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Original article

Presence of *Borrelia* in different populations of *Ixodes pararicinus* from northwestern Argentina

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ABSTRACT

This work was performed to evaluate the presence of *Borrelia* in different populations of *Ixodes pararicinus* from northwestern Argentina (Jujuy, Salta and Tucumán provinces). Questing adults and nymphs of *I. pararicinus* were collected from vegetation, and *I. pararicinus* nymphs were also collected on birds. Eighty-two ticks were tested for *Borrelia* presence by PCR targeting the gene flagellin and the *rrfA-rrlB* intergenic spacer region. Pools of ticks positive to *Borrelia* were formed by two nymphs collected on *Turdus rufiventris* in Tucumán, one nymph collected on *Syndactyla rufosuperciliata* in Jujuy, one nymph collected on *Turdus nigriceps* in Tucumán, three nymphs collected on *T. nigriceps* in Tucumán, and two females collected from vegetation in Salta. Two haplotypes of *Borrelia* sp. belonging to the *Borrelia burgdorferi* sensu lato complex were found. One of them is closely related to the haplotypes of *Borrelia* genospecies previously reported in *I. aragaoi* from Uruguay (haplotypes D and E) and in *I. pararicinus* from Jujuy Province in Argentina. The second haplotype (detected in the sample of Salta) is closely related to the haplotypes A, B and C associated with *I. aragaoi* from Uruguay. All these results suggest that the presence of *B. burgdorferi* s.l. genospecies in *I. pararicinus* ticks is widespread along the entire distribution of this tick species in northwestern Argentina. However, the *Borrelia* presence in *I. pararicinus* cannot be directly assumed as a phenomenon of medical relevance, because *Ixodes* ticks are not relevant as human parasites in South America, and none of the two *Borrelia* genospecies detected in this work is related to any of the *Borrelia* genospecies currently known to be pathogenic to humans.

1. Introduction

Arthropod-borne spirochaetes are known to cause different human diseases, and many of them are transmitted by ticks (Piesman and Gern, 2008). One of the most relevant group of tick-borne spirochaetes is the *Borrelia burgdorferi* sensu lato complex, which contains at least 20 genospecies (Casjens et al., 2011; Margos et al., 2011; Stanek and Reiter, 2011; Ivanova et al., 2013; Margos et al., 2014). Some of these genospecies, namely *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii*, are the major etiologic agents of Lyme borreliosis, an infectious disease transmitted by ticks to humans in the Holarctic region, although other *Borrelia* genospecies such as *Borrelia spielmanii*, *Borrelia bavariensis*, *Borrelia bissettii*, *Borellia valaisiana* and *Borrelia lusitaniae* have also been involved as etiologic agents of Lyme borreliosis in humans (Steere et al., 2005; Rudenko et al., 2011; Stanek and Reiter, 2011; Stanek et al., 2012). The remaining genospecies of the *B. burgdorferi* s.l. complex are considered of unknown pathogenicity. Most of the spirochaetes belonging to the *B. burgdorferi* s.l. complex are associated with ticks of the genus *Ixodes*, mainly with those species

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belonging to the *Ixodes ricinus* complex, but other ticks not included in the *I. ricinus* complex and non-*Ixodes* tick species were also associated with *B. burgdorferi* s.l. (Steere et al., 2005; Rudenko et al., 2011; Ivanova et al., 2013; Rudenko et al., 2016).

The only reliable records of *B. burgdorferi* s.l. infection in ticks from South America correspond to those achieved in Uruguay, Chile and Argentina (Barbieri et al., 2013; Ivanova et al., 2013; Nava et al., 2014; Sebastian et al., 2016). *Borrelia chilensis* was isolated from *Ixodes stilesi* ticks collected on small rodents and from vegetation (unfed specimens) in Chile (Ivanova et al., 2013). In Uruguay, specimens of *Ixodes aragaoi* (named as *Ixodes pararicinus*, see Onofrio et al., 2014) collected on deer, cattle and from vegetation (unfed specimens) were found to be infected with two new genospecies of *B. burgdorferi* s.l., one phylogenetically related to *B. bissettii* and the other to *Borrelia americana* (Barbieri et al., 2013). Two haplotypes of *B. burgdorferi* s.l. closely related to the haplotypes D and E of *B. burgdorferi* s.l. reported by Barbieri et al. (2013) in *I. aragaoi* from Uruguay, were detected in *I. pararicinus* ticks from Jujuy Province in Argentina (Nava et al., 2014). Finally also in Argentina, a genospecies belonging to the *B. burgdorferi* s.l. complex, denominated *Borrelia* sp. haplotype Patagonia, was detected in *Ixodes neuquenensis* and *Ixodes sigelos* ticks from the Patagonian region, and it was found to be phylogenetically closely related to *B. chilensis* (Sebastian et al., 2016). So far, none of these *Borrelia* genospecies reported from South America have been related to human disease.

In Argentina, *Ixodes pararicinus* is a tick species principally distributed in areas belonging to the Yungas Phytogeographic Province (YPP) sensu Cabrera (1994) (or Selva de las Yungas Ecoregion sensu Burkart et al., 1999) in Salta, Jujuy and Tucumán Provinces (Guglielmone and Nava, 2005). Two adult specimens of this tick were found infected with *B. burgdorferi* s.l. in a locality of Jujuy Province (Nava et al., 2014), but the geographical extent of the *Borrelia* infection in *I. pararicinus* along its geographical distribution in Argentina is unknown. The aim of this work was to evaluate the presence of *B. burgdorferi* s.l. in different populations of *I. pararicinus* distributed through the YPP in northwestern Argentina.

2. Materials and methods

Ixodes pararicinus ticks were collected in different localities which represent the distributional range of this tick species in the northwestern Argentina: I) Villa Monte, Jujuy Province (24°18′S, 64°31′W; 1900 m.a.s.l); II) Dique La Ciénaga, Jujuy Province (24°27′S, 65°15′W; 1200 m.a.s.l); III) Reserva Samay-huasi, Tucumán Province (27°19′S, 65°55′W; 1150 m.a.s.l); IV) El Siambón, Tucumán Province (26°46′S, 65°28′W; 1000 m.a.s.l), V) Parque Nacional El Rey, Salta Province (24°41′S, 64°36′W; 900 m.a.s.l) (Fig. 1). All localities belong to the YPP as defined by Cabrera (1994) or Selva de las Yungas Ecoregion according to Burkart et al. (1999) (Fig. 1). The YPP forms a narrow strip along

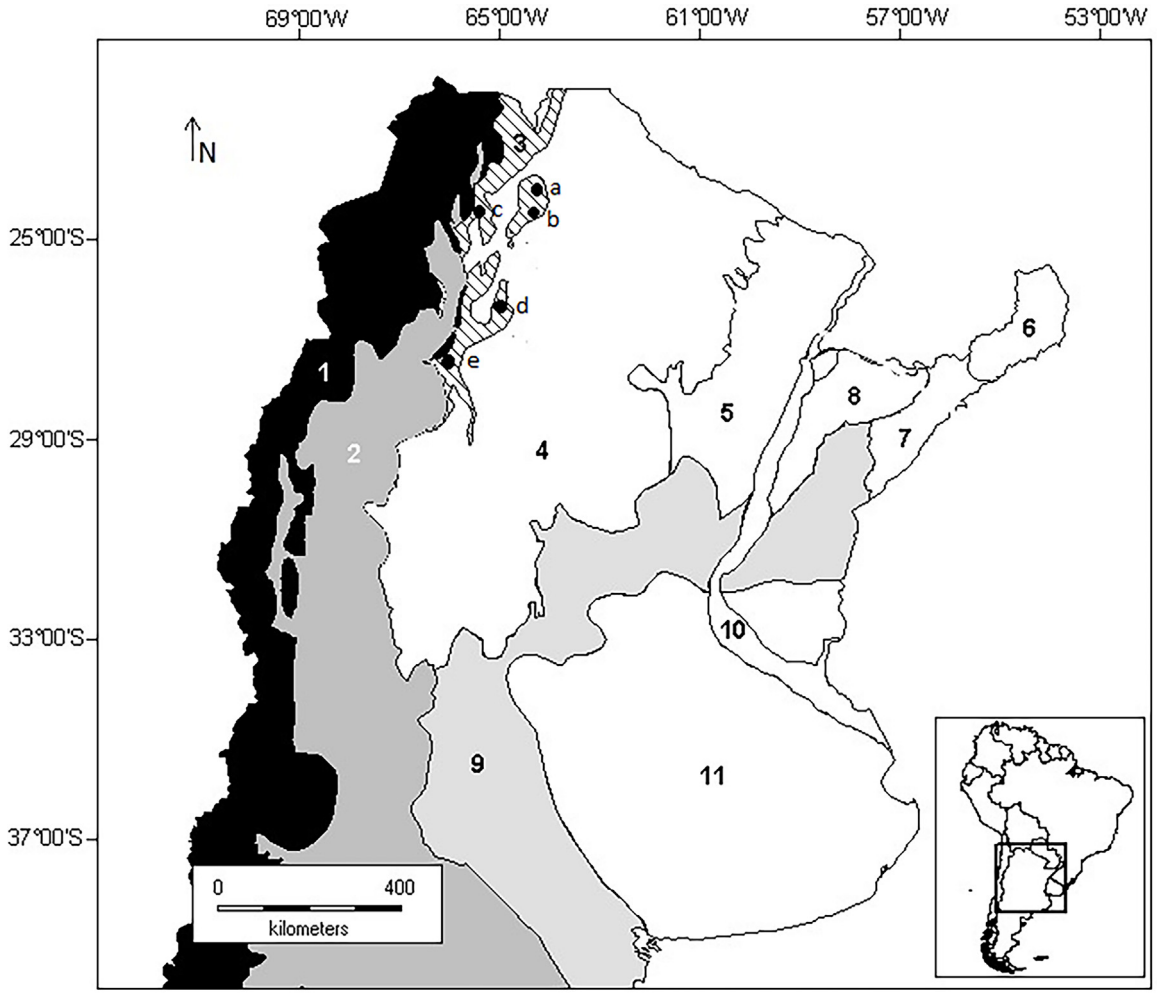

Fig. 1. Argentina ecoregions according to Burkart et al. (1999): (1) Altos Andes, (2) Puna, (3) Selva de las Yungas, (4) Chaco Seco, (5) Chaco Húmedo, (6) Selva Paranaense, (7) Campos y Malezales, (8) Esteros del Iberá, (9) Espinal, (10) Delta e Islas del Paraná, (11) Pampa. a) Villa Monte, Jujuy Province; b) Parque Nacional El Rey, Salta Province; c) Dique La Ciénaga, Jujuy Province; d) El Siambón, Tucumán Province; e) Reserva Samay-huasi, Tucumán Province.

Table 1
Data of the *Ixodes parauricinus* ticks processed for *Borrelia* detection. N: nymph; M: male; F: female.

ID POOLS	Tick species	Locality, Province	Host/Vegetation	Number of ticks
NP1	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	2NN
NP2	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	2MM
NP3	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	1FF
NP4	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	2MM
NP5	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	2NN
NP6	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	2NN
NP7	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	3MM
NP8	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	3FF
NP9	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	4MM
NP10	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	3FF
NP11	*Ixodes parauricinus*	Villa Monte, Jujuy	vegetation	3FF
NP12	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	7NN
NP13	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	1NN
NP14	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	4NN
NP15	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	4NN
NP16	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	2NN
NP17	*Ixodes parauricinus*	Villa Monte, Jujuy	*Syndactyla rufosuperciliata*	1NN
NP18	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus nigriceps*	1NN
NP19	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	1NN
NP20	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus nigriceps*	1NN
NP21	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	6NN
NP22	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus nigriceps*	4NN
NP23	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus nigriceps*	3NN
NP24	*Ixodes parauricinus*	Dique "la Cienaga", Jujuy	*Turdus nigriceps*	1NN
NP25	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	7NN
NP26	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus nigriceps*	2NN
NP27	*Ixodes parauricinus*	Reserva Samay-huasi, Tucuman	*Turdus rufiventris*	4NN
NP28	*Ixodes parauricinus*	El Siambon, Tucuman	vegetation	1NN
NP29	*Ixodes parauricinus*	El Siambon, Tucuman	vegetation	1NN
MP30	*Ixodes parauricinus*	Parque Nacional El Rey, Salta	vegetation	2FF
MP31	*Ixodes parauricinus*	Parque Nacional El Rey, Salta	vegetation	2MM

the eastern slopes of the mountains of northwestern Argentina, in areas with an altitudinal range from 500 to 2500 m.a.s.l. The climate is mountainous subtropical, the annual rainfall (concentrated in spring and summer, from November to March) varies from 900 to 2500 mm according to the locality, and the mean temperature fluctuates from 10 °C in winter to 26 °C in summer.

Questing adults and nymphs of *I. parauricinus* were collected by drag-sampling of the vegetation using a 1 × 1 m large white cloth flag. Nymphs of this tick species were also collected on birds, which were caught with mist-nest and released after examination for ticks around the eyes, neck, beak and ear opening. Details of the samples (tick stage, locality, vegetation/host) are shown in Table 1. Ticks were determined following Keirans et al. (1985) and Venzal et al. (2005), and were also compared with reference material deposited in the tick collection of Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela (INTA Rafaela), Argentina. Because the high morphological similarity with other *Ixodes* species as *I. aragaoi* (see Onofrio et al., 2014), sequences of a 410-bp fragment of the mitochondrial 16S rRNA gene were obtained from representative specimens to confirm the taxonomic determination with morphological characters. DNA was extracted and processed using a polymerase chain reaction (PCR) following the methodology described by Mangold et al. (1998a,b). Phylogenetic relationship among *Ixodes* species from the New World was analyzed using neighbour-joining distance (NJ) method. The tree was generated from the Tamura-Nei model with the program Mega version 4.0 (Tamura et al., 2011), gaps were excluded in the pairwise comparison, and support for the NJ topology was tested by bootstrapping over 1000 replications. Birds were determined by one of the authors (LGC) by using the guide of Narosky and Yzurieta (2003). Birds were caught with permissions of Administración de Parques Nacionales (number 55/08).

Ticks were processed for DNA extraction and screened by PCR for detection of *Borrelia* DNA following the methods detailed in Barbieri et al. (2013). Briefly, nested PCR was performed targeting the flagellin gene (*fla*) of *Borrelia* spp. with the primers presented by Barbour et al. (1996). The primers Fla LL (5′-ACA TAT TCAGAT GCA GAC AGA GGT-3′) and Fla RL (5′-GCA ATC ATA GCC ATTGCA GAT TGT-3′) were used in the first reaction, and the primers Fla LS (5′-AAC AGC TGA AGA GCT TGG AAT G-3′) and Fla RS (5′-CTT TGATCA CTT TC ATT CTA ATA GC-3′) were employed in the second reaction. Additionally, positive samples were further used to amplify a 225- to 255-bp fragment of the rrfA-rrlB intergenic spacer region (IGS) with the primers IGSb (5′-GTT AAG CTC TTA TTC GCT GAT GGT A-3′) and IGSa (5′-CGA CCT TCT TCG CCT TAA AGC-3′) (Derdáková et al., 2003). Nuclease free water was used as negative control and DNA of *Borrelia anserina* served as positive control in the PCR reactions. All positive samples were purified and afterwards sequenced using the appropriate primers. 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 (Thompson et al., 1994). Phylogenetic analyses were carried out with the maximum-likelihood (ML) method. 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 tree based on *Borrelia fla* partial 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. The number of variable nucleotide positions between *fla* and IGS sequences were used to calculate pairwise estimates of percent sequence divergence among sequences of *Borrelia* spp. found in different areas of the world. Gaps were excluded from the pairwise distance estimation.

3. Results and discussion

Eighty-two *I. parauricinus* ticks belonging to five different populations were analyzed to detect DNA of *Borrelia*. The morphological determination of ticks was confirmed by the phylogenetic analysis

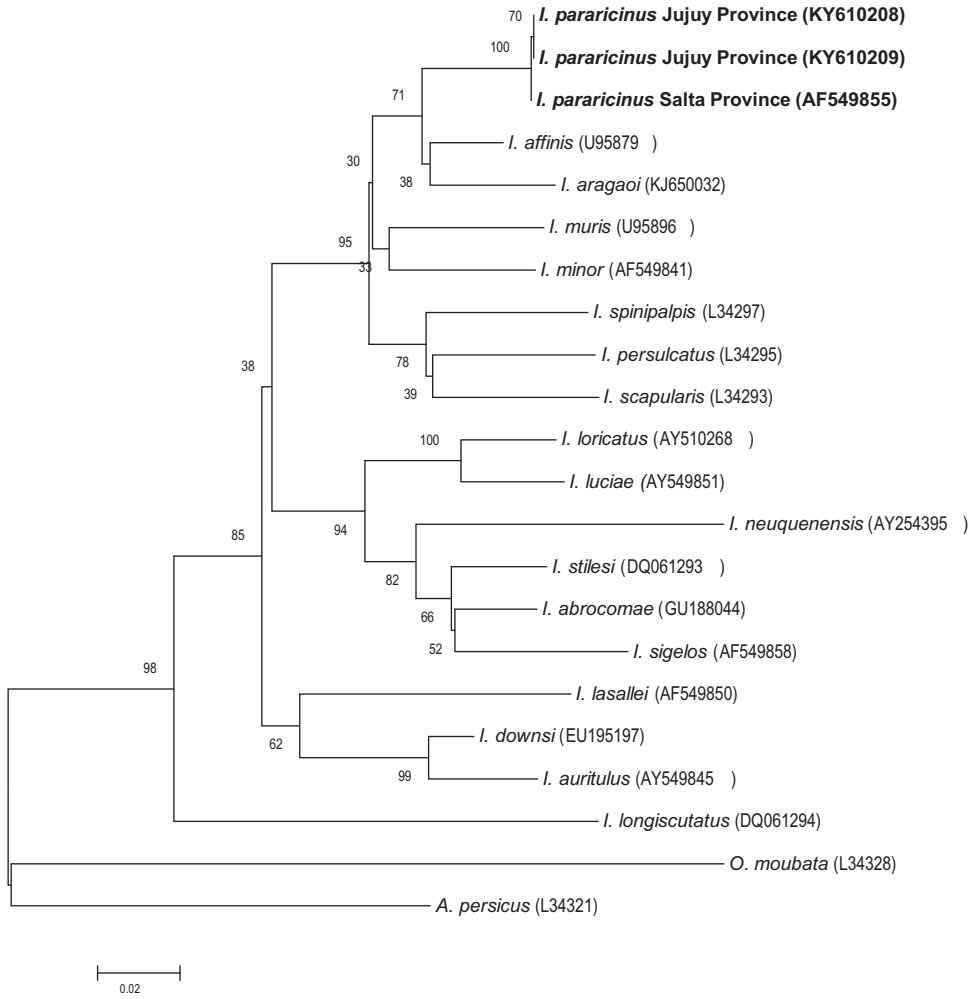

Fig. 2. Neighbour-joining tree constructed with mitochondrial 16S rDNA sequences of *Ixodes* species. Numbers on the branches represent bootstrap support and codes following each species name correspond to GenBank accession numbers.

of 16S rDNA sequences of representative specimens of *I. pararicinus* from Jujuy and Salta Provinces (Fig. 2). *Ixodes pararicinus* ticks were grouped in 31 pools (Table 1). Five of these 31 pools were found to be positive to *Borrelia* after the screening test performed with the PCR targeting the flagellin gene (*fla*). The positive samples corresponded to the following pools: NP16 (two nymphs collected on *Turdus rufiventris* (rufous-bellied) in Reserva Samay-huasi, Tucumán; GenBank accession number: KY595464), NP17 (one nymph collected on *Syndactyla rufosuperciliata* (buff-browed foliage-gleaner) in Villa Monte, Jujuy; GenBank accession number: KY595465), NP20 (one nymph collected on *Turdus nigriceps* (Andean slaty-thrush) in Reserva Samay-huasi, Tucumán; GenBank accession number: KY595466), NP23 (three nymphs collected on *T. nigriceps* in Reserva Samay-huasi, Tucumán; GenBank accession number: KY595467) and MP 30 (two females collected from vegetation in Parque Nacional El Rey, Salta; GenBank accession number: KY595468) (see Table 1).

The ML phylogenetic tree generated with *fla* sequences shows that all *Borrelia* haplotypes found in this work belong to the *B. burgdorferi* s.l. complex (Fig. 3). The haplotypes corresponding to the samples NP16, NP17, NP20 and NP23 were closely related to the haplotypes D and E of the *Borrelia* genospecies reported in *I. aragaoi* (named as *I. pararicinus;* see Onofrio et al., 2014) from Uruguay (Barbieri et al., 2013) and to the haplotypes I and II of the same *Borrelia* genospecies previously detected in *I. pararicinus* ticks from Jujuy Province in Argentina (Nava et al., 2014) (Fig. 3). The divergence of *fla* sequences within this group ranged from 0 to 2.0%. The analysis of IGS sequences has confirmed these results since the genetic divergence between the four IGS sequences of the *Borrelia* haplotypes detected in the samples from Samay-huasi (n: 3; GenBank accession numbers: submitted) and Villa Monte (n: 1; GenBank accession number: submitted) and the haplotype previously detected in *I. pararicinus* from Jujuy by Nava et al. (2014) ranged only from 0 to 1%. Also, the phylogenetic analysis of *fla* sequences (Fig. 3) has shown that the *Borrelia* haplotype detected in the pool of two *I. pararicinus* females from Parque Nacional El Rey in Salta Province (GenBank accession number: submitted) is evolutionary related to the *Borrelia* haplotypes A, B and C associated with *I. aragaoi* (named as *I. pararicinus*) from Uruguay (see Barbieri et al., 2013). The genetic divergence in the *fla* sequences among these haplotypes varied from 0.3 to 0.9%. Unfortunately, no IGS sequence of this sample from Parque Nacional El Rey was obtained.

The results herein presented together with those previously obtained by Nava et al. (2014) suggest that the presence of *Borrelia* in *I. pararicinus* ticks (fed and unfed specimens) is widespread along the entire distribution of this tick species in the YPP of Argentina. Therefore, it can be concluded that at least two genospecies belonging to the *B. burgdorferi* s.l. complex are associated to *I. pararcinus* in the YPP of Argentina. The same two genospecies were originally described infecting *I. aragaoi* ticks from Uruguay (Barbieri et al., 2013). This strict coincidence in the pattern of *Borrelia* infection between *I. aragaoi* and *I. pararicinus* is not unexpected because

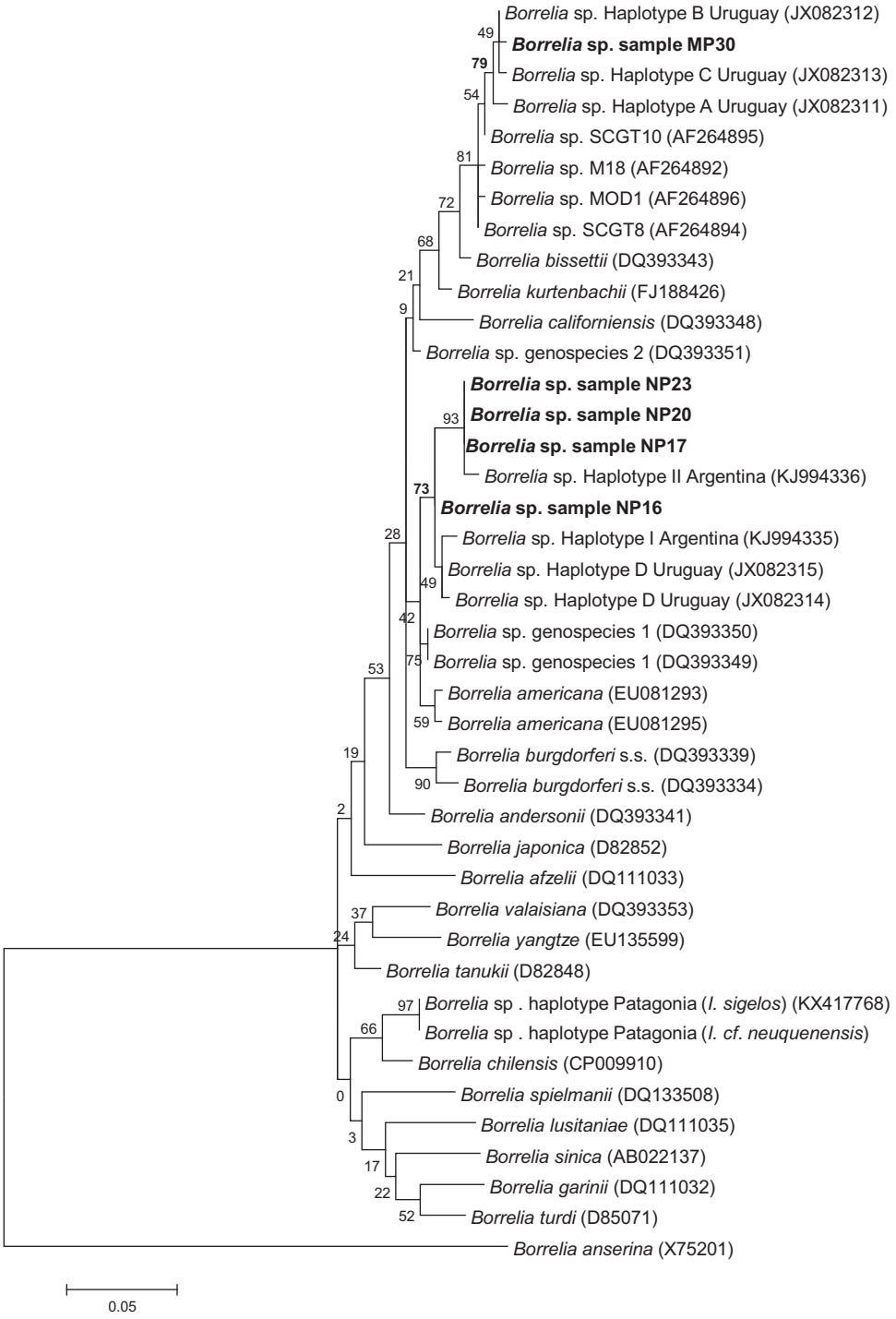

Fig. 3. Maximum-likelihood tree constructed from *Borrelia fla* partial sequences. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets.

they are two tick species very closely related from an evolutionary perspective (Venzal et al., 2005; Onofrio et al., 2014). But here, however, it is important to realize that the *Borrelia* infection in *I. pararicinus* and *I. aragaoi* ticks cannot be directly assumed as a phenomenon of medical relevance. First, none of the two *Borrelia* genospecies detected in *I. pararicinus* and *I. aragaoi* is related to any of the *Borrelia* genospecies currently known to be pathogenic to humans (see Fig. 3). Second, unlike the situation in the Holarctic region, the species of the genus *Ixodes* are not relevant as human parasites in South American countries (Guglielmone et al., 2006, 2014). Considering as a whole, the evidence presented by Barbieri et al. (2013), Ivanova et al. (2013). Nava et al. (2014), Sebastian et al. (2016) and in the current work allow to conclude that, although the presence of *B. burgdorferi* s.l. in species of the genus *Ixodes* from the Southern Cone of America appears to be more common than previously suspected, the probability of transmission of these spirochaetes to humans by *Ixodes* ticks in this region of the world is low. But of course much more investigations are needed to understand more profoundly the eco-epidemiology of the genospecies belonging to the *B. burgdorferi* s.l. complex in South America.

To date, the *I. pararicinus* ticks in which *Borrelia* haplotypes were detected corresponded to semi-engorged nymphs collected

on birds and unfed adults collected on vegetation (Nava et al., 2014; this study). The *Borrelia*-positive samples from Jujuy and Tucumán Provinces were formed by nymphs of *I. pararicinus* collected on the bird species *T. rufiventris, T. nigriceps* and *S. rufosuperciliata.* The role of birds, including those belonging to the genus *Turdus*, as reservoirs of *Borrelia* spp. and in the spread of infected ticks is well documented (Anderson et al., 1990; Hamer et al., 2012; Scott et al., 2012; Rudenko et al., 2014; Scott and Foley, 2016). Considering that birds are relevant hosts for the immature stages of *I. pararicinus* (Flores et al., 2014), it can be suggested that birds play a key role in the enzootic cycle of *B. burgdorferi* s.l. in Argentina, but further investigations are needed to understand in a proper way the ecology of tick-associated spirochaetes of the genus *Borrelia* in South America.

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