Original article

Geographical distribution and phylogenetic analysis of *Rhipicephalus sanguineus* sensu lato in northern and central Chile

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**ABSTRACT**

The presented study analyzed the presence and geographical distribution of the tropical and temperate lineages of *Rhipicephalus sanguineus* sensu lato in Chile. *R. sanguineus* s.l. ticks were collected from dogs at 14 sites in northern and central Chile for morphological and genetic analysis based on the 16S rDNA gene. Phylogenetic studies proved the existence of both, the tropical and the temperate lineages. The former was represented by a single haplotype and occurred in the far north; the latter included four haplotypes and was observed from the Tarapacá Region southwards. In four sites at latitudes from 20°S to 22°S, both lineages were found to coexist. Our study discovered for the first time the existence of the tropical lineage in Chile and demonstrated that distributions of the tropical and temperate lineages overlap, forming a transitional zone of approximately 200 km in northern coastal Chile.

1. Introduction

*Rhipicephalus sanguineus* sensu lato (s.l.) is a cluster of species containing some of the most widespread tick species in the world, with remarkable economical, medical, and veterinary importance (Nava et al., 2015; Hekimoğlu et al., 2016). Despite its known relevance as a vector of important parasitic and bacterial pathogens such as *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis*, *Babesia vogeli*, and *Rickettsia* spp. (Dantas-Torres, 2008; Dantas-Torres and Otranto, 2015), the exact taxonomic differences and relationships among *R. sanguineus* s.l. remain uncertain. The main reasons for these controversies are the unreliable original description of *R. sanguineus* (Latreille, 1806), the loss of the type specimen, and the high morphological similarity among the members of the species complex (Dantas-Torres and Otranto, 2015; Nava et al., 2015).

To address these taxonomic uncertainties, various molecular methods, mainly analyzing mitochondrial 16S and 12S rDNA, have been developed. These studies have supported the existence of various, genetically divergent *R. sanguineus* s.l. lineages (Burlini et al., 2010; Dantas-Torres et al., 2013; Liu et al., 2013; Moraes-Filho et al., 2011; Nava et al., 2012; Oliveira et al., 2005; Sanches et al., 2016; Szabó et al., 2005; Chitimia-Dobler et al., 2017). In South America, two lineages have been identified: a tropical lineage in tropical areas in Brazil, Paraguay, Colombia, Peru, and Argentina, and a temperate lineage in subtropical and temperate regions in Brazil, Uruguay, Argentina, and Chile (Moraes-Filho et al., 2011; Nava et al., 2012). Comparative studies of specimens from a wider geographical range suggest the existence of further lineages in Europe, Africa, and Asia (Dantas-Torres et al., 2013; Hekimoğlu et al., 2016; Chitimia-Dobler et al., 2017). Besides its taxonomic relevance, the recognition of different lineages might also have important clinical implications, since, as recently demonstrated, both tropical and temperate lineages have different vectorial competence to transmit the canine pathogen *E. canis* (Moraes-Filho et al., 2015; Cicutti et al., 2017).

In Chile, ticks of the *R. sanguineus* complex were first described in the central Metropolitan Region by the mid-1970s (Tagle, 1976).
Currently, this species complex is known to be endemic from Arica in the far north (18°29′01″S) to Valdivia in southern Chile (39°49′11″S) (Abarca et al., 2016; González-Acuña and Guglielmone, 2005; López et al., 2015), but the presence and distribution of different lineages of *R. sanguineus* s.l. have not been studied systematically yet. The presented study aimed to analyze the spatial distribution and limits of *R. sanguineus* s.l. lineages and haplotypes in northern and central Chile.

### 2. Material & methods

#### 2.1. Tick sampling

Ticks from five administrative regions in northern and central Chile, ranging from Arica (18°29′01″S) to Santiago (33°8′43″S), were included. Specimens (maximum three per dog) were collected from stray dogs in 12 study sites from Arica to Antofagasta during January to February 2016 and from household dogs in Coquimbo and Santiago in 2014 and 2015 (Table 1). Dogs were restrained and examined by a veterinarian, which carefully removed ticks with tweezers and placed them in ethanol 70%. Samples were kept at room temperature and sent to the Laboratorio de Infectología y Virología Molecular, Pontificia Universidad Católica de Chile in Santiago, Chile, for taxonomical identification according to Walker et al. (2000) and subsequent molecular analysis.

The study protocol was reviewed and approved by the Comité Ético Científico (Approval N°12-170), and by the Comité de Bienestar Animal (Approval N°12-033) of the Faculty of Medicine, Pontificia Universidad Católica de Chile in Santiago, Chile.

#### 2.2. Molecular analysis and sequencing

Subgroups of 5–10 male ticks per site were used for further molecular analysis by partial amplification of 16sDNA gene. Dried ticks were mechanically triturated and re-suspended in PBSTX DNAase free buffer. DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Subsequently, a partial fragment of 405 base pairs (bp) of the constitutive mitochondrial gene 16S rDNA was amplified using 5′-CGG-GTC-TGA-CTG-ATG-ACG-T-3′ and 5′-GGT-CGA-TAA-TTT-TTT-AAA-TTG-CGG-3′ as forward and reverse primers, respectively (Mangold et al., 1998). The PCR protocol included the following steps: 2 min at 94 °C, followed by 7 cycles of 30 s at 94 °C – 30 s at 45 °C – 45 s at 72 °C continued by 28 cycles of 30 s at 94 °C – 30 s at 47 °C – 45 s at 72 °C, with increasing annealing temperatures (0.3 °C per cycle), and a final step of 10 min at 72 °C. For amplification, Platinum® PCR SuperMix High Fidelity (cat: 12523016) (Thermo Fisher Scientific, Waltham, MA, USA) was used. All PCRs were performed in a ProFlex 3 × 32 well PCR system (Thermo Fisher Scientific). PCR products were separated in 2% agarose gel stained with SYBR® Safe DNA gel stain (Thermo Fisher Scientific) and visualized in a trans-illuminator. Sequencing was carried out by Macrogen Corp. (Bethesda, MD, USA).

#### 2.3. Phylogenetic analysis

The obtained sequences were manually edited and aligned in BioEdit 7.2.5 version (Hall, 1999). The phylogenetic analysis was inferred by Neighbor-Join (NJ), Maximum Likelihood (ML), and Maximum Parsimony (MP) methods using the software MEGA6 (Tamura et al., 2013). The search of the most appropriate model of nucleotide substitution for phylogenetic analysis was performed in MEGA6, according to the Bayesian information criterion (BIC), which was applied to the methods of NJ and ML. For the ML method, initial trees for the heuristic search were obtained automatically by applying NJ and Bio NJ algorithms to a matrix of pairwise distances estimated using the
Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. In addition, MP method was used to corroborate the topology of the obtained phylogenetic tree. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm with level 1 search in which the initial trees were obtained by random addition of sequences (10 replicates). For all the methods, the support of the topology was based on a bootstrapping of 1000 replicates, and the positions equivalent to gaps or missing data were deleted. Additionally, we included sequences available in GenBank of *R. sanguineus* s.l. from other regions in South America as well as other species belonging to the *R. sanguineus* group such as *R. camacasi, R. guilhoni, R. turanicus, R. pussillus*, and *R. rossicus* (Table 2). A sequence of *R. appendiculatus* was used as an outgroup.

### 3. Results

A total of 136 ticks were collected, which were all morphologically identified as *R. sanguineus* s.l.; after amplification, sufficient genetic material was available from 70 of those specimens (Table 1). The phylogenetic analysis included a total of 84 partial sequences of the mitochondrial gene 16S rDNA, 70 from our samples and 14 from sequences published in GenBank. The T92 model (Tamura, 1992) was chosen (using MEGA 6), as the most accurate nucleotide substitution model for our dataset. Based on this model, we were able to determine six haplotypes, which grouped in two phylogenetic clades with marked divergence. In accordance with the reference sequences, these clades grouped in two phylogenetic clades with marked divergence. In accordance with the reference sequences, these clades clustered within the *R. sanguineus* clade, which is in accordance with a recent analysis from northern Africa (Chitimia-Dobler et al., 2017). The temperate lineage derived from African populations, which colonized the New World during the period of Atlantic slave trade (Burlini et al., 2010). As suggested by in vivo experiments of Labruna et al. (2017), the further spatial establishment of the lineage in South America might be related to their ecological preferences. The pronounced behavioral diapause of the temperate lineage might lead to a better adaptation and higher survival in colder regions, while the lack of diapause of the tropical lineage could be beneficial in tropical climates with less seasonal temperature variations.

In Chile, only the temperate lineage has so far been reported, but the studies included only few samples from the central region (Santiago, Viña del Mar) as well as from Easter Island (Moraes-Filho et al., 2011; Nava et al., 2012). Our study, providing the first comprehensive molecular data over a wide geographical range, proved that both lineages are endemic in Chile and that the genetic differences among and between lineages and haplotypes are similar to those observed in other South American regions (Dantas-Torres et al., 2013; Moraes-Filho et al., 2011; Nava et al., 2012). The tropical lineage was detected in the far north; it was defined by a single haplotype (Temp2), which clustered with a haplotype with a wide distribution in Latin America (Argentina, Brazil, Venezuela, Panama, Costa Rica, and Mexico) (Cicuttin et al., 2015; Dolz et al., 2015; Moraes-Filho et al., 2011). Interestingly, the included sequence of *Rhipicephalus camacasi* from Africa (Kenya) also grouped within this cluster, which is in accordance with a recent analysis from northern Africa (Chitimia-Dobler et al., 2017). The temperate lineage inhabited a wider geographical range and was represented by four haplotypes, indicating a wider genetic diversity. Temp2 showed the widest geographical distribution extending from far north to central Chile; this haplotype was phylogenetically closely related to the haplotype II described by Nava et al. in Argentina (Nava et al., 2012). Temp 3 haplotype was much less prevalent and Temp4 was represented by a single tick specimen, collected from a locality where both lineages co-existed. This haplotype showed the highest divergence from the tropical

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Genbank accession number</th>
<th>Lineage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. sanguineus</em></td>
<td>Chile (Metropolitan Region)</td>
<td>GU553077.1</td>
<td>Temperate</td>
<td>Moraes-Filho et al. (2011)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Argentina (Chaco Province)</td>
<td>KR909456.1</td>
<td>Temperate</td>
<td>Cicuttin et al. (2015)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Argentina (Santa Fé Province)</td>
<td>JX195168.1</td>
<td>Temperate</td>
<td>Nava et al. (2012)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Argentina (Santa Fé Province)</td>
<td>JX195167.1</td>
<td>Temperate</td>
<td>Nava et al. (2012)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Argentina (Formosa Province)</td>
<td>JX206980.1</td>
<td>Tropical</td>
<td>Nava et al. (2012)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Brazil (Northeast)</td>
<td>GU553077.1</td>
<td>Tropical</td>
<td>Moraes-Filho et al. (2011)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>Brazil (Northeast)</td>
<td>GU553075.1</td>
<td>Tropical</td>
<td>Moraes-Filho et al. (2011)</td>
</tr>
<tr>
<td><em>R. camacasi</em></td>
<td>Kenya</td>
<td>KJ746973.1</td>
<td>NA</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>R. turanicus</em></td>
<td>Greece</td>
<td>KC243867.1</td>
<td>NA</td>
<td>Dantas-Torres et al. (2013)</td>
</tr>
<tr>
<td><em>R. turanicus</em></td>
<td>Turkmenistan</td>
<td>KF145150.1</td>
<td>NA</td>
<td>Dantas-Torres et al. (2013)</td>
</tr>
<tr>
<td><em>R. pussillus</em></td>
<td>Spain</td>
<td>KU513962.1</td>
<td>NA</td>
<td>Almeida et al. (2017)</td>
</tr>
<tr>
<td><em>R. rossicus</em></td>
<td>Romania</td>
<td>KY111472.1</td>
<td>NA</td>
<td>Unpublished</td>
</tr>
<tr>
<td><em>R. guilhoni</em></td>
<td>Nigeria</td>
<td>KC243854.1</td>
<td>NA</td>
<td>Dantas-Torres et al. (2013)</td>
</tr>
<tr>
<td><em>R. appendiculatus</em></td>
<td>Unknown</td>
<td>L34301.1</td>
<td>NA</td>
<td>Black and Piesman (1994)</td>
</tr>
</tbody>
</table>

4. Discussion

The genetic diversity of the morphotype *R. sanguineus* has raised controversies on its taxonomic position and systematic relationships, leading to the idea of a complex of various morphologically indistinguishable but genetically distinct lineages and/or species (Dantas-Torres and Otranto, 2015; Nava et al., 2015; Sanches et al., 2016). In South America, a tropical and a temperate lineage have been reported (Moraes-Filho et al., 2011; Nava et al., 2012). Ancestral biogeographic reconstructions of *R. sanguineus* s.l. suggest that the American continent was independently colonized by European and African tick populations (Hekimoğlu et al., 2016). European ancestors of the temperate lineage could have been introduced during the “Colombian exchange”, while the tropical lineage derived from African populations, which colonized the New World during the period of Atlantic slave trade (Burlini et al., 2010). As suggested by in vivo experiments of Labruna et al. (2017), the further spatial establishment of the lineage in South America might be related to their ecological preferences. The pronounced behavioral diapause of the temperate lineage might lead to a better adaptation and higher survival in colder regions, while the lack of diapause of the tropical lineage could be beneficial in tropical climates with less seasonal temperature variations.
Fig. 1. Maximum likelihood tree based on partial sequences of *R. sanguineus* s.l. 16S rDNA gene included in this study. Numbers at internal nodes represent support values from bootstrap based on 1000 replications.
The epidemiology of the different lineages has also important
implications for veterinary medicine. As suggested by experimental studies of Morales-Fló et al. (2015), only the tropical lineage is capable to transmit E. canis. This concept is in concordance with epidemiological findings from Uruguay, Brazil, and northern Argentina (Nava et al., 2012, Cicuttin et al., 2015), but was recently questioned by observations from Buenos Aires, Argentina, where the template lineage is endemic (Cicuttin et al., 2016; Cicuttin et al., 2017). These studies molecularly identified E. canis in 6.7% of dog samples from this region and also from 11 of 32 tick specimens, all belonging to the template lineage. The authors propose three possible explanations for these findings, I) the introduction of E. canis by chronically infected dogs, which acquired the pathogen in other areas; II) the transmission of E. canis within the region by transient populations of the tropical lineage, which yet not have been detected; and III) the fact that the template lineage of this region has a low vector capacity to transmit E. canis.

In Chile, canine infections by E. canis have only been reported in Arica in the far north (López et al., 2012, Dantas-Torres et al., 2008), indicating that the template lineage haplotype in this region (Temp2) is an effective vector of this pathogen.

In conclusion, our study added important epidemiological data on the geographical distribution of R. sanguineus s.l. lineages and haplotypes in northern and central Chile, proving the endemity of both, the tropical and temperate lineages, with a zone of coexistence and potential hybridization at latitudes between 20’S and 22’S. Further studies are necessary to better understand the geoclimatic factors including temperature and altitude that determine the endemicity and coexistence of the different lineages of this important ectoparasite in South America.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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References


