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A relapsing fever *Borrelia* and spotted fever *Rickettsia* in ticks from an Andean valley, central Chile

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Abstract

In humans, emerging infectious diseases are mostly zoonoses with ticks playing an important role as vectors. Tick-borne relapsing fever *Borrelia* and spotted fever *Rickettsia* occur in endemic foci along tropical and subtropical regions of the globe. However, both are widely neglected etiologic agents. In this study, we performed molecular analyses in order to assess the presence of *Borrelia* and *Rickettsia* DNA in ticks infesting small-mammals within a National Reserve located in the Andes Mountains, central Chile. While hard ticks were negative for the presence of both agents, sequences of four rickettsial (*gltA*, *htrA*, *ompA*, *ompB*) and two borrelial (16S rRNA and *flaB*) genes were obtained from larvae of an *Ornithodoros* sp. morphologically related with *Ornithodoros atacamensis*. Phylogenetic analyses indicated that the detected *Borrelia* and *Rickettsia* spp. belong to the relapsing fever and spotted fever groups, respectively. Moreover, the agents formed monophyletic clades with *Rickettsia amblyommatis* and "*Candidatus* Borrelia johnsonii." As positive ticks parasitize rodents within a highly visited National Reserve where outdoor activities are of common practice, the risk for human parasitism should not be discarded.

Keywords Tick-borne diseases · *Ornithodoros* · "*Candidatus* Borrelia johnsonii" · *Rickettsia amblyommatis* · Chile

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Introduction

Ticks are common parasites of terrestrial vertebrates (Sonenshine and Roe 2014) and harbor the greatest plethora of microorganisms in terms of diversity (Jongejan and Uilenberg 2004). As bloodsucking arthropods, ticks can transmit bacteria to their hosts and eventually to humans (Jongejan and Uilenberg 2004). Current research on tick-borne diseases has led to the discovery of pathogenic agents previously unrecognized for a determined region (Pritt et al. 2016; Muñoz-Leal et al. 2018; Kingry et al. 2018). As a result, an ongoing increase in the specific diversity of pathogens associated with ticks is now emerging (Parola et al. 2013; Cutler et al. 2017), and new areas of infection risk in nature have been identified.

Relapsing fever borreliae (RFB) are actively motile spirochetes that maintain chronic infections in ticks and vertebrates parasitized by these arthropods (Cutler 2015). RFB have distinct lineages: one associated with the Ixodidae (i.e. genera *Amblyomma*, *Bothriocroton*, *Hyalomma*, *Ixodes*, *Rhipicephalus*), another to the Argasidae (i.e. *Argas*, *Ornithodoros*), and a single species to the human body louse (Barbour and Schwan 2018). Particularly in South America, only one species, *Borrelia venezuelensis,* has been associated with human relapsing fever (Faccini-Martínez et al. 2018). However, RFB of unknown pathogenic roles have been detected in further South American *Ornithodoros* (Davis 1952; Marinkelle and Grose 1968; Parola et al. 2011; Muñoz-Leal et al. 2019), suggesting that these ticks constitute understudied reservoirs.

Bacteria of the genus *Rickettsia* are obligatory intracellular coccobacilli that infect soft ticks (Argasidae), hard ticks (Ixodidae), and other terrestrial and aquatic invertebrates (Weinert et al. 2009). Human-pathogenic rickettsiae belong to the spotted fever group and have been detected almost exclusively in hard ticks (Parola et al. 2013). Notwithstanding, *Rickettsia rickettsii*, the most pathogenic species of the group (Parola et al. 2013), was once isolated from *Otobius lagophilus* and *Ornithodoros nicollei* (Silva-Goytia and Elizondo 1952), showing that soft ticks do harbor spotted fever rickettsiae (SFR) in nature.

RFB and SFR are recognized as emerging agents globally (Parola et al. 2013; Cutler et al. 2017). While some tick species are well-known reservoirs and hold endemic foci of pathogenic strains in certain ecosystems (Parola et al. 2013; Cutler et al. 2017), RFB and SFR are still widely neglected (Chikeka and Dumler 2015). A risk to acquire both diseases relies on the exposure to the milieu where infected ticks occur in nature.

Along its territory, Chile holds large preserved areas proper for outdoor activities, yet the role of autochthonous ticks as vectors of RFB or SFR remains almost unstudied. The purpose of this study was to assess the presence of *Borrelia* and *Rickettsia* in ticks collected on wild vertebrates in a National Reserve emplaced along an Andean valley of central Chile.

Materials and methods

Site of study, capture of vertebrates and collection of ticks

Between 19 and 26 February 2018 (austral summer), we captured small-mammals and birds in order to collect ticks engorging on them in three areas emplaced at an average altitude of 1072 m within "Río Los Cipreses National Reserve" (RCNR), located in the higher

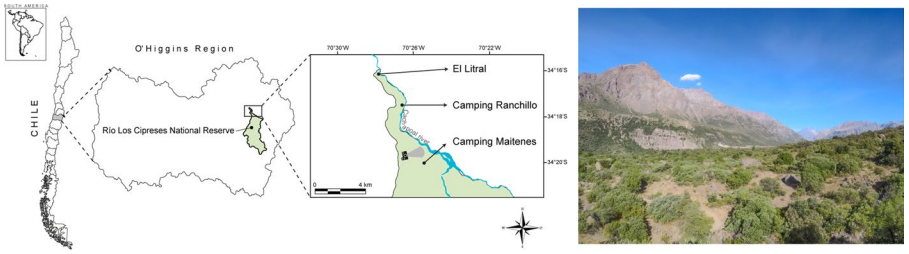

Fig. 1 Map showing the points of small-mammal and bird captures within Río Los Cipreses National Reserve (RCNR): El Litral, 34°16′19″S, 70°27′35″W, elevation 963 m; Camping Ranchillo 34°18′09″S, 70°26′56″W, elevation 1124 m; Camping Maitenes 34°20′10″S, 70°24′16″W, elevation 1130 m. The right-hand photograph was taken from the point and orientation given in the map (camera draw), and exemplifies the typical vegetation and topography of the ecosystem at sampling sites

basin of the Cachapoal River, Andes Mountains, central Chile (Fig. 1). We chose RCNR because it represents a highly visited protected area: ~28,000 visitors annually between the years 2015–2017 (http://www.conaf.cl/parques-nacionales/visitanos/estadisticas-de-visitacion/). RCNR was created to preserve a typical valley of central Chilean Andes, in which ecosystems are shaped by a temperate climate with dry and hot summers (December to March), and rainy winters (June to September) (Millán and Peña 2000). Small-mammals and birds were captured using 80 Sherman-like traps during five nights and three mist nets for 6 days, respectively. Traps were baited with oat and vanilla extract. While birds were manually restrained, small-mammals were weighed using a 300 g Pesola scale, and then anesthetized with an intraperitoneal injection of a ketamine (60 mg/kg)-xylazine (3 mg/kg) solution (Carpenter and Marion 2017). Ticks were visually screened upon hosts' skins, manually detached with tweezers and stored in 100% ethanol vials. Animals were identified based on morphology using taxonomic guides and then released in the site of capture.

Identification of ticks, DNA extraction and sequencing

All ticks were identified to the genus level using morphological keys (Nava et al. 2017) complemented with original descriptions of Chilean species (Keirans et al. 1976; Muñoz-Leal et al. 2016). For hard ticks, taxonomically relevant characters were photographed with the software ZEN Pro 2 implemented in a Stereo Discovery V12 stereomicroscope (Carl Zeiss, Munich, Germany). On the other hand, collected soft ticks were clarified in 30% KOH (w/v), hydrated in distilled water and mounted on slides using Hoyer's medium. A detailed observation of slide-mounted specimens was performed through optical microscopy (BX40 microscope, Olympus, Tokyo, Japan), and photographs were taken with an Olympus DP70A camera. The edition of micrographs was made with the software Image-Plus Pro v5.1.

After morphological identification, pools and individual tick specimens were submitted to DNA extraction using the guanidine isothiocyanate and phenol/chloroform protocol (Sangioni et al. 2005). Depending on the size of each tick, the final pelleted DNA was eluted in 10–30 µl of Tris EDTA buffer solution (TE) and stored at − 20 °C until tested. To check a successful DNA extraction and genetically identify collected specimens, a conventional polymerase chain reaction (PCR) using primers 5′-CCGGTCTGAACTCAGATCAAGT-3′ (forward) and 5′-GCTCAATGATTTTTTAAATTGCTG-3′ (reverse) targeting

a ≈ 460-bp fragment of the tick mitochondrial 16S rRNA gene was implemented following Mangold et al. (1998). PCR products were resolved in 1.5% agarose gels stained with SybrSafe (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA, USA) and results captured by imaging (AlphaImager HP, ProteinSimple). Amplicons of expected size were treated with ExoSAP-IT (Affimetryx/Thermo Fisher Scientific, Santa Clara, CA), prepared for sequencing with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and sequenced in an ABI 3500 automatic device (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA). Obtained sequences were de novo assembled and trimmed with Geneious R9 (Kearse et al. 2012). Edited sequences were submitted to BLASTn analyses (www.ncbi.nlm.nih.gov/blast) in order to infer genetic similarities with congeneric organisms available in GenBank (Altschul et al. 1990).

Borrelia and *Rickettsia* PCR

For determining the presence of *Borrelia* and *Rickettsia* DNA, two initial PCRs were performed: (1) a nested reaction targeting the *Borrelia flaB* gene, and (2) a conventional reaction for amplifying a fragment of the *Rickettsia gltA* gene. Positive samples were then submitted to a battery of conventional and semi-nested protocols to amplify *Borrelia flaB* and 16S rRNA, and *Rickettsia gltA*, *htrA*, *ompA,* and *ompB* genes. References for thermal conditions and primers employed in each protocol are listed in Table 1. Conventional PCRs were performed using a mix of 25 µl, composed by 12.5 µl of DreamTaq Green PCR master mix (2X, Thermo Scientific, Baltics UAB, Vilnius, Lithuania), 1 µl of each primer (10 pmol/µl), 8 µl of ultrapure water and 2.5 µl of template DNA. For nested and semi-nested rounds, we employed 1 µl of the first reaction product and 9.5 µl of ultrapure water. *Borrelia anserina* strain PB (Ataliba et al. 2007) and *Rickettsia vini* strain Breclav (Nováková et al. 2016). Ultrapure water was employed in negative controls for each reaction. Mix preparation, pipetting of nested reactions, and electrophoresis were performed in separated rooms. Sequencing and further analyses of the obtained sequences were performed as above.

Phylogenetic analyses

Independent alignments using obtained and GenBank-retrieved sequences were constructed for each sequenced gene using CLUSTAL W (Thompson et al. 1994) implemented MEGA 5 (Tamura et al. 2011), and manually adjusted with GeneDoc (Nicholas et al. 1997). Two phylogenetic analyses were inferred with obtained sequences of tick 16S mitochondrial rDNA, one for soft ticks, joining 65 sequences, and another for hard ticks including 48 sequences. *Ixodes uriae* (AB030017) and *Ixodes holocyclus* (AB051084) were used as outgroups for the Argasidae tree, and *Argas persicus* (AF001402) rooted the Ixodidae tree. Nucleotide substitution models for all phylogenetic analyses were calculated with MEGA 5 (Tamura et al. 2011). Each phylogeny was inferred by two methods. A maximum parsimony (MP) analysis was implemented in PAUP 4.0b10 (Swofford 2002) with 500 bootstrap replicates, random stepwise addition of starting trees (with random addition of sequences), and TBR branch swapping. Then, a bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with four independent Markov chain runs for 5,000,000 metropolis-coupled MCMC generations, sampling a tree every 100 generations. The first 25% of the trees represented burn-in, and the remaining trees

Table 1 Primers used to amplify *Borrelia* (*flaB*, 16S rRNA) and *Rickettsia* (*gltA, htrA, ompA, ompB*) genes

Targeted gene	Primer name	Sequence (5′ to 3′)	References	PCR protocol (primer forward/primer reverse): amplicon size
flaB	FLA LL[a]	ACATATTCAGATGCAGACAGAGGT	Stromdahl et al. (2003)	1st round PCR: FlaRL/FlaLL; 665 bp
	FLA RL[a]	GCAATCATAGCCATTGCAGATTGT	Stromdahl et al. (2003)	2nd round nested: FlaRS/FlaLS; 354 bp
	FLA LS[a]	AACAGCTGAAGAGCTTGGAATG	Stromdahl et al. (2003)	
	FLA RS[a]	CTTTGATCACTTATCATTCTAATAGC	Stromdahl et al. (2003)	
	Fla-ans5′	TGTGATATCCTTTTAAAGAGACAAATGG	Schwan et al. (2005)	1st round PCR: Fla-ans5′/Btur-fla3′; 1246 bp
	Btur-fla3′	GGTTCCTGACTTTAACACTAGCC	Schwan et al. (2005)	2nd round semi-nested: 1Fla-ans5′/Fla-1B; 633 bp
	Fla + 1A	AGAGCTTGGAATGCAACCTG	Schwan et al. (2005)	2nd round semi-nested: Fla + 1A/Btur-fla3′; 799 bp
	Fla–1B	TGCTTCATCCTGATTTGC	Schwan et al. (2005)	
16S rRNA	FD3	AGAGTTTGATCCTGGCTTAG	Ras et al. (1996)	1st round PCR: FD3/T50; 1489 bp
	T50	GTTACGACTTCACCCTCCT	Ras et al. (1996)	2nd round semi-nested: FD3/16s-1; 730 bp
	Rec4	ATGCTAGAAACTGCATGA	Ras et al. (1996)	2nd round semi-nested: 16s-2/T50; 550 bp
	Rec9	TCGTCTGAGTCCCCATCT	Ras et al. (1996)	2nd round nested: Rec4/Rec9; 519 bp
	16s-1	TAGAAGTTCGCCTTCGCCTCTG	Schwan et al. (2005)	
	16s-2	TACAGGTGCTGCATGGTTGTCG	Schwan et al. (2005)	
gltA	CS-78[a]	GCAAGTATCGGTGAGGATGTAAT	Labruna et al. (2004a, b)	PCR: CS-78/CS-323; 401 bp
	CS-323[a]	GCTTCCTTAAAATTCAATAAATCAGGAT	Labruna et al. (2004a, b)	PCR: CS-239/CS-1069; 830 bp
	CS-239	GCTCTTCTCATCCTATGGCTATTAT	Labruna et al. (2004a, b)	
	CS-1069	CAGGGTCTTCGTGCATTTCTT	Labruna et al. (2004a, b)	
htrA	17k-5	GCTTTACAAAATTCTAAAAACCATATA	Labruna et al. (2004b)	PCR: 17k-5/17kD2; 526 bp
	17kD2	CATTGTTCGTCAGGTTGGCG	Webb et al. (1990)	
ompA	*Rr*190.70p	ATGGCGAATATTTCTCCAAAA	Regnery et al. (1991)	PCR: *Rr*190.70p/190.701; 617 bp
	190.701	GTTCCGTTAATGGCAGCATCT	Roux et al. (1996)	
ompB	120.M59	CCGCAGGGTTGGTAACTGC	Roux and Raoult (2000)	PCR: 120.M59/120.807; 862 bp
	120.807	CCTTTTAGATTACCGCCTAA	Roux and Raoult (2000)	

[a]Primers used in the initial PCR screening

were used to calculate bayesian posterior probability values. GenBank accession numbers of sequences used for tick phylogeny are embedded in the tree.

Alignments of *Borrelia* 16S rRNA and *flaB* genes were constructed including sequences for 29 strains of the genus. Alignments of *gltA, htrA, ompA,* and *ompB* genes joined other 35 homologous sequences belonging to 28 *Rickettsia* spp. Borrelial and rickettsial genes were manually concatenated in the order 16S rRNA-*flaB* and *gltA*-*htrA*-*ompA*-*ompB*, respectively. Resulting matrixes were submitted to a bayesian and maximum likelihood (ML) phylogenetic analyses using the general time reversible (GTR) substitution model and a gamma variation rate between sites. The bayesian phylogeny for both agents was implemented as above stated. The ML tree was constructed with PhyML (Guindon and Gascuel 2003), with five substitution rate categories and 1000 bootstrap replicates. The nearest neighbour interchange (NNI) distance was used to improve the tree topology (Li et al. 1996). *Borrelia coriaceae* Co53 (CP005745) and *Rickettsia canadensis* McKiel (CP000409) rooted each tree, respectively. GenBank accession numbers for *Borrelia* and *Rickettsia* sequences used to construct both phylogenies are specified in Suppl. 1.

Results

Capture of vertebrates, collection and identification of ticks

A total of 46 animals (23 birds and 23 small-mammals) were captured totalizing a capture effort of 49 h for birds and 84 h for small-mammals. The numbers of captured specimens (birds–small-mammals) per each site were as follows: 0–5 at El Litral, 9–3 at Camping Ranchillo and 14–15 at Camping Maitenes. All birds were negative to tick infestation. A total of seven larvae and 12 nymphs of the Ixodidae family and eight larvae of the Argasidae family were collected upon rodents (Table 2).

All nymphs and larvae of the Ixodidae family were initially identified as *Ixodes* by the presence of an anal groove curved anteriorly to the anus. Nymphal characters observed through optical microscopy unveiled that these ticks matched the morphology of *Ixodes sigelos* (Keirans et al. 1976; Guglielmone et al. 2005) (Fig. 2a–d). All four slide-mounted argasid larvae were determined as an unidentified *Ornithodoros* sp. by sharing the following traits: dorsal surface provided with 18 pairs of setae, seven anterolateral, five central (one specimen with six central) and six posterolateral pairs; dorsal plate pyriform in shape; hypostome dental formula: 3/3 from the apex until one-fourth of its total length, then 2/2 towards the base; first, second and third files provided with 22, 20 and 8 denticles respectively (Fig. 2e–h). One nymph collected on *Octodon bridgesi* at Camping Ranchillo, one larva collected on *Abrothrix olivaceus* and four slide-mounted larvae collected on *Phyllotis darwini* at Camping Maitenes were deposited in the tick collection "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva" under CNC-3838 and -3839.

All 20 ticks submitted to DNA extraction were positive to mitochondrial 16S rRNA gene PCR. Obtained sequences confirmed morphological diagnoses of hard ticks partially since a single haplotype of 407 bp was only 97.05% (395/407 bp) identical with *I. sigelos* from Argentina (HM014413, Sanchez et al. 2010). Due to this percentage of similarity, we opted to treat our species as an *Ixodes* sp. belonging to the *I. sigelos* group. On the other hand, *Ornithodoros* larvae yielded a trimmed sequence 94.96% (377/397 bp) identical with *Ornithodoros atacamensis*, a parasite of lizards in the Atacama Desert of northern Chile (Muñoz-Leal et al. 2016). Sequences of ticks obtained in this study were deposited

Table 2 Captures of birds and small-mammals per locality and date

Species	n	Collected ticks	Local	Date of capture
Aves				
Anairetes parulus	1	–	Camping Ranchillo	22/02/18
	2	–	Camping Maitenes	24/02/18
	1	–	Camping Maitenes	25/02/18
Asthenes humicola	1	–	Camping Maitenes	23/02/18
Columbina picui	1	–	Camping Maitenes	25/02/18
Curaeus curaeus	1	–	Camping Maitenes	25/02/18
	2	–	Camping Maitenes	26/02/18
Diuca diuca	1	–	Camping Ranchillo	20/02/18
	1	–	Camping Ranchillo	21/02/18
	1	–	Camping Maitenes	23/02/18
Elaenia albiceps	1	–	Camping Ranchillo	20/02/18
	1	–	Camping Ranchillo	21/02/18
	1	–	Camping Maitenes	23/02/18
	1	–	Camping Maitenes	26/02/18
Mimus thenca	1	–	Camping Ranchillo	20/02/18
	1	–	Camping Ranchillo	21/02/18
Molothrus bonariensis	1	–	Camping Ranchillo	21/02/18
Sephanoides sephaniodes	1	–	Camping Maitenes	26/02/18
Turdus falcklandii	1	–	Camping Ranchillo	21/02/18
	2	–	Camping Maitenes	25/02/18
Mammalia (Marsupialia)				
Thylamys elegans	1	–	El Litral	20/02/18
	1	–	El Litral	21/02/18
	2	–	Camping Maitenes	23/02/18
	2	–	Camping Maitenes	24/02/18
	1	–	Camping Maitenes	25/02/18
Mammalia (Rodentia)				
Abrothirx olivaceus	2	–	El Litral	20/02/18
	1	–	Camping Ranchillo	21/02/18
	1	1L *Ixodes* sp.	Camping Ranchillo	21/02/18
	1	–	Camping Maitenes	22/02/18
	1	–	Camping Maitenes	23/02/18
Octodon bridgesi	1	7 N *Ixodes* sp.	Camping Maitenes	22/02/18
	1	1 N *Ixodes* sp.	Camping Maitenes	24/02/18
Phyllotis darwini	1	6L, 2 N *Ixodes* sp.	Camping Maitenes	24/02/18
	1	8L *Ornithodoros* sp.	Camping Maitenes	25/02/18
Rattus norvegicus	1	–	El Litral	20/02/18
	1	–	Camping Ranchillo	21/02/18
Rattus norvegicus	1	1 N *Ixodes* sp.	Camping Maitenes	22/02/18
	1	1 N *Ixodes* sp.	Camping Maitenes	23/02/18
	2	–	Camping Maitenes	25/02/18

Species positive to tick infestation are specified

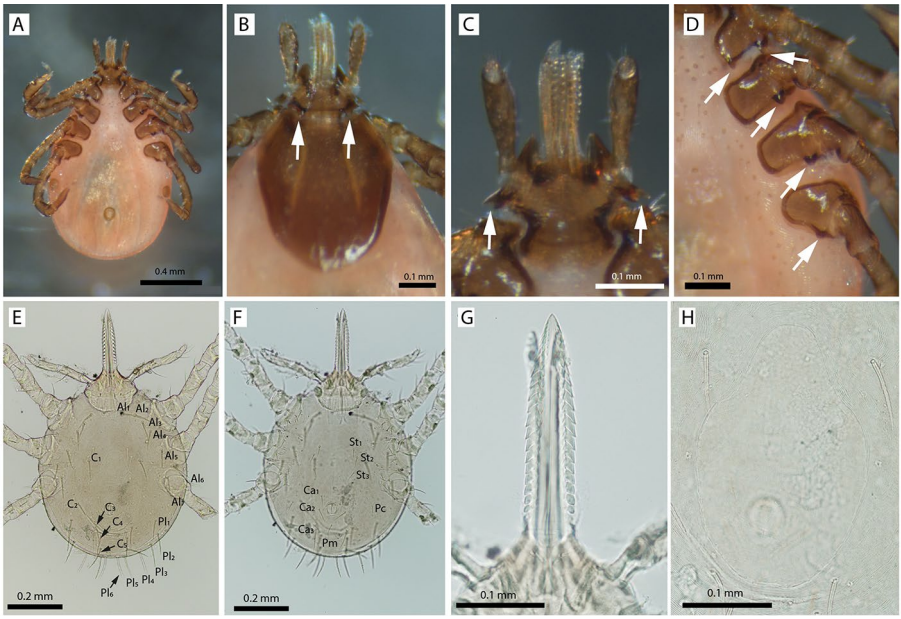

Fig. 2 Micrographs of the *Ixodes* sp. nymph and the larva of *Ornithodoros* sp. collected in this study. Nymph of *Ixodes* sp.: **a** ventral view; **b** dorsal capitulum: wing shaped lateral protuberances (arrowed) and scutum; **c** ventral capitulum: posterior processes in palpal article I (arrowed); **d** coxae I, II, III and IV: note the number of spurs (arrowed) in each coxa. Larva of *Ornithodoros* sp.: **e** dorsal view (number of setal pairs indicated); **f** ventral view (number of setal pairs indicated); **g** hypostome; **h** dorsal plate. *Al* anterolateral, *C* central, *Ca* circumanal, *Pl* posterolateral, *Pm* posteromedian, *Pl* posterolateral

in GenBank under accession numbers MK110659 (*Ornithodoros* sp. Cachapoal) and MK110660 (*Ixodes* sp. *sigelos* group Cachapoal).

Borrelia and *Rickettsia* PCR

While ten nymphs and six larvae of an *Ixodes* sp. of the *I. sigelos* group, and four larvae of an *Ornithodoros* sp. collected from different hosts were screened to detect *Borrelia* and *Rickettsia* DNA, only soft ticks yielded positive amplicons (Table 3). While trimmed sequences for *Borrelia* 16S rRNA and *flaB* genes resulted into fragments of 1272 bp and 1005 bp each, *Rickettsia gtlA*, *htrA*, *ompA*, *ompB* genes yielded partial fragments of 992 bp, 479 bp, 587 bp, and 811 bp, respectively.

After BLASTn comparisons, sequences of *Borrelia* obtained in the current study were the most identical with "*Candidatus* Borrelia johnsonii" strains IA-1 and 15-3581 as follows: 16S rRNA gene 99.92% identical (1271/1272 bp, 0 gaps) to both strains (EU492388, MF062083); *flaB* gene 99.80% identical (1000/1002 bp, 0 gaps) to strain IA-1 (EU492387), and 100% identical (980/980 bp, 0 gaps) to strain 15-3581 (MF062084). Sequences of *Rickettsia* obtained in this study showed the highest identity percentages with two rickettsiae of the spotted fever group. On the one hand, *gltA* and *htrA* genes were 99.59% (988/992 bp, 0 gaps) and 99.37% (476/479 bp, 0 gaps) identical with homologous sequences of *Rickettsia raoultii* strain IM16 (CP019435). On the other hand, *ompA* and *ompB* sequences were 98.12% (576/587 bp, 0 gaps)

Table 3 Ticks submitted to PCR for *Borrelia* (gene *flaB*) and *Rickettsia* (genes *gltA*, *htrA*, *ompA*, *ompB*) screenings

Tick species	Host	Ticks submitted to PCR	Genes				
			flaB	*gltA*	*htrA*	*ompA*	*ompB*
Ixodes sigelos	*Rattus norvegicus*	1N	–	–	–	–	–
		1N	–	–	–	–	–
	Octodon bridgesi	3N (pool)	–	–	–	–	–
		3N (pool)	–	–	–	–	–
	Phyllotis darwini	2N (pool)	–	–	–	–	–
		6L (pool)	–	–	–	–	–
Ornithodoros sp.	*Phyllotis darwini*	1L	+	+	+	+	+
		1L	+	+	+	+	+
		2L	+	+	+	+	+

Negative and positive detections are indicated with "–" and "+", respectively

N nymph, *L* larva

and 97.79% (799/817 bp, 6 gaps) identical with *Rickettsia amblyommatis* strain Ac37 (CP012420). We tentatively named both detected agents as *Borrelia* sp. Cachapoal and *Rickettsia* sp. Cachapoal. Their respective sequences were deposited in GenBank (*Borrelia*: MK106060, MK112520; *Rickettsia*: MK112516, MK112517, MK112518, MK112519).

Phylogenies

The morphological identity of the collected *Ixodes* specimens was considered inconclusive when compared with sequences of other congeneric species available in GenBank. In line with the previous statement, the *Ixodes* sp. herein collected clusters within a monophyletic group composed by sequences of other morphologically closely related species from Argentina and Chile, and also with *Ixodes abrocomae* and *Ixodes nuttalli*. The sequence of an *Ixodes* sp. from Mocha Island (Chile) appears as its closest relative (Fig. 3). However, support values for this group of *Ixodes* spp. is low. On the other hand, with consistent bayesian and MP support values, the phylogeny for soft ticks indicates that the undetermined *Ornithodoros* sp. collected in RCNR corresponds to a sister taxon of *O. atacamensis* (Fig. 4). Additionally, another yet-to-be-described species recently reported in association with small-mammals from northeastern Brazil (Maia et al. 2018) appears closely related to both *Ornithodoros* sp. Cachapoal and *O. atacamensis* (Fig. 4).

Supported by high bayesian posterior probability and ML bootstrap values, *Borrelia* sp. Cachapoal formed a monophyletic group with "*Ca*. B. johnsonii." Moreover, both species clustered as a sister group to a clade composed by *Borrelia parkeri*, *Borrelia turicatae,* and *Borrelia venezuelensis* (Fig. 5). On the other hand, *Rickettsia* sp. Cachapoal clustered between a group of *R. amblyommatis* strains and a clade formed by *Rickettsia* sp. ARANHA and *Rickettsia* sp. AL, two putative new rickettsial agents detected in *Amblyomma longirostre* from Brazil (Labruna et al. 2004a; Ogrzewalska et al. 2008) (Fig. 6).

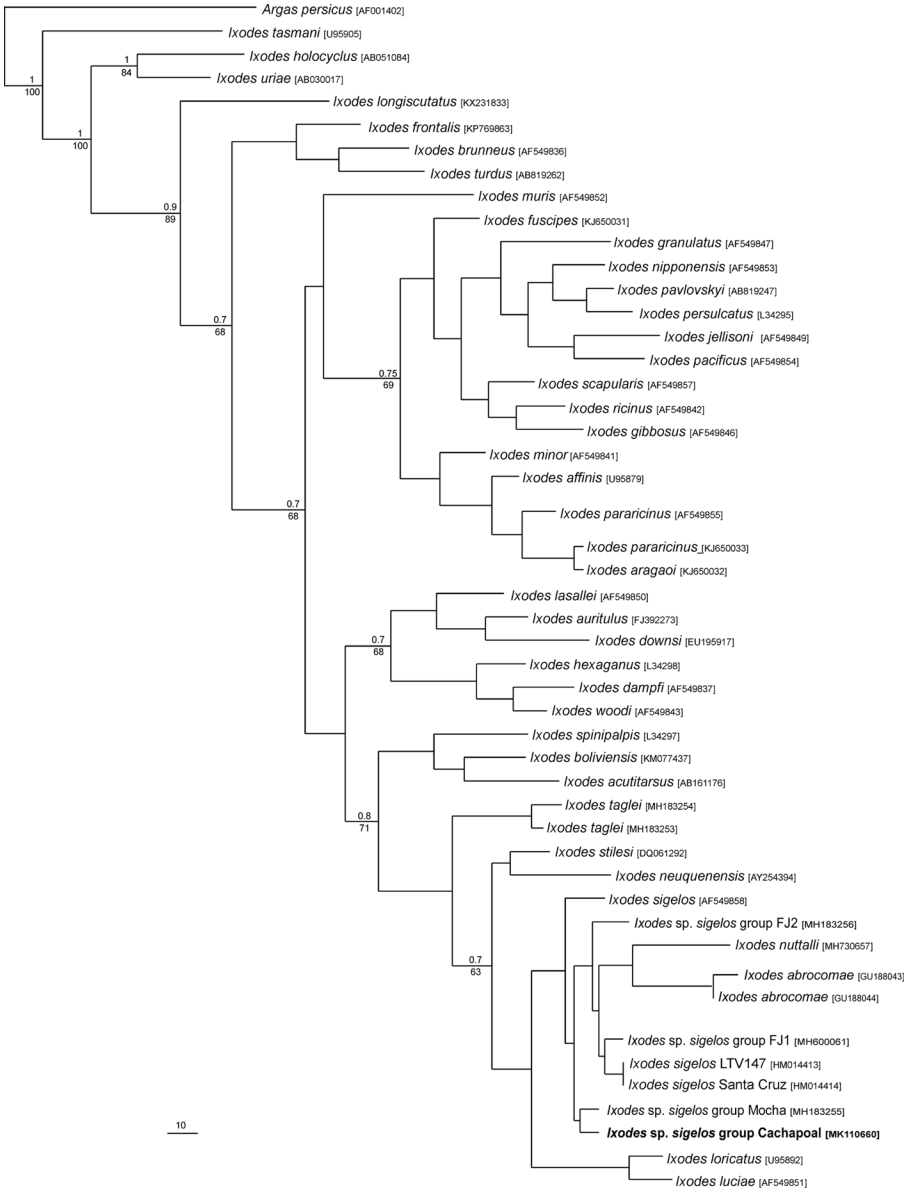

Fig. 3 Maximum parsimony (MP) and bayesian inferred phylogenetic tree constructed for ticks of the genus *Ixodes*. The tree is drawn to scale and scale bar indicates nucleotide substitutions per site. Otherwise omitted, numbers above and below branches represent bayesian posterior probability and MP bootstrap values ≥ 0.70 and $\geq 70\%$, respectively. The position of the *Ixodes* sp. collected in this study is highlighted in bold as "*Ixodes* sp. *sigelos* group Cachapoal."

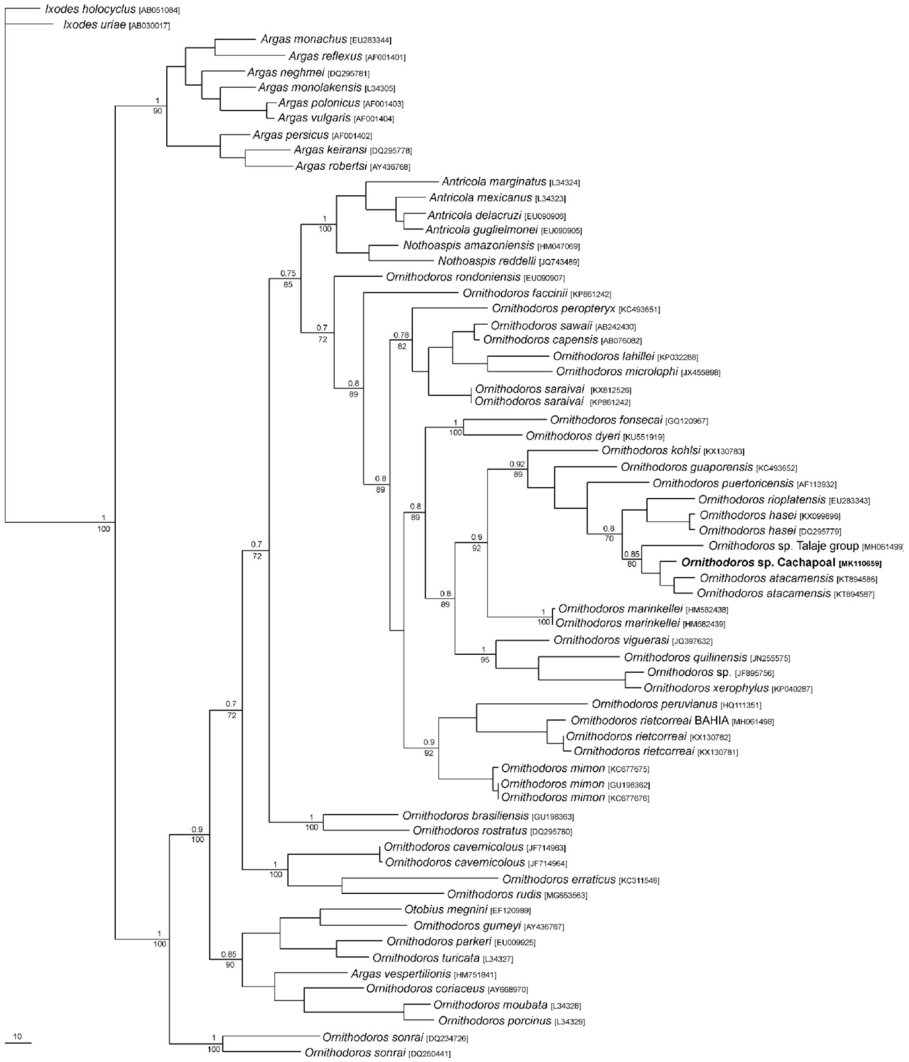

Fig. 4 Maximum parsimony (MP) and bayesian inferred phylogenetic tree for Argasidae family. The tree is drawn to scale and scale bar indicates nucleotide substitutions per site. Otherwise omitted, numbers above and below branches represent bayesian posterior probability and MP bootstrap values ≥ 0.70 and $\geq 70\%$, respectively. The position of the *Ornithodoros* sp. collected in this study is highlighted in bold as "*Ornithodoros* sp. Cachapoal."

Discussion

Vertebrate hosts and tick identities

Although capture efforts allowed the examination of ten species of birds and one marsupial, ticks were found parasitizing rodents only. An *Ixodes* sp. of the *I. sigelos* group was the sole hard tick identified in the current study. *Ixodes sigelos* was originally described from

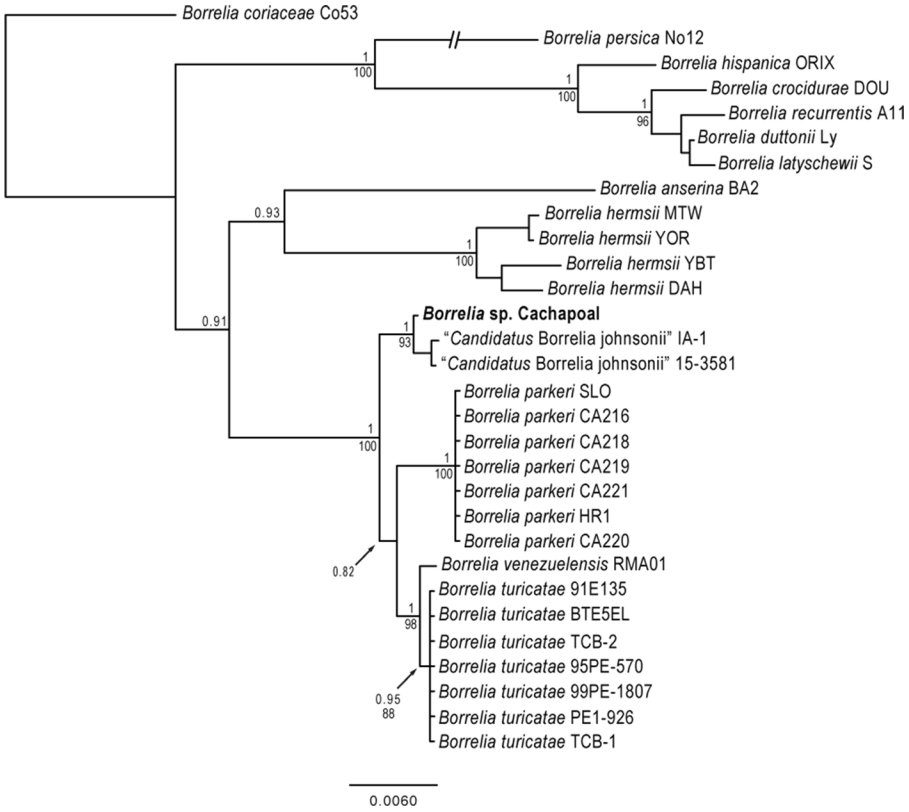

Fig. 5 Maximum likelihood (ML) and bayesian inferred phylogenetic tree for *Borrelia* spp. The tree is drawn to scale and scale bar indicates nucleotide substitutions per site. Otherwise omitted, numbers above and below the nodes represent bayesian posterior probability and ML bootstrap values ≥0.70 and ≥70%, respectively. The position of *Borrelia* sp. Cachapoal is highlighted in bold

specimens collected along Chile (i.e. Metropolitan, Maule, and Biobío regions) in association with abrocomid (Abrocomidae), cricetid (Cricetidae) and octodontid (Octodontidae) rodents (Keirans et al. 1976). Further collections identified this tick in the Patagonia region from Argentina and Chile (Guglielmone et al. 2005; Sebastian et al. 2016). Here, our results point that the *Ixodes* sp. collected from cricetid and octodontid rodents in the RCNR would correspond to a species morphologically and phylogenetically related with *I. sigelos*. Remarkably, phylogenetic analyses including several sequences identified as *I. sigelos* from Argentina and Chile (Fig. 3) indicate that this taxon is paraphyletic. This fact reinforces the hypothesis that ticks morphologically *affinis* to *I. sigelos* could correspond to a group of cryptic species (Sanchez et al. 2010).

Engorged larval stages of an unidentified *Ornithodoros* sp. were collected on *P. darwini*, which corresponds to the first time that soft tick larvae are found parasitizing this rodent species. Slide-mounted specimens revealed a phenotype highly similar to *O. atacamensis*, a tick described in association with reptiles from the barren ecosystems of the Atacama Desert in northern Chile (Muñoz-Leal et al. 2016). Slight but evident differences separate currently collected *Ornithodoros* sp. from *O. atacamensis* larvae: six instead of five posterolateral pairs of setae, and the third row of denticles reaching a fourth rather than a third

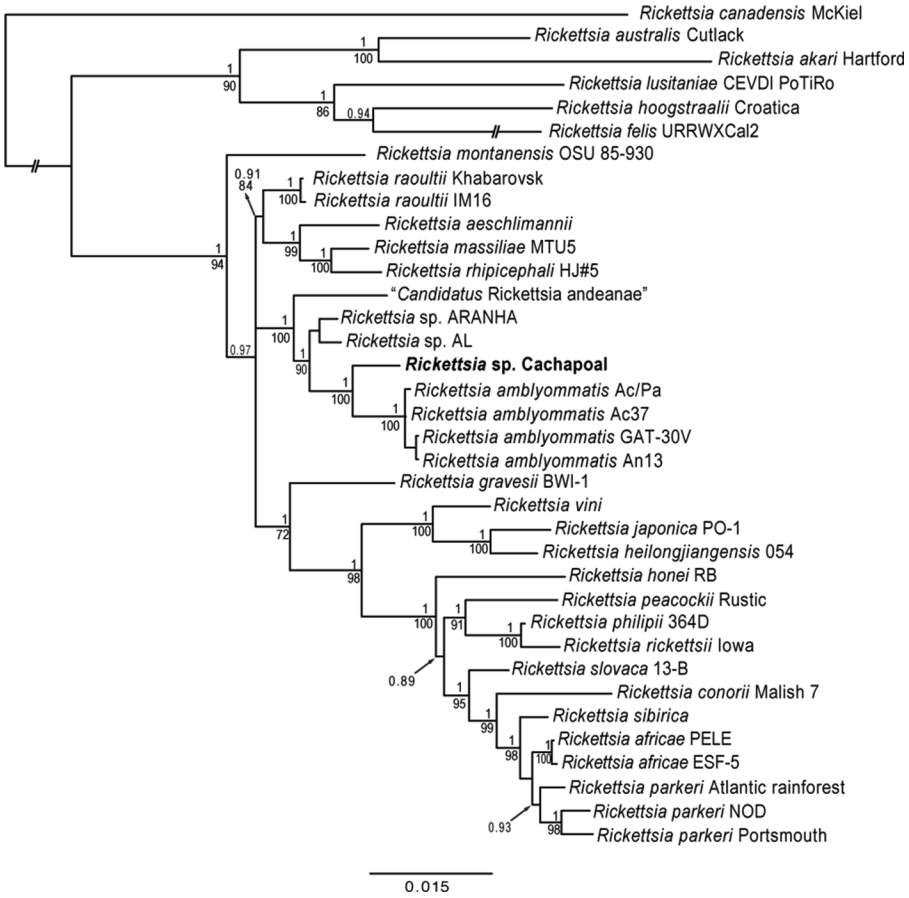

Fig. 6 Maximum likelihood (ML) and bayesian inferred phylogenetic tree for *Rickettsia* spp. The tree is drawn to scale and scale bar indicates nucleotide substitutions per site. Strain names are indicated immediately after each species. Otherwise omitted, numbers above and below the nodes represent bayesian posterior probability and ML bootstrap values ≥0.70 and ≥70%, respectively. The position of *Rickettsia* sp. Cachapoal is highlighted in bold

of the hypostome, respectively. This morphological relatedness seems to be explained after assessing the phylogenetic positions of both tick species. Indeed, the tree topology suggests that genetic divergence observed between them (> 5%) would be sufficient enough to consider the collected *Ornithodoros* sp. as a putative new taxon. However, the sequencing of additional loci, the examination of a major number of larvae, collecting material from other localities, including adult forms, is now needed to assess this hypothesis accurately.

Borrelia

Recently, a phylogenetic analysis of 16S rRNA, *flaB* and *glpQ* genes unveiled a close relatedness between a Brazilian strain of *B. venezuelensis* and *B. turicatae* (Muñoz-Leal et al. 2018), a human-pathogenic species associated with *Ornithodoros turicata* in North

America (Dworkin et al. 2008). This scenario, in which two genetically similar *Borrelia* spp. are associated with vectors with distanced distributions is impressive, yet it constitutes an unsolved question. Similarly, genetic identities and phylogenetic analyses of 16S rRNA and *flaB* sequences of *Borrelia* sp. Cachapoal indicate a close relatedness with "*Ca*. B. johnsonii", another North American species harbored by the bat tick *Ornithodoros kelleyi* (Schwan et al. 2009). Although the sequencing of more loci of both South American borreliae is undoubtedly necessary before stating any conclusion, the occurrence of close genetic relationship between North and South American RFB suggests a common origin at some point in their evolutionary history. This fact warrants special interest since northern and southern parts of the American continent were once isolated and joined only nearly three Mya (Estrada-Peña et al. 2018).

Recently, a sound molecular survey for *Borrelia* spp. in patients with symptoms of tick-borne illness detected "*Ca*. B. johnsonii" DNA in human blood, introduced this species as a previously unrecognized pathogenic agent (Kingry et al. 2018). Even if the role of *Borrelia* sp. Cachapoal as an etiologic agent of relapsing fever in humans remains unsolved, its phylogenetic position as a sister clade of "*Ca*. B. johnsonii" suggests that a pathogenic nature cannot be discarded.

For the moment, the role of *P. darwini* as a vertebrate reservoir of *Borrelia* sp. Cachapoal remains unknown. However, considering that ticks were collected feeding upon and that rodents are recognized natural reservoirs of other RFB (Cutler 2015), it is highly possible that this Chilean species could play a role in the cycle of *Borrelia* sp. Cachapoal in RCNR.

Rickettsia

Rickettsiae are common microorganisms among the South American Ixodidae (Labruna 2009; Parola et al. 2013). The study of *Rickettsia* has been largely biased towards ixodid ticks because of the well-known vector roles these parasites hold. In turn, limited attention has been paid to rickettsiae associated with the Argasidae in South America (but see Tahir et al. 2016). Here, larvae of an undetermined *Ornithodoros* sp. were positive to *Rickettsia* detection, suggesting that this species naturally harbor bacteria of this genus. Though, a blood origin for this molecular detection cannot be discarded since all collected ticks were almost fully engorged.

After sequencing partial fragments of *gltA*, *htrA*, *ompA*, and *ompB* loci observed genetic divergences of *Rickettsia* sp. Cachapoal exceeded the divergence limits considered when inferring identities for a determined species within the genus (Raoult et al. 2005). Moreover, phylogenetic analyses inferred by bayesian and ML methods emplaced *Rickettsia* sp. Cachapoal as a well-supported branch, sister to *R. amblyommatis* (Fig. 6). A monophyletic clade of *R. amblyommatis*-related rickettsiae appears composed by the other two species, *Rickettsia* sp. ARANHA and *Rickettsia* sp. AL, both characterized from *A. longirostre* ticks collected in Brazilian Amazon and Atlantic rainforest (Labruna et al. 2004a; Ogrzewalska et al. 2008). With the current evidence, to propose any explanation between phylogenetically related, yet biogeographically vastly distanced *Rickettsia* spp. would be rather speculative. However, examples of phylogenetically related strains of *Rickettsia* associated with soft ticks and hard ticks occurring in distanced regions of the world have been already demonstrated (Izzard et al. 2018). This fact suggests that the study of soft tick-associated rickettsiae would bring valuable information in order to unveil evolutionary pathways of these bacteria among the Ixodoidea.

Although the role of *R. amblyommatis* as an etiologic agent is still unclear, this species does correspond to an SFR, and therefore it should be considered as a potentially pathogenic organism (Parola et al. 2013). Consequently, a pathogenic role of phylogenetically closely related *Rickettsia* sp. Cachapoal should not be ignored.

Final remarks

At least two species of ticks were identified parasitizing rodents in the RNRC. Two putative new agents, a relapsing fever *Borrelia* and a spotted fever *Rickettsia,* were characterized upon DNA extracted from a yet-to-be-identified soft tick species of the genus *Ornithodoros*. Although to date human cases of tick-borne relapsing or spotted fever are inexistent in Chile, and records of ticks parasitizing humans lack in recent times for the country (Guglielmone and Robbins 2018), it must not been overlooked that both diseases are still neglected in regions where they are endemic worldwide (Chikeka and Dumler 2015). From this point of view, our results call for the attention into scenarios gathering epidemiological factors compatible for both maladies to occur, such as outdoor enthusiasts exposed to ticks while entering Andean environments in central Chile.

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Author contributions SML and MBL conceived the study. SML, DFC, and MA made vertebrate capture and tick collection in the field. SML identified ticks, performed laboratory work, and drafted the manuscript. SML and AM performed phylogenetic analyses. All authors contributed to reviewing the manuscript, read, and approved the final version.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval Animal captures, handling, and the collection of biological samples have been approved by the "Corporación Nacional Forestal" (CONAF) and by permit 417/2018 given by the "Servicio Agrícola y Ganadero" (SAG), Chile. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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