Veterinary Parasitology 215 (2016) 78–87

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Molecular, biological, and morphometric comparisons between different geographical populations of *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae)

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ARTICLE INFO

Article history:
Received 19 August 2015
Received in revised form 4 November 2015
Accepted 12 November 2015

Keywords:
Rhipicephalus sanguineus
Genetic analysis
Morphometry
Taxonomy

ABSTRACT

In this study, different geographical populations of *Rhipicephalus sanguineus* sensu lato were compared by molecular, biological, and morphometric methods. Phylogenetic trees were constructed using 12S and 16S rDNA sequences and showed two distinct clades: one composed of ticks from Brazil (Jaboticabal, SP), Cuba (Havana) Thailand (Bangkok) and the so-called "tropical strain" ticks. The second clade was composed of ticks from Spain (Zaragoza), Argentina (Rafaela, Santa Fe) and the so-called "temperate strain" ticks. Morphometric analysis showed good separation between females of the two clades and within the temperate clade. Males also exhibited separation between the two clades, but with some overlap. Multiple biological parameters revealed differences between the two clades, especially the weight of the engorged female. These results confirm the existence of at least two species under the name "*R. sanguineus*".

1. Introduction

The "*Rhipicephalus sanguineus* complex" includes 17 species: *Rhipicephalus aurantiacus* Neumann, 1907; *Rhipicephalus bergeoni* Morel and Balis, 1976, *Rhipicephalus boueti* Morel, 1957; *Rhipicephalus camicasi* Morel, Mouchet and Rodhain, 1976; *Rhipicephalus guilhoni* Morel and Vassiliades, 1963; *Rhipicephalus leporis* Pomerantzev, 1946; *Rhipicephalus moucheti* Morel, 1965; *Rhipicephalus pumilio* Schulze, 1935; *Rhipicephalus pusillus* Gil Collado, 1936; *Rhipicephalus ramachandrai* Dhanda, 1966; *Rhipicephalus rossicus* Yakimov and Kol-Yakimova, 1911; *R. sanguineus* sensu stricto (s.s.); *Rhipicephalus schulzei* Olenev, 1929; *Rhipicephalus sulcatus* Neumann, 1908; *Rhipicephalus tetracornus* Kitaoka and Suzuki, 1983; *Rhipicephalus turanicus* Pomerantzev, 1940; and *Rhipicephalus ziemanni* Neumann, 1904. Some of these are closely related, morphologically similar, and, consequently, have been misidentified (Walker et al., 2000 reviewed in Dantas-Torres and Otranto, 2015).

Historically, *R. sanguineus* sensu stricto (s.s) is the most controversial species in the "*R. sanguineus* complex". Originally, was classified as *Ixodes sanguineus* by Latreille (1806) and later transferred to the genus *Rhipicephalus* by Koch (1844). Moreover, the original description does not provide a definition of the morphological basis for the species. According to Nava et al. (2015), in light of these data, *R. sanguineus* s.s. could be relegated to a *nomen nudum*. Following this description, many species and subspecies belonging to the "*R. sanguineus* complex" were synonymized as *R. sanguineus* s.s. around the world (Camicas et al., 1998; Walker et al., 2000). The type locality is Gallia (France). In this context, Guglielmone et al. (2014) deemed *R. sanguineus* s.s. a Palearctic species, considering all other records of this species around the world as speculative.

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http://dx.doi.org/10.1016/j.vetpar.2015.11.007
0304-4017/

Over the last decade, some studies started to indicate that what was known until the moment as *R. sanguineus* (s.s.) could be represented by more than one species. Szabó et al. (2005) and Oliveira et al. (2005) suggested that the taxon of *R. sanguineus* would be composed of at least two morphologically and genetically distinct strains in the Neotropics. Moraes-Filho et al. (2011) proposed a so-called "southern lineage," located in temperate localities (Argentina, Uruguay, Chile, Italy, and south Brazil), and a "northern lineage," located in tropical and subtropical localities (Brazil, Paraguay, Colombia, South Africa, Mozambique, and northern Argentina). Nava et al. (2012) observed these same lineages in the Southern Cone of South America. Dantas-Torres et al. (2013) also recognized these lineages in the Old World and suggested the possibility of other genetic lineages under the name "*R. sanguineus*." Despite these findings, the taxonomy status of this species is far from resolved. Along this line, a consensual redescription of *R. sanguineus* s.s. and a description of the other(s) species under this name are required, after an exhaustive worldwide revision of this species complex (Dantas-Torres et al., 2013). However, morphological variations within the same genetic strain of *R. sanguineus* (Pegram et al., 1987; Dantas-Torres et al., 2013) are quite common, which is the main current taxonomic issue. Levin et al. (2012) and Gray et al. (2013) drew attention to the need of studies addressing morphology, genetic and biological aspects, considering variations of these ticks over a large geographical range.

In view of these data, the present study aimed to compare, genetically, morphometrically and biologically, the different geographical populations of *R. sanguineus* sensu lato (s.l.) from the so-called tropical (Brazil, Cuba, and Thailand) and temperate (Argentina and Spain) strains. The results obtained in this study may contribute to a better understanding of *R. sanguineus*' biosystematic status.

2. Materials and methods

2.1. Ticks

The specimens used in this study were obtained from colonies established at the Department of Veterinary Pathology, Universidade Estadual Paulista—UNESP, Campus of Jaboticabal, São Paulo State, Brazil from isolates made in Cuba, Thailand, Argentina and Spain (Table 1 and Fig. 1). The identification of isolates was confirmed by each provider according to Walker et al. (2000). To maintain colonies, pools of ticks were periodically fed on 5–8 month-old New Zealand white rabbits. Non parasitic stages were kept under controlled conditions to 27 °C, 80% relative humidity, and 12-h photoperiod for tropical strains and to 20 °C, 80% relative humidity, and 12-h photoperiod for temperate strains.

2.2. Molecular analysis

Phylogenetic analyses were performed from mitochondrial DNA of ticks from the colonies described in Table 1. A sample of *R. sanguineus* from La Libertad, Magdalena, Colombia (4°35′N; 74°04′W), kindly provided by Dr. Efrain Benavides Ortiz (University of La Salle, Bogotá, Colombia), was added to the molecular analysis. From each strain, DNA extraction was separately performed using two individual adult ticks, according to a previously described protocol (Mangold et al., 1998). A 380 base pair (bp) fragment of the 12S rDNA gene and a 460 bp fragment of the 16S rDNA gene were amplified by PCR using previously described primers (Black and Piesman, 1994; Szabó et al., 2005). Amplified DNA was purified using a Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's recommendations. Purified PCR products were submitted for sequencing using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit in an Applied Biosystems 373A gene sequencer. Sequences were manually edited using Bioedit Sequence Aligment Editor (Hall, 1999) and aligned using Clustal W software (Larkin et al., 2007). Additionally, GenBank available *Rhipicephalus* spp. 12S and 16S rDNA partial sequences were included in the molecular analysis. Only sequences published in reference's studies or unpublished sequences with host and geographical origin information were used. The GenBank accession numbers of these sequences and the geographical origins are presented in the phylogenetic trees. GenBank available partial 12S rDNA (AF150034) and 16S rDNA (L34307) sequences of *Hyalomma marginatum* were used as outgroups. The nucleotide sequences obtained in this study were deposited in the GenBank database (12S rDNA: KC018070, KC018072, KC018074, KC018075, KC018076; 16S rDNA: JX997387, JX997389, JX997390, JX997391, JX997393). The percentage of nucleotide variation among sequences of a given species was calculated by pairwise comparison (Kimura 2-parameter model) using the MEGA 5.0 software (Tamura et al., 2007). The formula $D = 1 - (M/L)$ was used to compare the sequences obtained in this work with the *Rhipicephalus* spp. consensus sequence. In this formula D is the sequence difference, M is the number of alignment positions at which the two sequences have a base in common and L is the total number of alignment positions over which the two sequences are compared (Chilton et al., 1995). The maximum likelihood (ML) method was used to make the phylogenetic analysis, which was also conducted in MEGA 6.0 Program. ML trees were generated using the Tamura–Nei substitution model with uniform rates among sites. The partial deletion option was used for gap analysis in MP trees with 95% of site coverage cutoff. A bootstrap test with 1000 replications was applied to estimate the confidence of the tree branching patterns.

2.3. Morphometric comparison

For morphometric comparisons, 10 couples of each *R. sanguineus* strain were slide-mounted according to the method of Famadas et al. (1996). Measurements were performed using a MC80DX light microscope coupled with a digital camera (Leica Microsystems). The following characteristics were measured: basis capituli (length and width); palps (length); tarsus I (length and width); dorsal scutum (length and width); idiosoma (length from scapular apices to posterior idiosomal margin and width); spiracular plates (length and width); and male adanal plates (length and width at base). All measurements are in millimeters and expressed as mean ± standard deviation. Voucher tick specimens were deposited in the Laboratory of Imunopathology, Department

Table 1
Rhipicephalus sanguineus strains used in the present study.

Species	Location	Coordinates	Provided by
1. *R. sanguineus* s.l.	Havana, Cuba	23°07′N; 82°22′W	Dr. Alina R. Mallon
2. *R. sanguineus* s.l.	Jaboticabal, SP, Brazil	21° 15′S; 48° 18′W	Dr. Gervásio H. Bechara
3. *R. sanguineus* s.l.	Bangkok, Thailand	7° 59′N; 98° 20′E	Dr. Sathaporn Jittapalapong
4. *R. sanguineus* s.l.	Rafaela, Santa Fe, Argentina	31° 15′S; 61° 29′W	Dr. Santiago Nava
5. *R. sanguineus* s.l.	Zaragoza, Spain	41° 39′N; 00° 52′W	Dr. Agustín Estrada-Peña