed along cervical fields, in scapular areas and around eyes; few short setae, more numerous in anterior field of *scutum*; eyes flat at about mid-length of *scutum*; scapulae rounded (Fig. 2A, C). **Alloscutum** with three vertical linear grooves (inconspicuous in fed females) and covered with scattered whitish and short setae. **Spiracular plate:** broadly elongated with narrow dorsal prolongation visible dorsally (Fig. 2E). **Capitulum:** *basis capituli* dorsally hexagonal, clearly wider than long, *basis capituli* width 0.69 ± 0.02 (0.58–0.74), *basis capituli* length 0.19 ± 0.01 (0.15–0.24) (dorsal view), length from palpal apices to cornua apices 0.53 ± 0.01 (0.48–0.58), lateral angles broad, cornua small, palps short and narrowly rounded apically; porose areas small, oval, separated from one another by an interval slightly larger than their diameter; hypostome short, blunt, dental formula 3/3 in 6–7 rows, apex with corona of fine denticles; palps total length 0.26 ± 0.007 (0.24–0.28), segment I length 0.03 ± 0.003 (0.03–0.05), segment II length 0.11 ± 0.005 (0.10–0.13), segment III length 0.12 ± 0.003 (0.11–0.13) (Fig. 2A, C, D). **Legs:** coxa I with two long triangular spurs, subparallel, the external narrower than the internal; coxae II–IV with a single short external spur each; coxae increasing in size from I to IV (Fig. 2B). Genital aperture broadly U-shaped, lateral margins diverging anteriorly, located between coxae II (Fig. 2B).

3.1.3. Nymph

Body: outline oval, broadest at level of insertion of legs IV, total length 1.34 ± 0.01 (1.30–1.36), length from apices of scapulae to posterior body margin 1.09 ± 0.03 (1.00–1.16), maximum width 0.70 ± 0.02 (0.66–0.76) (Fig. 3A). **Scutum:** inornate, approximately as long as broad, lateral margins nearly straight, posterior margin broadly rounded, length 0.47 ± 0.02 (0.40–0.50), maximum width 0.48 ± 0.03 (0.40–0.53), ratio length to width 0.98 ± 0.01 (0.94–1.00); cervical grooves short, deep anteriorly, shallow posteriorly, sigmoid in shape, extending posteriorly to the level of the eyes; scapulae rounded and short; few and shallow punctations on *scutum*, barely visible, and few short setae; eyes flat at the level of posterior third of *scutum* (Fig. 3B, C). **Alloscutum** and ventral surface covered by scattered whitish setae (Fig. 3A, B). **Capitulum:** *basis capituli* sub-triangular dorsally, *basis capituli* width 0.30 ± 0.01 (0.27–0.33), *basis capituli* length 0.12 ± 0.007 (0.10–0.13), length from palpal apices to posterior margin of *basis capituli* 0.24 ± 0.01 (0.21–0.27), cornua absent, lateral angles slightly curved, ventral processes present, palps short and apically acute (Fig. 3C, D); hypostome short, blunt, dental

⁴ The body wall of males expands laterally and posteriorly in fed specimens.

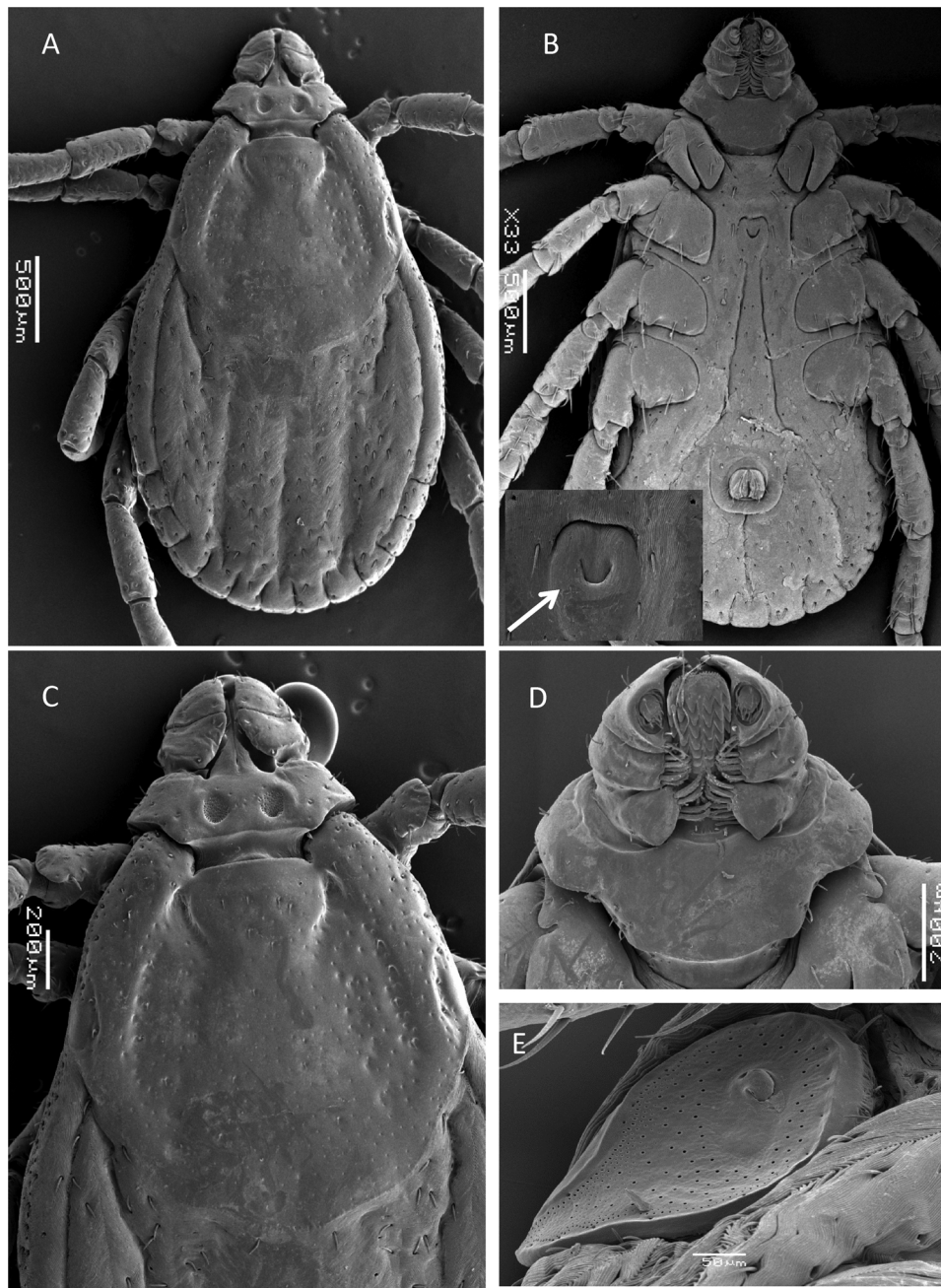


Fig. 2. *Rhipicephalus sanguineus* sensu stricto. Female. A. Body, dorsal view. B. Body, ventral view. C. Scutum and dorsal view of capitulum. D. Capitulum, ventral view. E. Spiracular plate. White arrow in B shows the genital aperture.

formula 2/2 (Fig. 3D). *Legs*: coxa I with two triangular spurs, widely divergent, the external slightly longer than the internal; coxa II–IV each with a single, short, acute, external spur, decreasing in size from coxa II to coxa IV (Fig. 3B).

3.1.4. Larva

Body: outline sub-oval, broadest at level of insertion of legs III, total length including capitulum 605.91 ± 24.52 (568–647), length from apices of scapulae to posterior body margin 501.50 ± 21.91 (460–529), maximum width 413.54 ± 25.40 (382–470) (Fig. 4A). *Scutum*: broader than long, length 218.82 ± 12.61 (202–245), maximum width 370.00 ± 9.86 (353–392), ratio width to length 1.69 ± 0.09 (1.53–1.83); posterior margin slightly sinuous; cervical grooves shallow, ending posteriorly before the level of eyes; scapulae rounded; presence of few and very shallow punctations, barely visible;

3 pairs of short scutal setae (Sc): $Sc_1 28.50 \pm 3.20$ (24.4–34), $Sc_2 22.50 \pm 1.80$ (19.5–24.4), $Sc_3 22.40 \pm 2.13$ (19.5–24.4); eyes flat at the level of posterior third of scutum (Fig. 4A, F). *Alloscutum* and ventral surface covered by scattered whitish setae (Fig. 4A, B, F, G). Ten pairs of dorsal body setae - 2 pairs of central dorsal (Cd): $Cd_1 26.73 \pm 4.99$ (20–24.4), $Cd_2 26.73 \pm 1.40$ (20–24.4), and 8 marginal dorsal (Md): $Md_1 26.50 \pm 2.33$ (24.4–31.7), $Md_2 27.60 \pm 3.66$ (19.5–34), $Md_3 27.20 \pm 3.70$ (19.5–31.7), $Md_4 24.60 \pm 2.50$ (19.5–29), $Md_5 25.13 \pm 2.13$ (22–29), $Md_6 25.59 \pm 1.99$ (22–29), $Md_7 24.33 \pm 2.76$ (19.5–26.8), $Md_8 24.81 \pm 3.29$ (19.5–29) (Fig. 4F). Fifteen pairs of ventral setae - 3 pairs of sternal setae (St): $St_1 42 \pm 4.70$ (34–49), $St_2 38.51 \pm 2.71$ (34–41.5), $St_3 39.01 \pm 1.91$ (36.6–41.5); 2 pre-anal pairs (Pa): $Pa_1 33 \pm 2.33$ (29–36.6), $Pa_2 32.11 \pm 3.22$ (29–39); 4 premarginal pairs (Pm): $Pm_1 33.84 \pm 3.42$ (29–41.5), $Pm_2 36.20 \pm 4.11$ (29–44), $Pm_3 32.81 \pm 3.71$ (26.8–39),

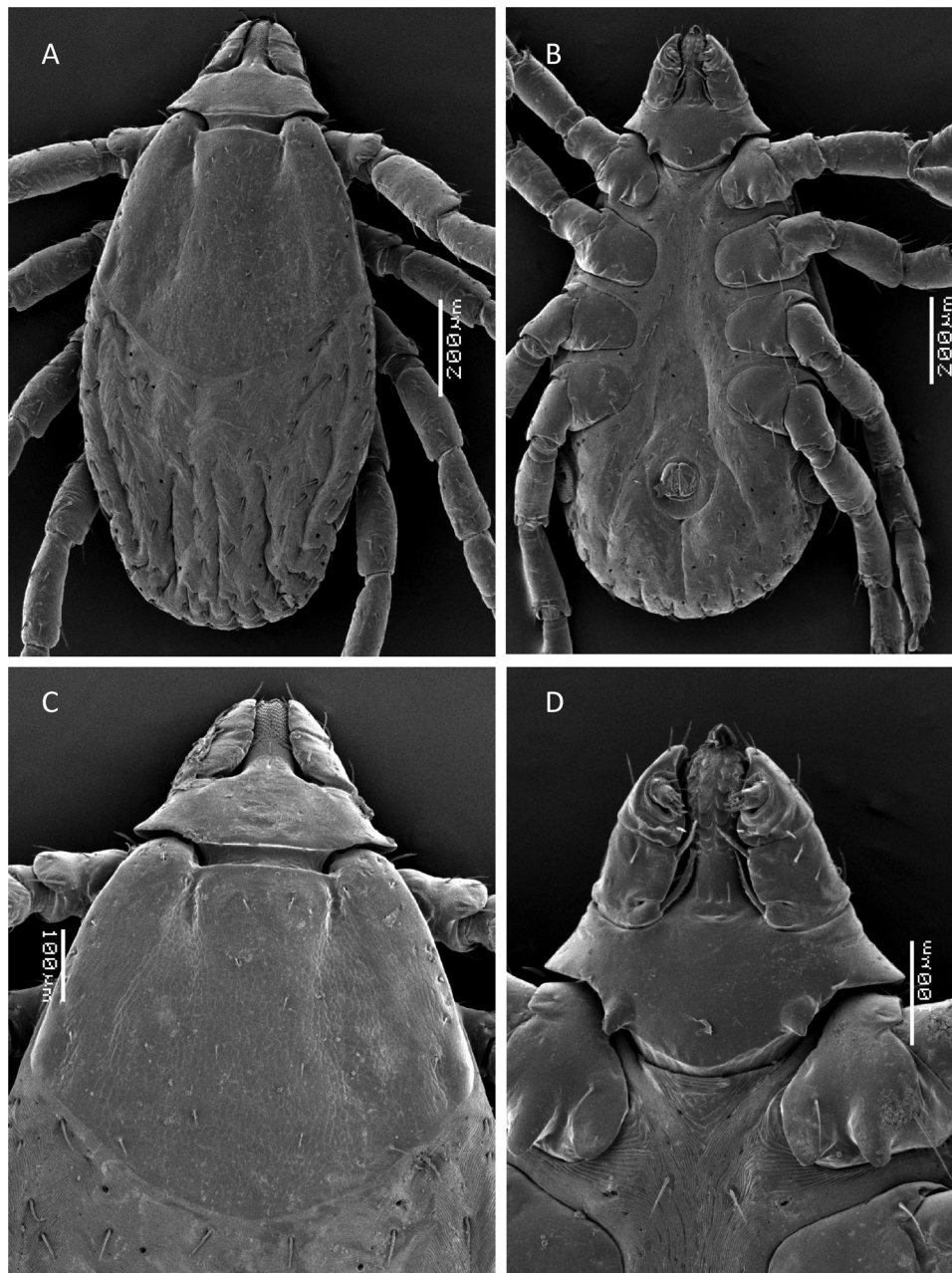


Fig. 3. *Rhipicephalus sanguineus* sensu stricto. nymph. A. Body, dorsal view. B. Body, ventral view. C. Scutum and dorsal view of capitulum. D. Capitulum, ventral view.

Pm_4 30.63 ± 4.00 (24.4–36.6); 5 marginal ventral pairs (Mv): Mv_1 26.70 ± 3.85 (22–34), Mv_2 24.66 ± 1.91 (22–29), Mv_3 24.33 ± 2.61 (19.5–29), Mv_4 24.33 ± 2.41 (19.5–26.8), Mv_5 26.10 ± 1.71 (24.4–29) and 1 pair on anal valves (A): A 21.99 ± 1.51 (19.5–24.4) (Fig. 4G). **Capitulum:** *basis capituli* almost two times broader than long, *basis capituli* width 153 ± 4.51 (146–158), *basis capituli* length from posterior margin to posthypostomal setae (Ph) 81.11 ± 7.70 (68–95), length from palpal apices to posterior margin of *basis capituli* 141 ± 7.10 (130–153), lateral angles short and slightly curved, posterior margin slightly convex; palps short, length 83 ± 2.77 (78–88), and apically acute; hypostome short, length from pH to apex 55.21 ± 1.51 (53.6–58.5), width 33.77 ± 3.10 (29–39), blunt, dental formula 2/2 with five to six denticles per file (Fig. 4C, D). **Legs:** coxa I–III each with a single, short, rounded, external spur, decreasing in size from coxa I to coxa III (Fig. 4B, E). Tarsus I long, length 188 ± 5.40 (180–195).

3.1.5. Neotype

A male collected in Montpellier (43.33°N, 3.50°E), France, was designated as neotype of *R. sanguineus* s.s. The specimen is deposited in the United States National Tick Collection, Institute for Coastal Plain Science, Georgia Southern University, USA (USNMENT00862715). The principal measurements (in millimetres) of the male neotype are: total body length 3.71; length from apices of scapulae to posterior body margin 3.10, maximum body width 2.13, length from palpal apices to cornua apices 0.59, *basis capituli* width 0.72, and *basis capituli* length 0.25. The morphology of the male neotype matches with the description above.

3.2. Molecular characterization

Because not all gene fragments were available for each specimen and because we used many sequences deposited in GenBank, we could

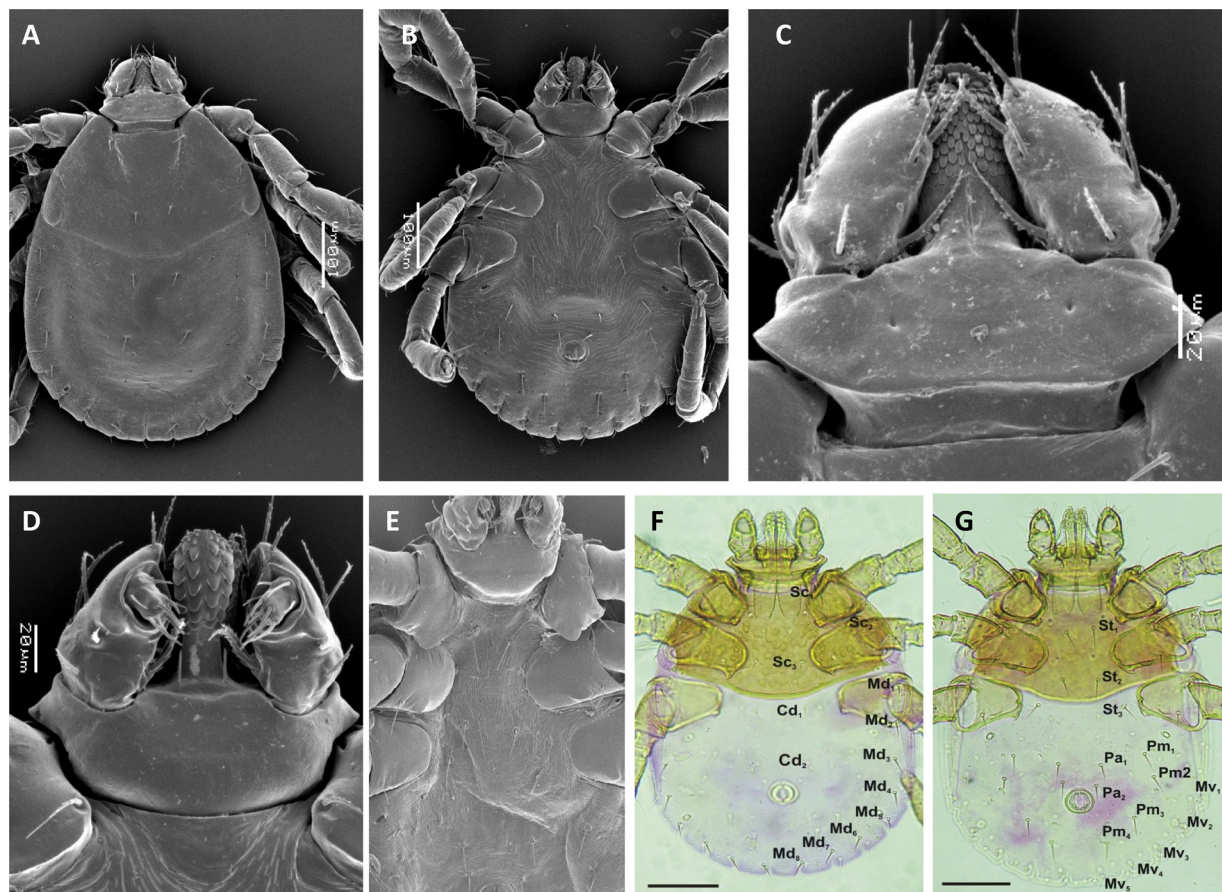


Fig. 4. *Rhipicephalus sanguineus* sensu stricto. Larva. A. Body, dorsal view. B. Body, ventral view. C. Capitulum, dorsal view. D. Capitulum, ventral view. E. Coxae. F. Setae pattern, dorsal view (scale bar 0.1 mm). G. Setae pattern, ventral view (scale bar 0.1 mm).

not concatenate sequence datasets for total evidence analysis, which would have been preferable. Figs. 5–8 represent, therefore, the separate phylogenetic analyses of the 4 gene datasets. The overall phylogenetic topologies obtained with the sequences of the three mitochondrial genes (16S rRNA, 12S rRNA and COI) were similar (Figs. 5–7). The sequences belonging to the *R. sanguineus* s.s. ticks from Montpellier (France) formed a clade together with *R. sanguineus* from Italy, Spain, Portugal, Switzerland and the American *R. sanguineus* “temperate lineage”, represented by sequences (16S and 12S) obtained from ticks of Argentina, Chile, USA and Uruguay (Figs. 5–7). The bootstrap values supporting this lineage are high in all the phylogenetic trees (see Figs. 5–7). This clade is clearly separated from the branches containing the other species of the *R. sanguineus* group (see Figs. 5–7), with the exception of “*R. turanicus*-like” ticks from the western Mediterranean region of Europe and southern Switzerland (from now on *R. turanicus* sensu lato) which are known to cluster phylogenetically with *R. sanguineus* s.s. while being morphologically distinct.

Unlike the results for mitochondrial DNA sequences, the pattern obtained with ITS2 sequences does not show a split between *R. sanguineus* s.s. and other species and lineages of the *R. sanguineus* group (Fig. 8). In fact, sequences belonging to a *R. sanguineus* s.s. tick from Montpellier and to a *R. sanguineus* s.s. tick from Southern Brazil (“temperate lineage”) are grouped in the same clade with *R. turanicus* sensu stricto and *R. sanguineus* s.l. from different regions of the world, including some sequences obtained from ticks known to belong to the “tropical lineage” from Colombia, Costa Rica, Honduras and Egypt (Fig. 8).

The pairwise differences within the clade containing *R. sanguineus* s.s. from France (see Figs. 5–8) ranged from 0.0 to 1.2%, 0.0 to 2%, 0 to 1% and 0.2 to 0.8%, for the 16S, 12S, COI and ITS2 datasets,

respectively. The nucleotide divergence between the sequences of *R. sanguineus* s.s. from France and those included in the other clades of *R. sanguineus* s.l. (see Figs. 5–8) were always higher than 3.7% (16S), 4.3% (12S), 9.9% (COI) and 2.7% (ITS2).

The sequences of the *R. sanguineus* s.s. ticks belonging to the colony from Montpellier are deposited in Genbank with the following accession numbers: 16S: MH630342 (F1 generation), MH630343 (F2 generation), MH630344 (F3 generation); 12S: MH630345; COI: MH630346 (F1 generation), MH630347 (F2 generation); ITS2: MH616087.

4. Discussion

The diagnostic characters unique to the male of *R. sanguineus* s.s. are given by the following combination: *spiracular plate* elongated and subtriangular in shape with a dorsal prolongation narrow and usually visible dorsally, with the dorsal prolongation narrower than the width of the adjacent festoon; punctations of the *scutum* moderate in number and unequal in size; marginal groove conspicuous, deep and punctate; posteromedian groove distinct and elongated, and posterolateral grooves often sub-circular, shorter than posteromedian groove; adanal plates long, wide, and subtriangular in shape, with a clear concavity in its inner margin and posterior margin broadly rounded or truncated; accessory adanal with the posterior end narrower than the width of adjacent festoon. The female of *R. sanguineus* s.s. can be diagnosed by a combination of broadly U-shaped genital aperture, *spiracular plate* with a narrow dorsal prolongation visible dorsally, *basis capituli* hexagonal with broad lateral angles, and *scutum* barely longer than broad with posterior margin sinuous and punctations moderate in number and unequal in size, larger and more numerous along cervical fields.

In the comparison with the remaining species of the *R. sanguineus*

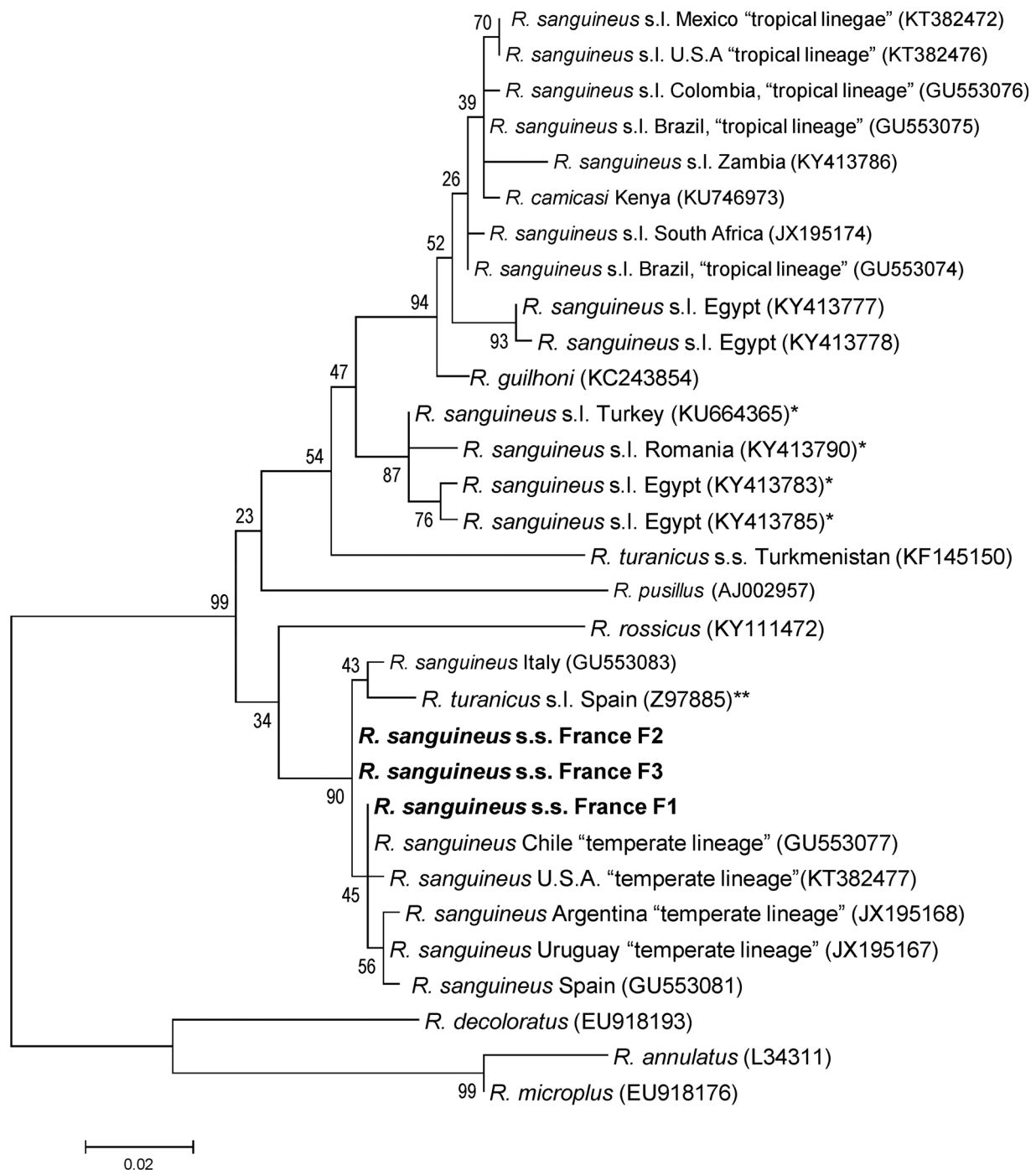


Fig. 5. Maximum-likelihood tree based on partial 16S rRNA gene sequences of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets. The tree was constructed with the general time reversible model (GTR + G).* “Morphotype I” sensu Dantas-Torres et al. (2013) or lineage “southeastern Europe” sensu Chitimia-Dobler et al. (2017). ** See discussion on the *R. turanicus* sensu lato.

group (based on the descriptions and figures of Pomerantzev (1950); Morel and Vassiliades (1962); Filippova (1997); Walker et al. (2000); Estrada-Peña et al. (2004, 2017) and Horak et al. (2018)) the male of *R. sanguineus* s.s. can be differentiated from those of *R. guilhoni* and *R. turanicus* s.s. by the dorsal prolongation of the spiracular plate, which is narrower than the width of the adjacent festoon in *R. sanguineus* and equal or wider than the width of the adjacent festoon in the other two species. Also, the male of *R. turanicus* s.s. has a cusp on the internal margin of the adanal plates, which is absent in *R. sanguineus* s.s. The male of *R. sanguineus* s.s. can be distinguished from the male of *R.*

camikasi by the marginal groove, which is deeper and more punctate in *R. sanguineus* s.s. The male of *R. moucheti* has sickle-shaped adanal plates that allows an easy differential diagnosis with *R. sanguineus* s.s. The punctations of the scutum in males of *R. sanguineus* s.s. are more numerous and conspicuous than in *R. rossicus*, and the punctations in *R. sulcatus* are more numerous and more dense than in *R. sanguineus* s.s. There is a small internal cusp in the adanal plate of *R. pumilio* which is absent in *R. sanguineus* s.s. In the males of *R. pusillus*, *R. leporis* and *R. schulzei* the marginal groove is shorter and the total size is notably smaller than in the males of *R. sanguineus* s.s. The female of *R.*

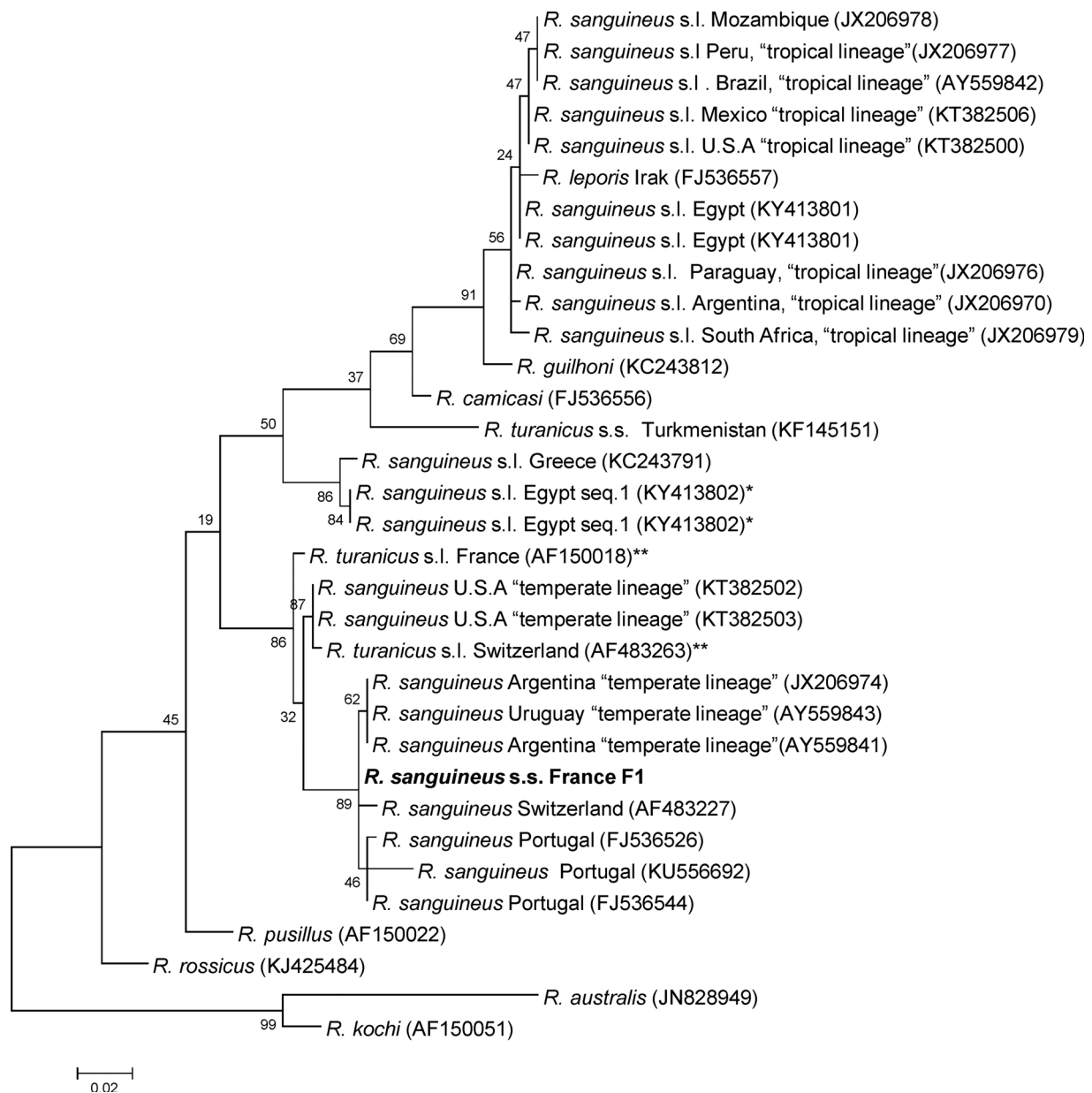


Fig. 6. Maximum-likelihood tree based on partial 12S rRNA gene sequences of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets. The tree was constructed with the general time reversible model (GTR + G).* "Morphotype I" sensu Dantas-Torres et al. (2013) or lineage "southeastern Europe" sensu Chitimia-Dobler et al. (2017). ** See discussion on the *R. turanicus* sensu lato.

sanguineus s.s. has a genital aperture broadly U-shaped, while the female of *R. turanicus* s.s. has a genital aperture narrowly U-shaped and the *scutum* with punctations more numerous and larger. The shape of the genital aperture is a character that also allows the differentiation of the female of *R. sanguineus* s.s. (genital aperture broadly U-shaped) from those of *R. camicasi* (genital aperture narrowly U-shaped) and *R. guilhoni* (genital aperture truncated V-shaped with distinct hyaline flaps). The female of *R. moucheti* can be distinguished from that of *R. sanguineus* s.s. by its genital aperture that is widely V-shaped and by having a *scutum* with punctations more numerous and larger than in *R. sanguineus* s.s. The *scutum* of the females of *R. sulcatus* and *R. pusillus* is smaller in size and more punctate than that of the female of *R. sanguineus* s.s. The female of *R. leporis* can also be separated from that of *R. sanguineus* s.s. by its smaller-sized *scutum* and larger punctations on the cervical fields. In the same way, females of *R. sanguineus* s.s. and *R. rossicus* differ in the punctuation pattern, because the latter species has a

scutum that is relatively impunctate. The lateral angles of the *basis capituli* are more prominent and pointed in the female of *R. pumilio* than in the female of *R. sanguineus* s.s., and the *basis capituli* of the female of *R. schulzei* differs from that of *R. sanguineus* s.s. by the presence in the former tick of long sharp lateral angles with a slight downward curvature. However, morphological diagnoses of ticks from this group will continue to be difficult, and molecular characterization of all species should be obtained to support morphological identifications.

The diagnostic characters for the nymph of *R. sanguineus* s.s. are: *basis capituli* sub-triangular dorsally with lateral angles slightly curved and presence of ventral processes, mean total length of the body of 1.34 mm (range: 1.30–1.36), *scutum* approximately as long as broad with lateral margins nearly straight and posterior margin broadly rounded, and cervical grooves short and sigmoid in shape extending posteriorly to the level of the eyes. The morphological features which characterize the larva of *R. sanguineus* s.s. are: *basis capituli* broader than

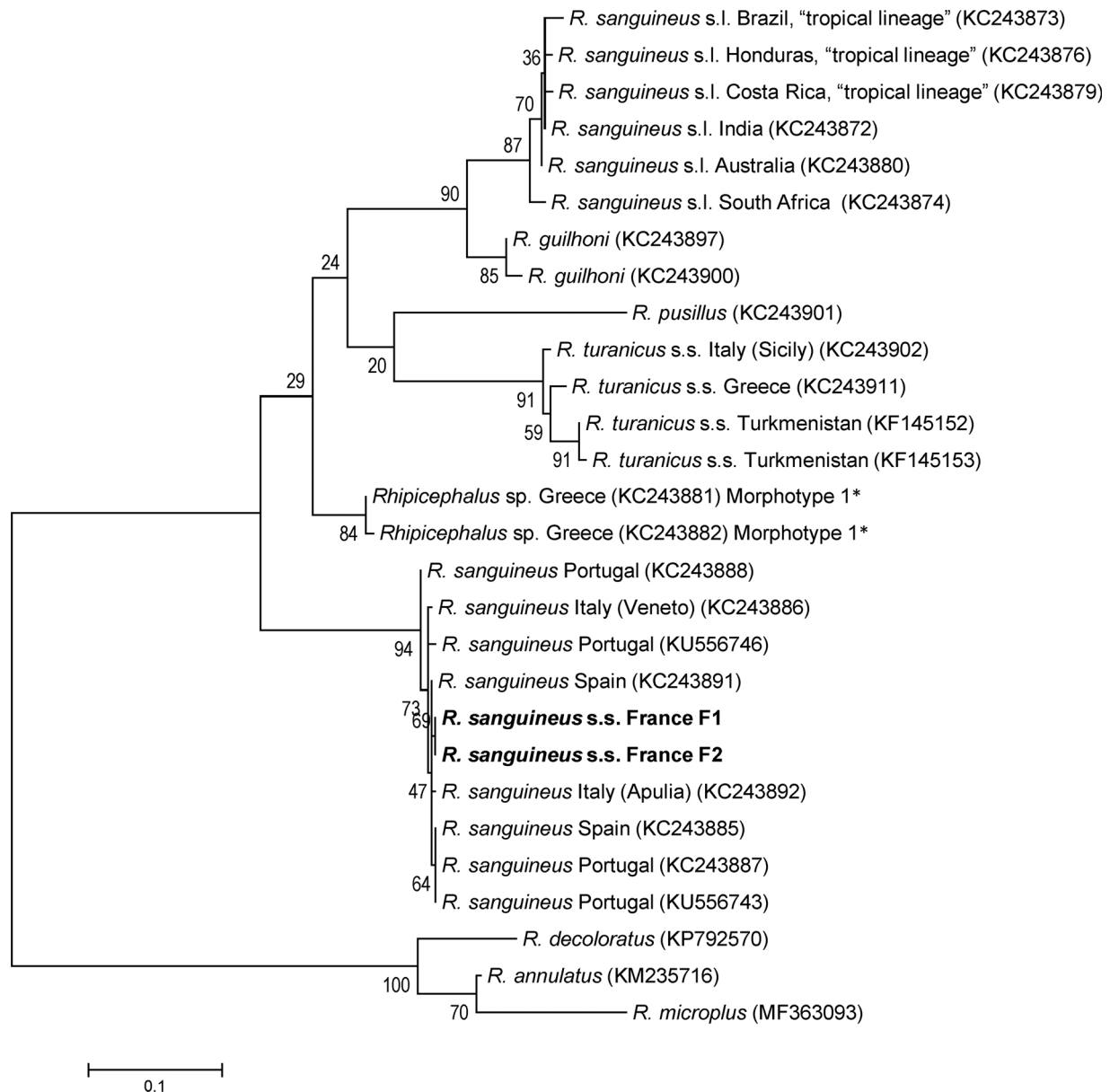


Fig. 7. Maximum-likelihood tree based on sequences of the mitochondrial COI gene of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets. The tree was constructed with the Hasegawa-Kishino-Yano model (HKY + G + I). * "Morphotype I" sensu Dantas-Torres et al. (2013).

long with lateral angles short and slightly curved and with posterior margin slightly convex, a mean total length of the body of 605.9 μm (range: 568–647), short cervical grooves shallow and subparallel, and *scutum* almost twice broader than long. The differential diagnosis among sympatric and morphologically related species of the *R. sanguineus* group when it is applied to immature stages is particularly difficult, as stated by Walker et al. (2000). The pattern of the morphological discrete characters and the number and disposition of setae (in the case of larvae) of the immature stages of *R. sanguineus* s.s. described in the current work, matches the description of larvae and nymphs of *R. sanguineus* presented in Filippova (1997) and Walker et al. (2000). However, the only way to solve the limitation related to the differential diagnosis of larvae and nymphs is by performing comparative studies using analogous material (unfed specimens) where both discrete and continuous morphological characters are analysed together along with molecular analysis. Future studies on this topic are needed.

The phylogenetic patterns obtained with mitochondrial DNA

sequences support *R. sanguineus* s.s. as a well-defined taxon when compared with other species of the *R. sanguineus* group: *R. turanicus* s.s., *R. camicasi*, *R. guilhoni*, *R. sulcatus*, *R. pusillus*, *R. rossicus* and *R. leporis*. In the phylogenetic trees and in the pairwise comparisons of genetic divergence, the taxon defined in this work as *R. sanguineus* s.s. constitutes an independent lineage within the *R. sanguineus* group. In contrast, ITS-2 sequences among the *R. sanguineus* group are homogeneous and their analyses do not provide informative phylogenetic reconstructions. This lack of polymorphism in ITS-2 sequences of species from the *R. sanguineus* group was already reported by Zahler et al. (1997) and Latrofa et al. (2013). Burger et al. (2014) also found that ITS-2 sequences within the *R. microplus* complex are highly conserved.

Molecularly *R. sanguineus* s.s. encompasses also to the so-called "temperate lineage" sensu Moraes-Filho et al. (2011) and Nava et al. (2012) from the New World, which includes populations of *R. sanguineus* s.l. ticks from temperate and cold areas of Argentina, southern Brazil, Chile, Uruguay, and USA. The genetic and morphological similarities (see diagnosis of *R. sanguineus* s.l. from "temperate lineage" in

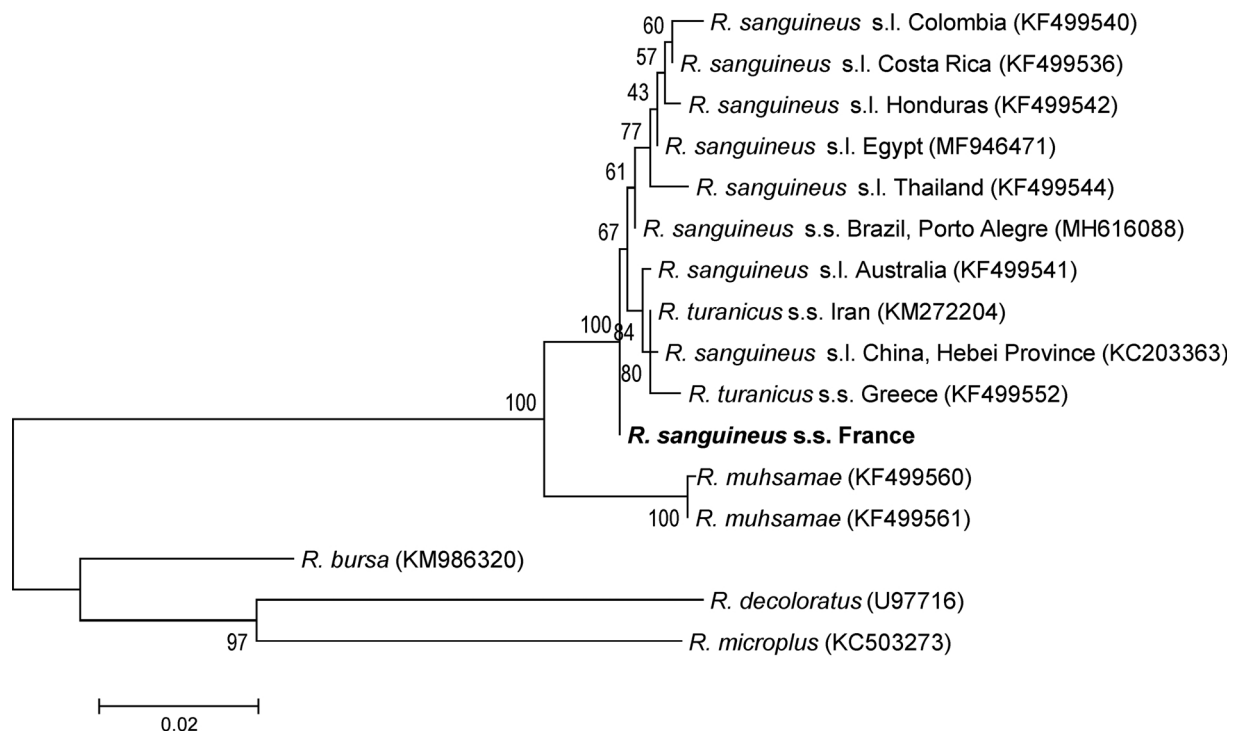


Fig. 8. Maximum-likelihood tree based on sequences of *ITS2* of species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are indicated in brackets. The tree was constructed with the Tamura 3-Parameter model (T3P) with gamma distribution (+G).

Nava et al. (2017)) and biological compatibility (crosses between *R. sanguineus* ticks from Esperanza (Argentina) and Montpellier (France) produced viable progeny (S. Nava and M.N. Saracho-Bottero, in preparation)) allow us to affirm that the *R. sanguineus* s.l. ticks previously included within the “temperate lineage” in the Americas (Moraes-Filho et al., 2011; Nava et al., 2012; Sanches et al., 2016; Zemtsova et al., 2016; Chitimia-Dobler et al., 2017; Díaz et al., 2018) in fact belong to *R. sanguineus* s.s. In contrast, the populations of *R. sanguineus* s.l. ticks from the American “tropical lineage” (Moraes-Filho et al., 2011; Nava et al., 2012; Sanches et al., 2016; Zemtsova et al., 2016; Chitimia-Dobler et al., 2017; Díaz et al., 2018) and those of the “*Rhipicephalus* sp. I” lineage of Dantas-Torres et al. (2013) or “southeastern European lineage” of Chitimia-Dobler et al. (2017), clearly do not belong to *R. sanguineus* s.s. (see Figs. 5 and 6). Indeed, crosses between *R. sanguineus* ticks from the “tropical lineage” from Sao Paulo (Brazil) and *R. sanguineus* s.s. ticks from Montpellier (France) produced sterile hybrids (M.B. Labruna and M.P.J. Szabó, in preparation).

Another relevant issue to be resolved is the relationship between *R. sanguineus* s.s. and the populations of a tick that has been called *R. turanicus* in parts of the Mediterranean basin of Spain, Portugal and France and in southern Switzerland (Morel and Vassiliades, 1962; Estrada-Peña and Sanchez, 1988; Bernasconi et al., 2002; Estrada-Peña et al. (2004, 2017) (named in this work as *R. turanicus* s.l.). The taxon *R. turanicus* s.s. is formally well defined (see Filippova (1997)) and phylogenetically represents an independent lineage (see Dantas-Torres et al. (2013), Chitimia-Dobler et al. (2017) and the Figs. 5–7 of this work). The type locality of *R. turanicus* s.s. is Tashkent, Uzbekistan (lectotype), and *bona fide* records of *R. turanicus* s.s. appear to be restricted to localities of Central Asia and southeastern Europe. The study by Estrada-Peña and Sanchez (1988) and Estrada-Peña et al. (2004) with specimens from Western Europe did not clarify the status of *R. turanicus* s.s. because there is no guarantee that these investigators were working with *bona fide* *R. turanicus* s.s. (Guglielmone and Nava, 2014). In conclusion, it can be stated that *R. turanicus* s.l. ticks from the Mediterranean basin and southern Switzerland do not belong to the

taxon *R. turanicus* s.s. The male of *R. sanguineus* s.s. differs morphologically from that of *R. turanicus* s.l. by the shape of the spiracle plate (dorsal prolongation sharper and narrower than the width of the adjacent festoon in *R. sanguineus* s.s. and as broad as the adjacent festoon in *R. turanicus* s.l.). The females of these two taxa differ at least in the shape of the genital aperture (broadly U-shaped in *R. sanguineus* s.s., narrow U-shaped in *R. turanicus* s.l.) and in the dorsal prolongation of the spiracular plate (narrower in *R. sanguineus* s.s. than in *R. turanicus* s.l.). The features of the dorsal grooves in the males and the dorsal punctations in both males and females are variable, and should not be used to separate *R. turanicus* s.l. from *R. sanguineus* s.s. These morphological differences contrast with the lack of significant genetic differentiation (Mangold et al., 1998; Beati and Keirans, 2001; Moraes-Filho et al., 2011; Santos-Silva et al., 2011; Almeida et al., 2017; this study). It is important to mention that intermediate forms were observed in areas of Western Europe, suggesting possible hybridization events in nature (Estrada-Peña and Sanchez, 1988). Further studies based on crosses and backcrosses and analyses of population genetics with polymorphic codominant markers are needed to determine whether *R. sanguineus* s.s. and *R. turanicus* s.l. are conspecific or not, and to evaluate the extent of gene flow between them. The *R. turanicus* s.l. specimens should be reported as *R. sanguineus* s.l. until the true identity of the taxon is confirmed.

It is pertinent to mention that with our molecular analyses, we did not pretend to resolve the whole taxonomic status of the group, but wanted merely to establish the phylogenetic placement of the neotype among closely related taxa. It is becoming increasingly evident that the tools used so far for unraveling relationships within the *R. sanguineus* group of species are insufficient: alternative fast-evolving nuclear markers, such as microsatellite loci, should be examined to better establish genetic structure between and within tick populations.

The evidence currently available supports the presence of *R. sanguineus* s.s. in Europe (France, Italy, Spain, Switzerland and Portugal) and America (Argentina, Brazil, Chile, Uruguay and U.S.A.). However, further studies are needed to determine the exact geographic range of

this taxon. Also, there are many taxa that were considered synonyms of *R. sanguineus* s.s., namely *Rhipicephalus beccarii* Pavesi, 1883, *Rhipicephalus bhamensis* Supino, 1897, *Rhipicephalus brevicollis* Neumann, 1897, *Rhipicephalus breviceps* Warburton, 1910, *Rhipicephalus bursa americanus* Neumann, 1897, *Rhipicephalus carinatus* Frauenfeld, 1867, *Rhipicephalus flavus* Supino, 1897, *Rhipicephalus intermedius* (Neumann, 1897), *Rhipicephalus limbatus* Koch, 1844, *Rhipicephalus macropis* Schulze, 1936, *Rhipicephalus punctatissimus* Gerstäcker, 1873³, *Rhipicephalus rubicundus* Frauenfeld, 1867, *Rhipicephalus rutilus* Koch, 1844, *Rhipicephalus sanguineus brevicollis* Neumann, 1897, *Rhipicephalus sanguineus punctatissimus* Gerstäcker, 1873⁴, *Rhipicephalus sanguineus sanguineus* (Latreille, 1806), *Rhipicephalus sculus* Koch, 1844, *Rhipicephalus stigmaticus* Gerstäcker, 1873, *Rhipicephalus texanus* Banks, 1908, *Ixodes linnaei* Audouin, 1826, *Ixodes plumbeus* Dugès, 1834⁶, *Boophilus dugesi*⁵ (Gervais, 1844), *Ixodes hexagonus sanguineus* Latreille, 1806⁷, and *Rhipicephalus dugesi* (Gervais, 1844)⁵ (Camicas et al., 1998; Walker et al., 2000). All of them were treated as *incertae sedis* by Guglielmone and Nava (2014) due to the uncertain morphological definition of *R. sanguineus* s.s., with the exception of *R. bursa americanus* and *I. linnaei* which were considered as *nomina dubia* by those authors. Although several of these names likely correspond to synonyms of *R. sanguineus* s.s., some of them whose type localities are from central and northern Africa (e.g. *R. brevicollis*, *R. limbatus*, *R. punctatissimus*, *R. rutilus* and *R. stigmaticus*), should be examined to determine whether they are synonyms or have priority in relation to the species and lineages included in the clades formed by *R. sanguineus* s.l. from Sub-Saharan Africa, *R. sanguineus* s.l. “tropical lineage” from America, *R. camicasi*, *R. guilhoni*, and *R. sanguineus* s.l. from Africa north of the Sahara, and by the lineages from Europe and Africa called “morphotype I” in Dantas-Torres et al. (2013) and “southeastern European” in Chitimia-Dobler et al. (2017) (see Figs. 5–7).

In this work, the neotype of *R. sanguineus* s.s. was designated by taking into account the rules of the International Code of Zoological Nomenclature listed in the introduction. All parasitic stages of *R. sanguineus* s.s. were morphologically described and DNA sequences of different molecular markers of this taxon are now available. The redefinition of *R. sanguineus* s.s., presented herein, should constitute a benchmark for further taxonomic investigations of the many different populations of *R. sanguineus* s.l. distributed worldwide.

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⁵ Treated as synonym of *R. sulcatus* in Camicas et al. (1998).

⁶ Treated as synonyms of *Ixodes dugesi* Gervais, 1844 (a name *incertae sedis*) in Guglielmone and Nava (2014).

⁷ Treated as *nomen nudum* by Guglielmone and Nava (2014).

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