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Molecular identification of *Bartonella* spp. and *Rickettsia felis* in fox fleas, Chile

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ABSTRACT

Seventy-five flea pools (one to ten fleas per pool) from 51 Andean foxes (*Lycalopex culpaeus*) and five South American grey foxes or chillas (*Lycalopex griseus*) from the Mediterranean region of Chile were analyzed for the presence of DNA of *Bartonella* spp. and *Rickettsia* spp. through quantitative real-time PCR for the *nouG* and *gltA* genes, respectively. Positive samples were further characterized by conventional PCR protocols, targeting *gltA* and ITS genes for *Bartonella*, and *gltA, ompA,* and *ompB* genes for *Rickettsia*. *Bartonella* was detected in 48 % of the *Pulex irritans* pools (*B. rochalimae* in three pools, *B. berkhoffii* in two pools, *B. henselae* in one pool), and 8 % of the *Ctenocephalides felis felis* pools (*B. rochalimae*, one pool). *Rickettsia* was confirmed in 11 % of *P. irritans* pools and 92 % of the *Ct. felis* pools. Characterization confirmed *R. felis* in all sequenced *Rickettsia*-positive pools. All *Ct. canis* pools were negative. A *Ct. felis* pool from a wild-found domestic ferret (*Mustela putorius furo*) also resulted positive for *R. felis*. Although opportunistic, this survey provides the first description of zoonotic pathogens naturally circulating in fleas parasitizing Chilean free-living carnivores.

1. Introduction

Fleas are known vectors of infectious pathogens, many of which are zoonotic. In Chile, a diversity of species of *Bartonella* have been described in dogs, cats, and their fleas, including *Bartonella clarridgeiae, Bartonella henselae, Bartonella berkhoffii, Bartonella vinsonii, and Bartonella koehlerae* [10,30,31,29,34,44]. *Bartonella clarridgeiae* was also identified in invasive American mink (*Neovison vison*) [41]. Regarding *Rickettsia*, DNA of *R. felis*, *Candidatus* Rickettsia senegalensis, and *Candidatus* Rickettsia asemboensis [10,31,35], and antibodies against spotted fever *Rickettsia* [13] were reported in domestic animals.

Wild canids are often infested with fleas that can carry flea-borne pathogens such as *Bartonella* spp. [27] and *Rickettsia* spp. [43]. In Chile, three species of wild foxes are present along the country: the Andean fox or culpeo (*Lycalopex culpaeus*), the South American grey fox or chilla (*L. griseus*), and the Darwin's fox (*L. fulvipes*). Nevertheless, there is insufficient information about flea-borne pathogens in Chilean wild canids. DNA of a *Rickettsia* sp. showing 97 % identity with *R. felis* was detected in a blood sample of a Darwin's fox [9]. On the other hand, Di Cataldo et al. [13] failed to detect antibodies against spotted fever *Rickettsia* in 32 serum samples from Andean and grey foxes. Fox fleas had only been investigated once before in the country by Poo-Muñoz et al. [35], who included fleas from two grey foxes that resulted negative for *Rickettsia*. The present survey aims to provide for the first time with

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Table 1
Numbers of pools of fleas retrieved on wild foxes analyzed for the presence of DNA of *Bartonella* spp. and *Rickettsia* spp. A further *Ct. felis* pool from a ferret was also analyzed.

	Pulex irritans Pools/ Foxes	*Ctenocephalides felis felis* Pools/Foxes	*Ctenocephalides canis* Pools/Foxes
Andean fox (*Lycalopex culpaeus*)	54/39	9/9	7/3
South American grey fox (*Lycalopex griseus*)	3/3	2/2	0/0

information regarding the presence of these important zoonotic vector-borne pathogens in fleas retrieved from free-living foxes in Chile. We also include in this survey the fleas opportunistically collected from a wild-found domestic ferret (*Mustela putorius furo*).

2. Material and methods

2.1. Flea sampling and identification

The study was performed on 51 Andean foxes and five South American grey foxes from the Metropolitan, O'Higgins, and Valparaiso Regions of Chile, all of them located in the Mediterranean climatic region of the country (Supplementary File 1). Fleas were retrieved from foxes captured in the wild (see [11] for details about the ectoparasite sampling) or foxes admitted to wildlife rescue centers (at the moment of admittance). Fleas were taxonomically identified based on previously described morphological keys [17,22,42]. After individual classification, fleas belonging to the same fox were pooled (one to ten fleas per pool). Overall, 75 pools from 56 different foxes were analyzed (Table 1). In addition, a *Ct. felis* pool from a wild-found domestic ferret admitted to a rescue center was included in the survey.

2.2. Extraction and DNA purification

Pools were submerged in liquid nitrogen and pulverized to perform

Table 2
Primers and protocols used for the molecular characterization of *Bartonella* sp. and *Rickettsia* sp.

Target	PRIMERS	AMPLIFICATION CYCLES	AMPLICON SIZE (PB)	REFERENCES
16S-23S rRna Intergenic Spacer Region (***ITS***) of *Bartonella* spp.	325s (5'-CTTCAGATGATGATCCCAAGCCTTYTG GCG -3') 1100as (5'- GAACCGACGACCCCCTGCTTGCAAAGC A-3')	95°C x 5min 94°C x 15s 66°C x 15s (55cycles) 72°C x 15s 72°C x 1min	453- 717	(Diniz et al., 2007)
Citrate synthase gene (*gltA*) of *Bartonella* spp.	CS443f (5'-GCTATGTCTGCATTCTATCA -3') CS1210r (5'- GATCYTCAATCATTTCTTTCCA -3')	94°C x 2min 94°C x 30s 48°C x 1min (45cycles) 72°C x 1min 72°C x 5min	767	(Billeter et al., 2011; Birtles and Raoult, 1996)
Citrate synthase (*gltA*) of *Rickettsia* spp.	CS-78 (5'-_GCAAGTATCGGTGAGGATGTAAT -3') CS-323 (5'-_GCTTCCTTAAAATTCAATAAATCAGGAT -3')	95°C x 3min 95°C x 15s 48°C x 30s (40cycles) 72°C x 30s 72°c x 7min	401	(Labruna et al., 2004).
Major outer membrane protein (*ompA*) *Rickettsia* spp.	*Rr*190.70p (5´- _ATGGCGAATATTTCTCCAAAA- 3´) *Rr*190.602n (5'- _AGTGCAGCATTCGCTCCCCCT- 3´)	94°C x 5min 94°C x 30s 46°C x 30s (30cycles) 72°C x 40s 72°c x 5min	532	(Regnery et al., 1991)
ompB Rickettsia spp. (Nested PCR)	Rc.rompB.4362p GTCAGCGTTACTTCTTCGATGC Rc.rompB.4,836n CCGTACTCCATCTTAGCATCAG *NESTED* Rc.rompB.4,496p CCAATGGCAGGACTTAGCTACT Rc.rompB.4,762n AGGCTGGCTGATACACGGAGTAA	95°C x 5min 95°C x 15s 54°C x 15s (35cycles) 72°C x 30s 72°c x 3min 95°C x 5min 95°C x 15s 56°C x 15s (35cycles) 72°C x 30s 72°c x 3min	453 245	(Choi et al., 2005)

Table 3
Bartonella spp. characterization in fleas from wild foxes, Chile.

Flea species (host)	No. pools	*ITS*			*gltA*		
		sp.	GenBank® ID	% identity	sp.	GenBank® ID	% identity
P. irritans (Andean fox)	1	*B. henselae*	MT095053	100	*B. henselae*	KY913621	99.87
	2	*B. rochalimae*	FN645466	99.40	*B. rochalimae*	FN645466	99.40–99.87
	1	*B. rochalimae*	FN645466	99.43	*B. rochalimae*	FN645459	99.87
	2	*B. vinsonii berkhofii*	DQ059764	95.38–95.40	Uncultured *Bartonella*	KT807806	99.18–99.26
	2	Negative			*B. vinsonii berkhofii*	CP003124	99.71–99.80
Ct. felis felis (Andean fox)	1	*B. rochalimae*	FN645466	99.40	*B. rochalimae*	FN645466	99.40

Genomic DNA extraction (DNeasy Blood and Tissue Kit, QIAGEN, Valencia, CA) according to manufacturing instructions. DNA concentration and purity were determined (NanoDrop ND-1000 spectrophotometer, Thermo Scientific, USA).

2.3. Endogenous control

All samples were submitted to conventional PCR (cPCR) (T100 BioRad thermocycler, BioRad, Hercules, CA) targeting the 18SrRNA flea gene as a control to check the integrity of the DNA template [38].

2.4. Bartonella sp. and Rickettsia sp. quantitative real time PCR

18SrDNA cPCR positive samples were subsequently submitted to a previously described quantitative real-time PCR (qPCR) for amplification of the *Bartonella* spp. *nuoG* gene [2] and for amplification of the *Rickettsia* spp. *gltA* gene. For *Bartonella* spp. amplification, reactions were performed in duplicate using 10 μL of PCR mixtures containing 5 μL of Go Taq® Probe qPCR Master Mix (Promega, Madison, WI, U.S.A.), 1.2 μM of each primer (F-Bart [5'-CAATCTTCTTTTGCTTCACC-3′] and R-Bart [5′- TCAGGGCTTTATGTGAATAC-3′], hydrolysis probe (Texas Red-5'- TTYGTCATTTGAACACG-3'[BHQ2a-Q]3′), and 1 μL of DNA sample. PCR amplifications were conducted in Low-Profile Multiplate™ Unskirted PCR Plates (BioRad©, Hercules, CA, U.S.A.) using a CFX96 Thermal Cycler (BioRad©, Hercules, CA, U.S.A.).

For citrate synthase (*gltA*) of *Rickettsia* spp. amplification, we used the primers CS-5 (GAGAGAAAATTATATATCCAAATGTTGAT), CS-6 (AGGGTCTTCGTGCATTTCTT) and a fluorogenic probe [5 6-FAM d (CATTGTGCCATCCAGCCTACGGT) BHQ-1 3] [21]. Real-time PCRs were performed in a Low-Profile Multiplate™ Unskirted PCR Plates (BioRad©, Hercules, CA, U.S.A.) using a CFX96 Thermal Cycler (BioRad©, Hercules, CA, U.S.A.). with 25 μL per reaction, which contained 12.5 μL of the Go Taq® Probe qPCR Master Mix (Promega, Madison, WI, U.S.A.), 0.75 μL of each primer at 15 μM, 0.25 μL of the probe at 15 μM, and 5.25 μL of molecular-grade water [21].

The qPCR was performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (M.I.Q.E.) [8]. Amplification efficiency (E) was calculated from the standard curve slope in each run using the following formula (E = 10–1/slope). Copy numbers were estimated using 10-fold serial dilutions of gBlock® (Integrated DNA Technologies, Coralville, IA, U.S.A.) encoding the *nuoG B. henselae* sequence (insert containing 83 bp) and other encoding the *gltA Rickettsia* spp. sequence (insert containing 147 bp). The number of gBlock® copies was determined according to the formula (Xg/μL DNA/[gBlock length in bp x 660]) x 6.022 × 10ˆ23 x gBlock copies/μL [26]. All PCR runs were performed with nuclease-free water (Promega®, Madison, WI, U.S.A.) as a negative control. Replicates showing a Cq difference higher than 0.5 were retested.

2.5. Conventional PCR for Bartonella sp. identification

For molecular characterization and species differentiation, *nuoG Bartonella* qPCR-positive samples were further tested by cPCR to amplify two loci: *gltA* [6,7] and ITS [14] (Table 2). For amplification reactions, each DNA sample (5 μL) was used as a template in a 25 μL reaction mixture containing 12.5 μL of a premixed GoTaq® G2 Green Master Mix DNA Polymerase 2X (Promega, Madison, U.S.A.), and 0.5 μM of each primer. All cPCR amplification reactions were performed in a T100 BioRAd thermocycler (BioRad©, Hercules, CA, U.S.A.). A DNA sample from a *Bartonella henselae*- naturally infected cat from a previous study was used as a positive control [41].

2.6. Conventional PCR for Rickettsia sp. identification

For molecular characterization and species differentiation, *gltA Rickettsia* qPCR positive samples were analyzed by cPCR to amplify three loci: *gltA* [21], *ompA* [37] and *ompB* [12] (Table 2). For amplification reactions, each DNA sample (5 μL) was used as a template in a 25 μL reaction mixture containing 12.5 μL of a premixed GoTaq® G2 Green Master Mix DNA Polymerase 2X (Promega, Madison, U.S.A.), and 0.5 μM of each primer. A DNA sample of a cat flea naturally infected with *Rickettsia felis* was used as a positive control [31].

2.7. DNA purification and sequencing

Only positive samples presenting strong band intensity were purified by enzymatic reaction using Exo-CIP™ Rapid PCR Cleanup Kit (New England Biolab inc., Ipswich, MA, USA.), following the manufacturer's instructions, and the purified DNA was sent to Macrogen (Seoul, Korea) for sequencing. Forward and reverse sequences were analyzed in Geneious Prime 7.1 to obtain consensus sequences. Identity percentages were obtained using BLASTn [1]. Representative sequences were deposited in GenBank® [5] including those with identity percentages lower than 100 % with other available sequences and those representing each haplotype resulting from the haplotype-polymorphism analysis of each gene by bacterial species. Accession numbers are OQ436432 – OQ436436 (*gltA Bartonella* spp. sequences) OQ435989 - OQ435990 (ITS *Bartonella* spp. sequences), OQ436437 (*gltA Rickettsia* sp. sequence), OQ436438 (*ompA Rickettsia* spp. sequences).

2.8. Haplotype analysis of gltA and ompA (Rickettsia sp.) sequences

For the genetic diversity analysis, the obtained sequences of each gene were aligned per gene/locus and bacterial species. The alignment was used to calculate the nucleotide diversity (π), polymorphism level (diversity haplotypes [dh], number of haplotypes [h], and the average number of nucleotide differences [K]), using DnaSP v5 software [24].

2.9. Phylogenetic analysis based on gltA (Bartonella sp.) and gltA and ompA (Rickettsia sp.) sequences

For the phylogenetic analysis, the best evolutionary model was selected according to the Bayesian Information Criterion (BIC) for each one of the codon positions (partition) for the encoded gene (*gltA* of *Bartonella* spp. and *gltA* and *ompA* for *Rickettsia* spp.) [40]. Thus, the best evolutionary model for *Bartonella* spp. *gltA* was TNe+G4 (partitions 1 and 2), and K2P+G4 (partition 3). The best evolutionary model for *Rickettsia* spp. *gltA* was K3Pu+F+G4 (partitions 1 and 2), K3Pu+F+G4

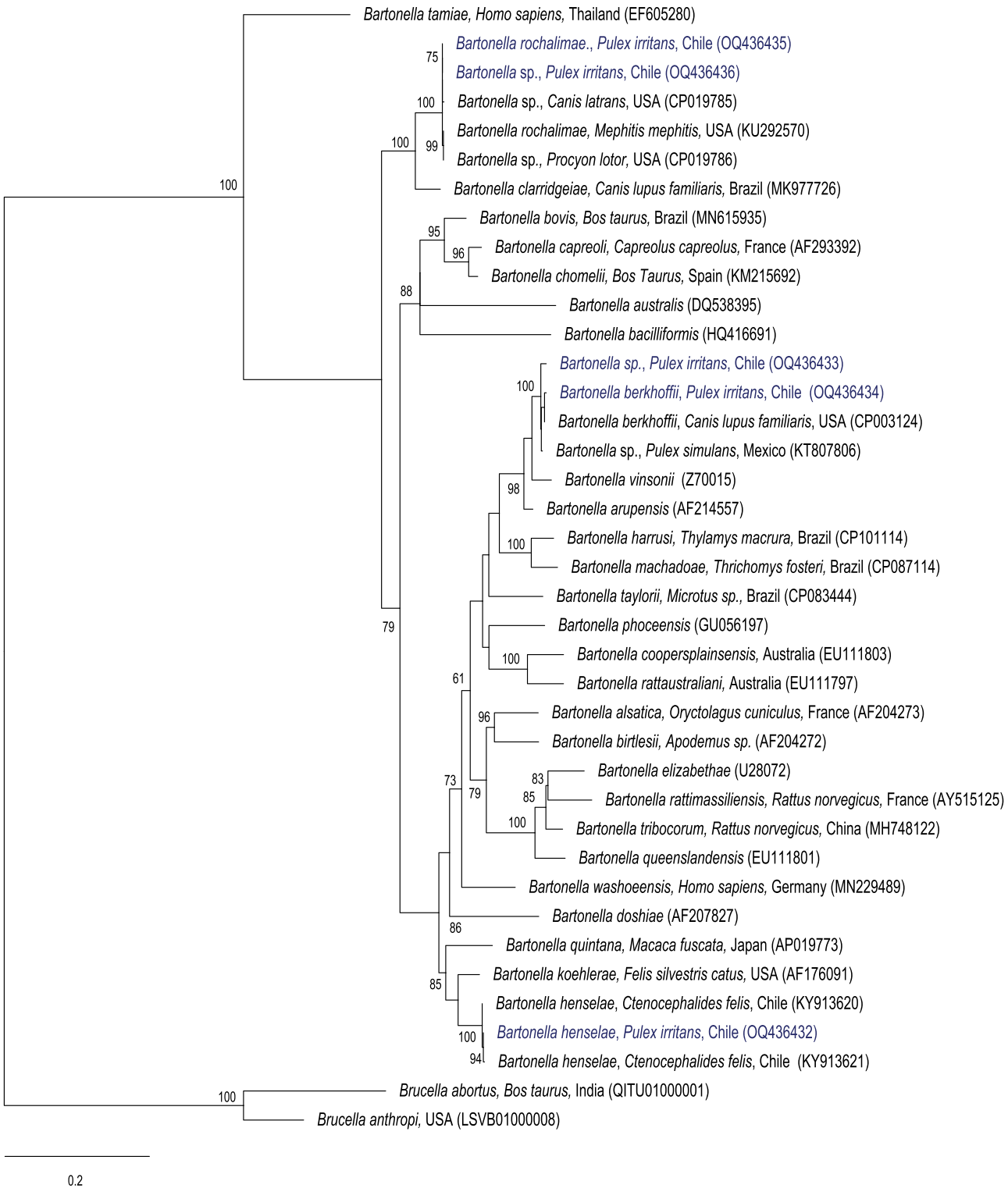

Fig. 1. Maximum Likelihood tree for a subset of *Bartonella* spp. inferred using an alignment (316 bp) of the gene encoding citrate synthase (*gltA*). Calculate substitution model were TNe+G4 (partitions 1 and 2), K2P+G4 (partition 3).

(partition 3). The best evolutionary model for *Rickettsia* spp. *ompA* was HKY+F+G4 (partition1 and 3), TPM3u+F+G4 (partition 2). For the non-coding gen (ITS) the best evolutionary model was selected according to the Akaike information criterion (AIC). The best model for *Bartonella* spp. ITS using the Akaike Information Criterion (AIC) [36] was GTR+F+G4. The selection of the best evolutionary model was assessed using ModelFinder [19]. Finally, the tree was inferred by the Maximum likelihood (ML) method on IQ-TREE [32].

3. Results

Forty-eight percent (26/54) of the *P. irritans* pools, belonging to 22 foxes, were positive for DNA of *Bartonella* according to the qPCR protocol (mean and SD of reactions' efficiency = 94.23 % ± 2.25 %; r^2 =

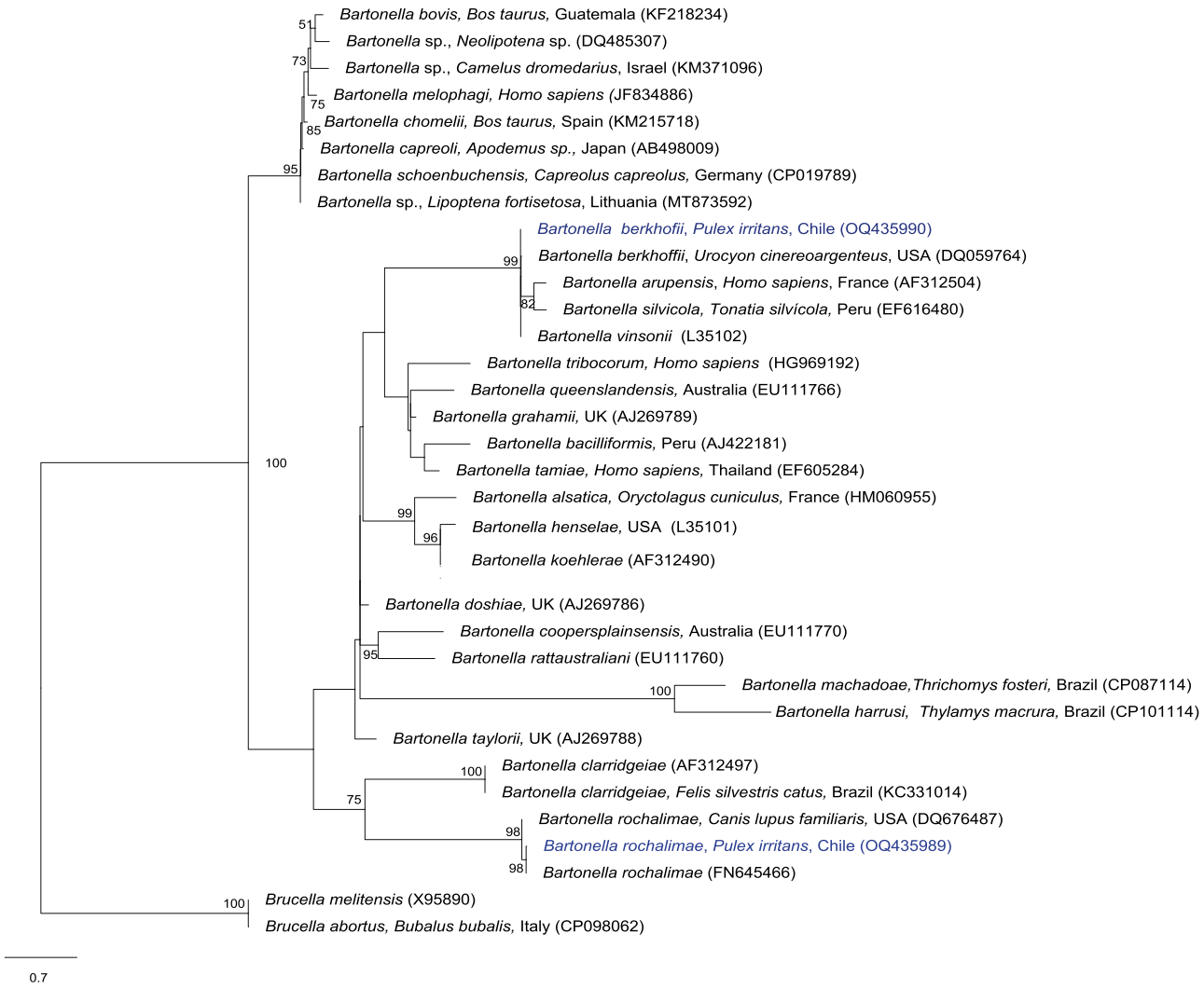

Fig. 2. Maximum Likelihood tree for a subset of *Bartonella* spp. inferred using an alignment (133 bp) of the ^{16}S–^{23}S rRNA gene internal transcribed spacer (*ITS*). Calculate substitution model were GTR+F+G4.

Table 4
Rickettsia spp. characterization in fleas from wild foxes, Chile.

Flea species (host)	No. pools	*gltA*			*ompA*			*ompB*		
		sp.	GenBank® ID	% identity	sp.	GenBank® ID	% identity	sp.	GenBank ® ID	% identity
P. irritans (Andean fox)	1	Negative			*Rickettsia felis*	MN267050	100	Negative		
Ct. felis felis (Andean fox and domestic ferret)	5	*Rickettsia felis*	MG952932	99.7–100	*Rickettsia felis*	MG818714	99.7–100	*Rickettsia felis*	ON053303	100

0.99 ± 0.002; slope = −3.46 ± 0.06; Y-intercept = 38.35 ± 0.71). From the amplification of *gltA* and ITS gene fragment of *Bartonella* spp., eight and six readable sequences were obtained, respectively (Table 3).

BLAST and phylogenetic analyses (Figs. 1 and 2) supported the identification of *B. henselae* in one pool and *B. rochalimae* in three pools. In addition, two pools showed identities around 95.4 % in the ITS sequence and 99 % in the *gltA* sequence with *B. berkhoffii*, and another two had around 99.8 % identity with this same species. Regarding *Ct. felis felis*, 8 % of the pools (2/12), belonging to two foxes, were positive for DNA of *Bartonella* according to the qPCR protocol. Only one pool was positive for the *gltA* and ITS c-PCR protocols, and both sequences had 99.4 % identity with *B. rochalimae*.

Eleven percent (6/54) of the *P. irritans* pools, belonging to four foxes, were positive for DNA of *Rickettsia* according to the qPCR protocol (mean and SD of reactions' efficiency = 105.9 % ± 9.75 %; r^2 = 0.99 ± 0.002; slope = −3.22 ± 0.24; Y-intercept = 33.6 ± 7.07). Of these, only one was positive for the *ompB*-cPCR (Table 4). In contrast, 92 % (11/12) of the *Ct. felis felis* pools, belonging to 1o foxes and the ferret, were positive for *Rickettsia* according with the qPCR protocol. A total of 11 sequences were obtained for *Rickettsia*; five from genes *gltA* and *ompA*, and one for *ompB*. The polymorphism-haplotype analysis denoted the absence of polymorphism among them, resulting in one haplotype. Sequences showed 99.7–100 % identity with *R. felis*, including the pool from the ferret. These identifications were confirmed by the phylogenetic analysis (Figs. 3 and 4).

Simultaneous presence of DNA of *Bartonella* and *Rickettsia* was

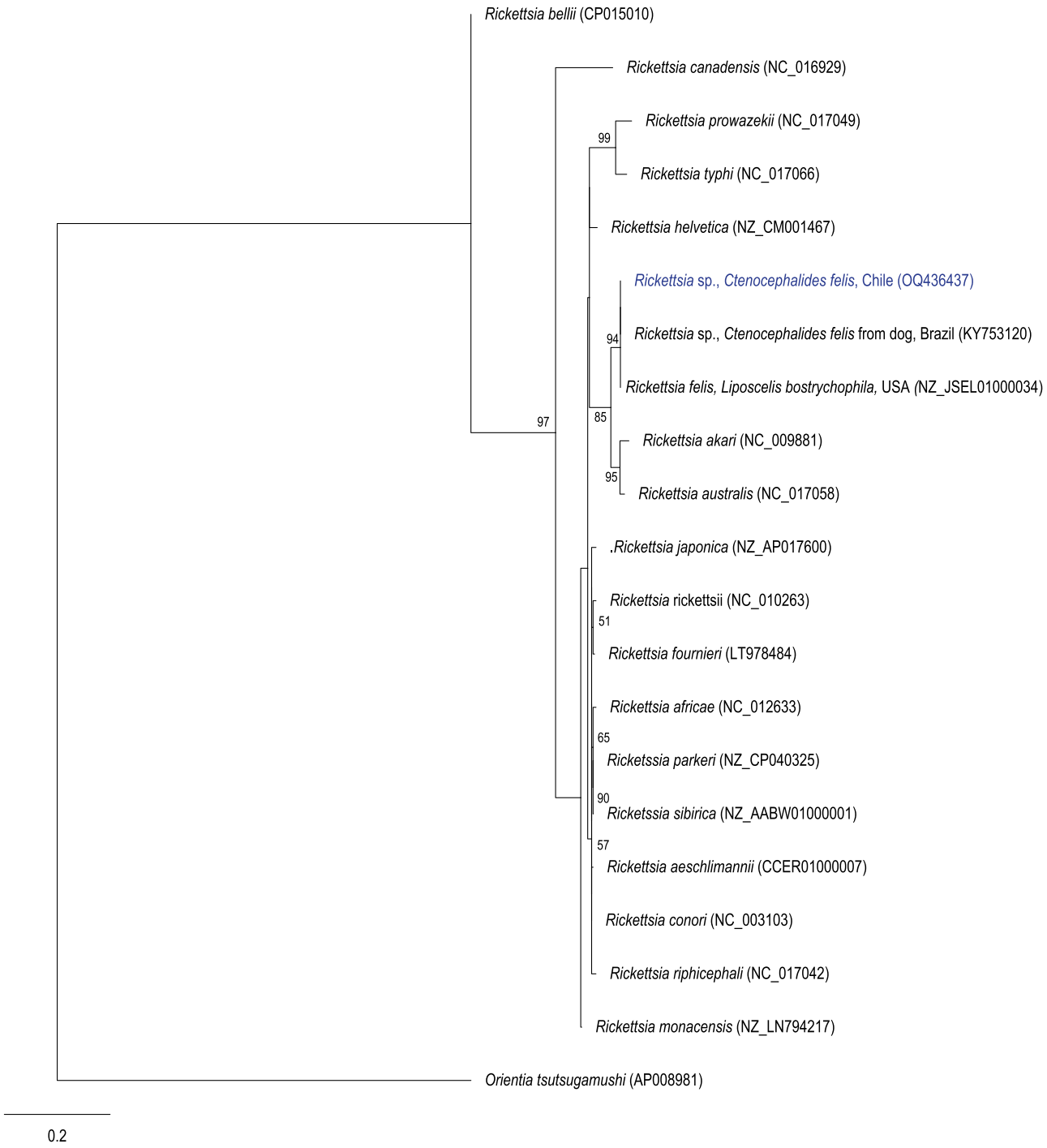

Fig. 3. Maximum Likelihood tree for a subset of *Rickettsia* spp. inferred using an alignment (377 bp) of the gene encoding citrate synthase (*gltA*). Calculate substitution model were K3Pu+F+G4 (partitions 1 and 2), K3Pu+F+G4 (partition 3).

detected in six *P. irritans* and two *Ct. felis* pools. Of these, only in one case the species involved were identified, with *B. rochalimae* and *R. felis* detected in one *Ct. felis* pool. On the other hand, all *Ct. canis* pools were negative for both *Bartonella* and *Rickettsia* qPCRs.

4. Discussion

We have herein confirmed the presence of potentially zoonotic pathogens in fleas collected from free-ranging foxes in Chile. We must emphasize that we used the fleas as sentinels for the presence of these pathogens in natural situations and that no conclusions can be drawn about their vectorial capacity [15].

All the species detected were known to occur in Chile in domestic animals and/or their fleas [10,30,31,29,34,35,44]. Both *B. rochalimae* and *B. berkhoffii* are worldwide distributed and likely have their natural reservoir in wild carnivores, especially canids, with fleas acting as vectors (e.g., [16,4,28,27]). Therefore, it is coherent that both species were the most prevalent among the *Bartonella* detected. Nevertheless, the prevalence detected here can be considered low when compared with other studies with these same species. In Argentina, up to 72 % of *P. irritans* pools from 76 % of the Andean foxes inhabiting natural areas were positive for *Bartonella* DNA, mostly *B. berkhoffii* but also

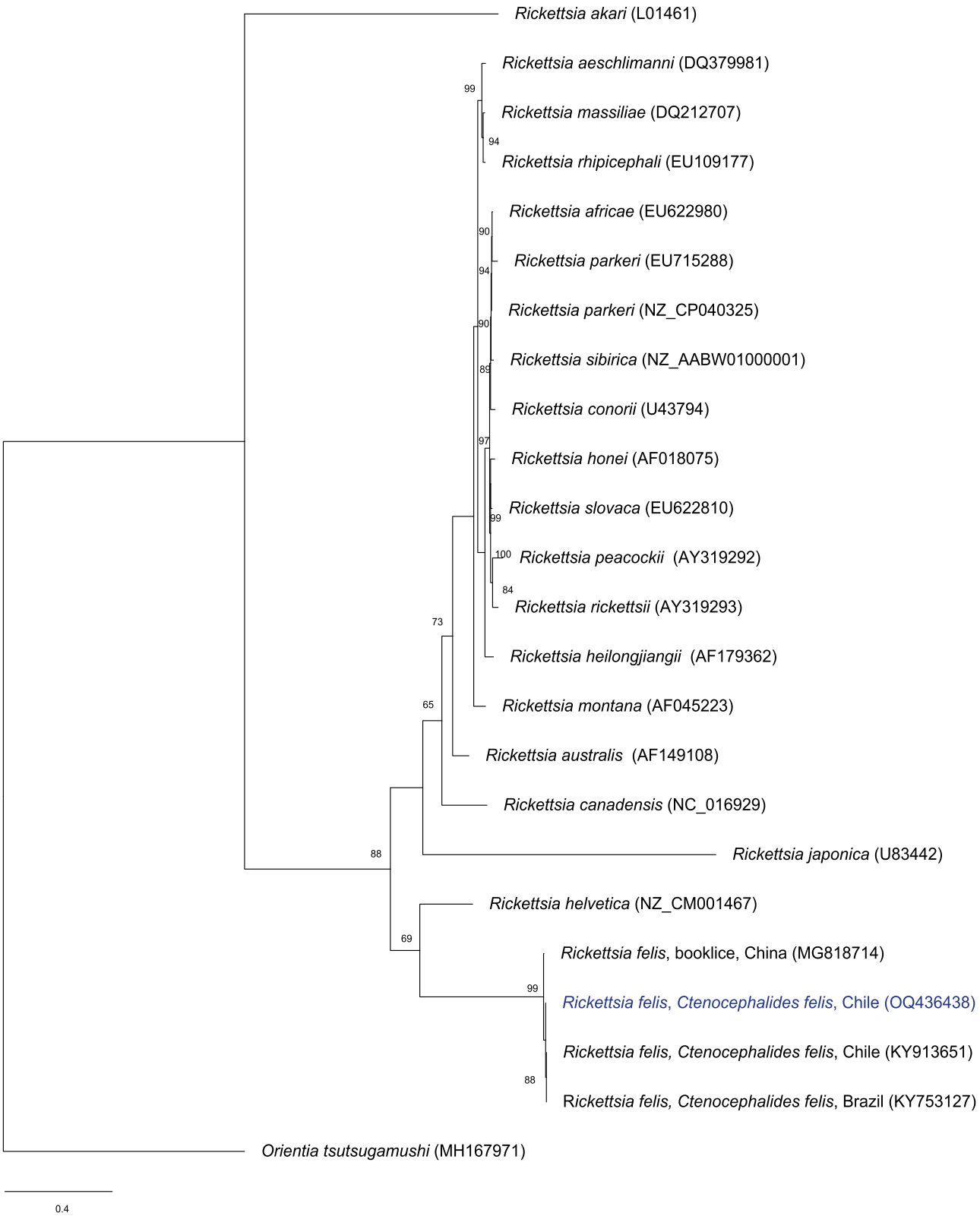

Fig. 4. Maximum Likelihood tree for a subset of *Rickettsia* spp. inferred using an alignment (469 bp) of the gene encoding major outer membrane protein (*ompA*) Calculate substitution model were HKY+F+G4 (partition 1 and 3), TPM3u+F+G4 (partition 2).

B. rochalimae [27]. Cevidanes et al. [11] demonstrated that dogs and foxes rarely share fleas in the study area where most of our foxes were surveyed. Therefore, both *B. rochalimae* and *B. berkhoffii* are probably naturally circulating in foxes and their fleas without dog participation in Chile.

We found *B. henselae* in *P. irritans* from a wild carnivore, which is a rare finding. It was reported once before in red foxes (*Vulpes vulpes*) fleas in Australia [18]. It was also unexpected the finding of *B. rochalimae* in *Ct. felis felis*. Rizzo et al. [39] also found this association in one out of 100 *Ct. felis* retrieved from Guinea pigs (*Cavia porcellus*) in Peru.

Nevertheless, it has to be kept in mind that the fleas were retrieved from the hosts, so we might be detecting DNA from the host blood ingested by the flea, and thus, no transmission competence is being proved here, as abovementioned.

A high proportion of *Ct. felis* pools were positive for *Rickettsia* spp., and those that were successfully sequenced were identified as *R. felis*, the causative agent of flea-borne spotted fever. Indeed, *Ct. felis* is its only known biological vector, although mosquitoes have been suspected to be able to transmit the bacterium [3]. *Rickettsia felis* has been previously detected in Chile in *Ct. felis* retrieved from cats and dogs [31,35,20]. It is worth mentioning that one of the positive *Ct. felis* pools was retrieved from the free-ranging domestic ferret. The pre-admission history of this animal was unknown, but likely escaped from an owner, as there are no naturalized populations of ferrets in the wild in Chile. We do not know either if this animal got parasitized before or after escaping. Nonetheless, the fact that the fleas it was hosting resulted positive for *R. felis* enhances the zoonotic risk at the domestic/wildlife interface.

The detection of a fragment of the *ompA* gene with 100 % identity with *R. felis* in a *P. irritans* pool was unexpected. Although it was previously confirmed in this a *P. irritans* retrieved from an Ugandan dog [33], the majority of studies investigating *R. felis* in *P. irritans* have usually resulted negative (e.g., [25,23]). Again, this DNA may belong to the flea blood meal.

In conclusion, although this was an opportunistic survey, with a limitation in space and time, we confirmed for the first time the presence of *B. henselae*, *B. rochalimae*, *B. berkhoffii* and *R. felis* in fox-associated fleas in Chile. We propose that there is a natural circulation of diverse flea-borne pathogens involving wild foxes and their fleas in Chile. Conversely, fleas are useful as sentinels for the presence and distribution of pathogens in the natural environment. Finally, veterinarians, researchers and other workers from wildlife rescue centers must be aware of the risk of contracting flea-borne pathogens during their professional activities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2023.101983.

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