RESEARCH

Molecular detection of *Rickettsia* in fleas from micromammals in Chile

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Abstract

Background: Rickettsial diseases are considered important in public health due to their dispersal capacity determined by the particular characteristics of their reservoirs and/or vectors. Among the latter, fleas play an important role, since the vast majority of species parasitize wild and invasive rodents, so their detection is relevant to be able to monitor potential emerging diseases. The aim of this study was to detect, characterize, and compare *Rickettsia* spp. from the fleas of micromammals in areas with different human population densities in Chile.

Methods: The presence of *Rickettsia* spp. was evaluated by standard polymerase chain reaction (PCR) and sequencing in 1315 fleas collected from 1512 micromammals in 29 locations, with different human population densities in Chile. A generalized linear model (GLM) was used to identify the variables that may explain *Rickettsia* prevalence in fleas.

Results: DNA of *Rickettsia* spp. was identified in 13.2% (174 of 1315) of fleas tested. Fifteen flea species were found to be *Rickettsia*-positive. The prevalence of *Rickettsia* spp. was higher in winter, semi-arid region and natural areas, and the infection levels in fleas varied between species of flea. The prevalence of *Rickettsia* among flea species ranged between 0–35.1%. Areas of lower human density showed the highest prevalence of *Rickettsia*. The phylogenetic tree showed two well-differentiated clades with *Rickettsia bellii* positioned as basal in one clade. The second clade was subdivided into two subclades of species related to *Rickettsia* of the spotted fever group.

Conclusions: To our knowledge, this is the first report of the occurrence and molecular characterization of *Rickettsia* spp. in 15 flea species of micromammals in Chile. In this study, fleas were detected carrying *Rickettsia* DNA with zoonotic potential, mainly in villages and natural areas of Chile. Considering that there are differences in the prevalence of *Rickettsia* in fleas associated with different factors, more investigations are needed to further understand the ecology of *Rickettsia* in fleas and their implications for human health.

Keywords: Pathogen, Bacteria, Vectors, Fleas, Rodents, Marsupials

Background

Rickettsia spp. are obligate intracellular microorganisms, Gram-negative coccobacilli, with the ability to reproduce, both in the nucleus and in the cytoplasm of infected cells [1]. These bacteria have a vertebrate reservoir and an arthropod vector (e.g. ticks, mites, fleas and lice); in some cases, the latter may be affected by these bacteria [2]. They have a worldwide distribution and are the causative agents of serious human infections [3].

Currently, 32 species are recognized (http://www.bacterio.net/-allnamesmr.html), and there are many strains that have not yet been characterized, while subspecies and uncultivated species are classified as "*Candidatus*" [4]. Recently, using new classification methods based on formal order analysis (FOA), which considers

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Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 2 of 21

whole-genome sequencing analysis, two groups are recognized within the genus *Rickettsia*: the major typhus group (MTG) and major spotted fever group (MSFG). The MTG is divided into the typhus group (TG) and ancestral group (AG) and is transmitted by insects. MSFG includes the *R. felis* group, *R. akari* group, and the "classical" spotted fever group that includes several species transmitted by mites and hard ticks, of which the most important are *R. rickettsii* and *R. conorii*, that cause Rocky Mountain spotted fever and Mediterranean spotted fever, respectively [4]. Since *Rickettsia* research has focused on species that affect humans, other species have received less attention [5]. Thus, there are several species of rickettsiae identified and are exclusively associated with arthropods. They are without known secondary hosts and associated with other organisms such as herbivorous insects, leeches, amoebas, inclusive algae, and plants, indicating that these are more common than suspected [5, 6], and that the effects they could cause in humans when contact is made are unknown.

Worldwide, micromammals, and especially rodents, are the main flea hosts. It is recognized that 74% of known flea species parasitize them; therefore, rodents play a fundamental role in the spread of flea-borne diseases, as various species of rodent fleas can also parasitize humans [7]. In addition to this, many rodent species are capable of inhabiting wild environments and adapting to rural and urban environments, which could favor a continuous gradient of transmission between domestic and wild species, and humans [8, 9]. In Chile, despite the great diversity of described fleas (114 species), which mainly parasitize rodents [10, 11], a scarce number of studies have detected *Rickettsia* in fleas [12–15]. These studies have focused on the molecular detection of pathogens in fleas of domestic mammals, identifying *R. felis* from cat and dog fleas (*Ctenocephalides felis* and *C. canis*) in central (Metropolitan region) and southern Chile (Valdivia) [12–14]. Recently, "*Candidatus* Rickettsia asembonensis", "*Candidatus* Rickettsia senegalensis", and *R. felis*, were detected in *C. felis* from cats in the Easter Island (Rapa Nui) [15]. No studies have shown their presence in rodent fleas. If this adds to the expansion of the human population invading wild areas, the chance of contacting fleas on infected rodents increases. Since, in some places, peri-urban rodents provide a link between wild rodent and human communities, humans are exposed to some zoonotic agents that circulate in these natural ecosystems [16, 17].

The aim of this study was to detect, characterize, and compare *Rickettsia* spp. from the fleas of micromammals in areas with different human population densities in Chile. The findings will provide the baseline for the future surveillance of *Rickettsia* spp. in Chile.

Methods

Sample localities and micromammal-trapping procedures

A total of 1512 micromammals belonging to 18 species (Table 1) were captured during a trapping effort of 11,034 trap/nights from 23 localities (9 cities, 6 villages and 8 natural areas) of the 29 sampled, covering 10 administrative regions in Chile and five bioclimatic regions (hyper-arid, arid, semi-arid, sub-humid and hyper-humid), latitude between −20.2167 and −53.1667 (Fig. 1). It was conducted from December 2015 to January 2018, during austral summer (December to February) and austral winter (July and September). These localities were selected based on the following demographic characteristics: (i) city, urban entity that has > 5000 inhabitants; (ii) village, urban entity with a population ranging between 2001–5000 inhabitants, or between 1001–2000 people, where less than 50% of the population that declares having worked, is engaged in primary activities (e.g. livestock, agriculture or fishing) [18]; and (iii) natural area, without human settlement, corresponding to national park (NP; unaltered areas of natural and biological diversity), and national reserves (NR; areas protecting wildlife populations or natural resources).

Micromammals were captured using a Sherman trap (23 × 7.5 × 9 cm, Sherman Co., Tallahassee, USA) and wire-mesh traps (30 × 10 × 11 cm; Forma Ltd., Santiago, Chile) baited with oats. The associated use of both types of traps strongly reduced the likelihood of a species being present but not captured. Each locality was sampled for two consecutive nights. In each sampling locality, the traps were placed in four parallel lines approximately 100 m from each other, and each line was equipped with 50 traps set 10 m apart from each other. Only in cities, traps were used along lines with a 5–10 m inter-trap space, and the traps were placed outside the buildings. The rodents were removed from the traps according to standard techniques [19], and were subsequently anesthetized with ketamine:xilazine (1:1) [20]. Flea samples from rodents were collected by hand or with forceps from the host and placed into sterile cryovials tubes with 95% ethanol. For each rodent, the total number of extracted fleas was recorded (abundance); with these data, the overall mean infection intensity (the number of fleas collected from all species/number of infested hosts), the overall mean abundance of infection (the number of collected fleas from all species/total number of hosts), and prevalence (the proportion of infected hosts) were calculated. The micromammals were identified following Iriarte [21]. Micromammals were released after sampling, except for invasive rodents [black rat (*Rattus rattus*), Norway rat (*Rattus norvegicus*), and house mouse (*Mus musculus*)] that were euthanized by cervical dislocation [19].

DNA extraction and PCR amplification

For DNA extraction, 5 fleas per host were selected, and when the number of fleas per host was less than 5, all the fleas were analyzed. Finally, DNA extraction was performed from 1315 fleas. Each flea was washed and cut between the third and fourth abdominal tergite with a scalpel. DNA was extracted from individual fleas using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The incubation time was 5 h; following DNA extraction, the flea's exoskeleton was recovered and stored in 96% ethanol to later mount and identify the flea species.

The presence of *Rickettsia* spp. was initially screened by polymerase chain reaction (PCR) using a short fragment of citrate synthase (*gltA*) gene (401 bp; Table 2) [22]. Thereafter, *gltA* positive samples were tested using three genes: *gltA* (830 bp) [22], *sca5* (*ompB*) [23], and we designed a set of primers for the β-subunit of RNA polymerase (*rpoB*) of *Rickettsia* sp. (GenBank: AF076436; Table 2). The amplification conditions were as follows: 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s of annealing temperature (see Table 2), 30 s at 72 °C, followed by a final extension of 5 min at 72 °C. The reactions were performed with GoTaq Green Master Mix 2X (Promega, Madison, USA) 12.5 µl, 5.5 µl of ultrapure nuclease-free water, 2 µl of forward primer (10 µM), 2 µl of reverse primer (10 µM), and 4 µl of DNA sample. The negative controls were carried out with ultrapure water, and the positive control was genomic DNA of *R. conorii* (AmpliRun® *Rickettsia conorii* DNA Control; Vircell, Granada, Spain). A selected number of *Rickettsia*-positive samples were purified and sequenced by the Macrogen Company (Seoul, Korea).

Table 1 Micromammal species captured, and fleas collected from 29 locations in Chile

Family and species of micromammal	No. micromammals collected	No. micromammals with fleas	No. of fleas collected	Prevalence (%) (95% CI)	Mean abundance (95% CI)	Mean intensity (95% CI)
Order Didelphimorphia						
Didelphidae						
Thylamys elegans	35	18	54	51.4 (33.98–68.62)	1.5 (0.83–2.97)	3.0 (1.83–5.22)
Order Rodentia						
Cricetidae						
Abrothrix hirta	319	191	643	59.9 (54.58–65.60)	2.0 (1.73–2.32)	3.4 (2.98–3.76)
Abrothrix lanosus	1	1	1	100	1.0	1.0
Abrothrix longipilis	5	4	9	80.0 (28.35–99.50)	1.8 (0.60–2.80)	2.3 (1.25–3.25)
Abrothrix olivacea	434	206	518	47.5 (42.68–52.29)	1.2 (1.03–1.37)	2.5 (2.27–2.80)
Chelemys macronyx	1	0	0	0	0	–
Irenomys tarsalis	1	0	0	0	0	–
Loxodontomys micropus	24	21	66	87.5 (67.63–97.35)	2.8 (1.96–3.79)	3.1 (2.38–4.24)
Oligoryzomys longicaudatus	229	81	162	35.4 (29.18–41.95)	0.7 (0.55–0.88)	2.0 (1.72–2.36)
Phyllotis darwini	120	49	133	40.8 (31.95–50.18)	1.1 (0.82–1.42)	2.7 (2.24–3.20)
Phyllotis limatus	2	0	0	0	0	–
Reithrodon physodes	5	2	6	40.0 (5.27–85.34)	1.2 (0.00–3.20)	3.0 (1.00–3.00)
Octodontidae						
Octodon bridgesi	1	1	2	100	2.0	2.0
Octodon degus	69	54	387	78.3 (66.69–87.30)	5.6 (4.20–7.78)	7.2 (5.56–9.93)
Abrocomidae						
Abrocoma bennetti	3	3	77	100	25.7 (5.00–45.00)	25.7 (5.00–45.00)
Muridae						
Mus musculus	11	2	0	18.2 (2.28–51.78)	0.2 (0.00–0.36)	1 (0.00–0.00)
Rattus norvegicus	2	0	0	0	0	–
Rattus rattus	250	73	214	29.2 (23.64–35.27)	0.9 (0.64–1.14)	2.9 (2.40–3.70)
Total	1512	706	2272	46.7 (44.12–49.20)	1.5 (1.38–1.66)	3.2 (2.99–3.59)

Note: The total number of rodents captured for each species, number of parasitized rodents, prevalence of fleas parasitizing rodents, total number of fleas collected, mean abundance, and mean intensity are indicated

Abbreviation: CI, confidence interval
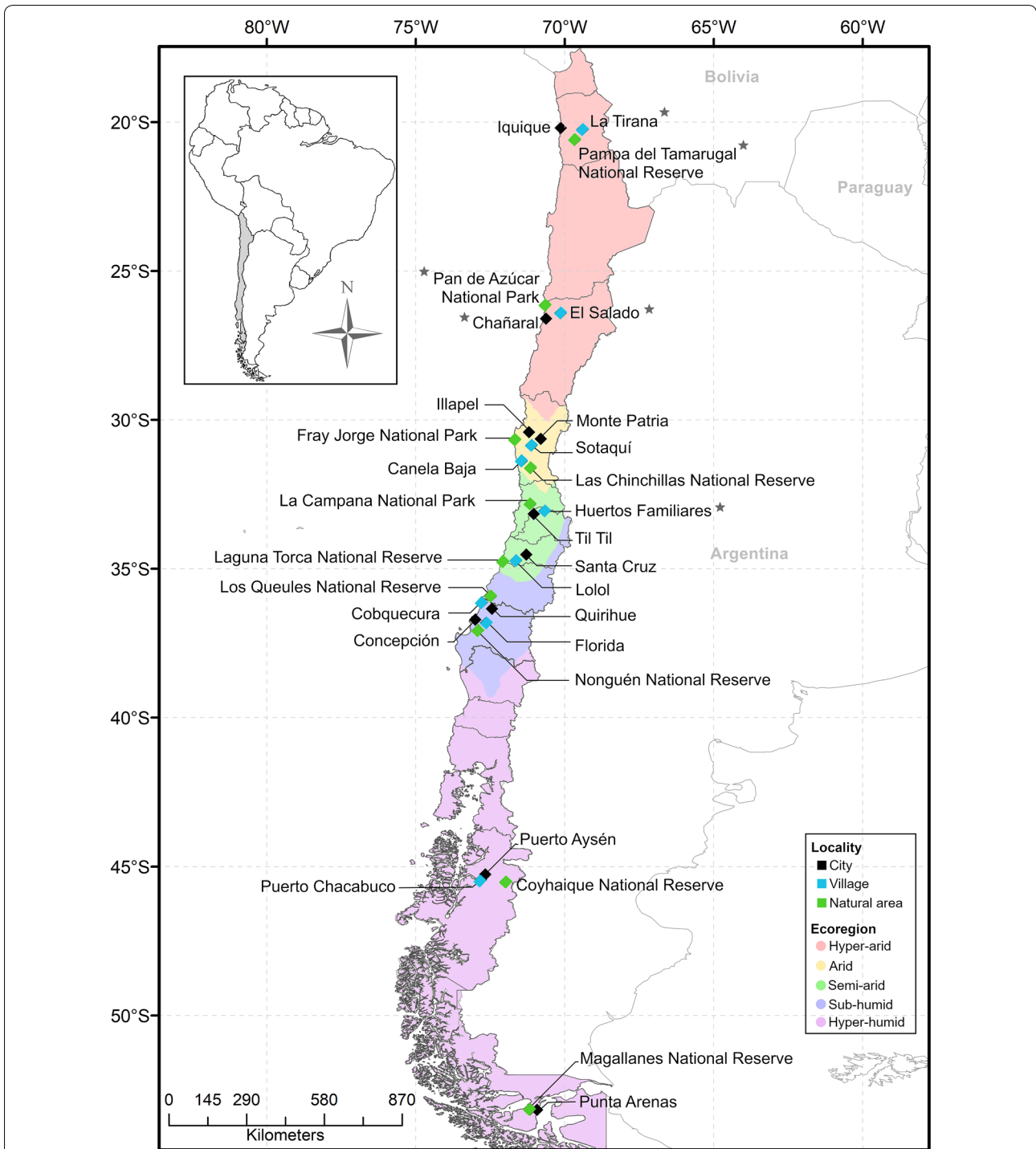
Fig. 1 Study area. There are indicated the type of locality where the micromammals were collected. The stars indicate the locations where rodents were not captured

Phylogenetic and BLAST analyses

All DNA sequences were edited and aligned using the Codon Code Aligner (CodonCode Corporation, Centerville, MA, USA). All sequences generated in this study were compared with those available on GenBank using the BLAST program (see http://www.ncbi.nlm.nih.gov/BLAST/). A Bayesian probabilities tree was created using MrBayes 3.2 based on *gltA* 830-bp gene fragment, using

Anaplasma phagocytophilum as the outgroup. We used the GTR + G substitution model to reconstruct the tree and 10,000,000 bootstrap trials.

Flea mounting and identification

After DNA extraction, each flea's exoskeleton was recovered and mounted on glass slides using conventional procedures. The fleas were identified using a light microscope, taxonomic keys, and the descriptions of Johnson [24], and Sanchez & Lareschi [25]. Voucher specimens (slides) were catalogued in the Museo de Zoología at Universidad de Concepción (MZUC-UCCC, Concepción, Chile) under the accession numbers 46647–46667.

Statistical analysis

The prevalence (percentage of micromammals parasitized with fleas) and abundance mean (mean number of fleas per host) in species of micromammals was calculated with total of samples of fleas collected ($n = 2272$), and confidence intervals (95% CI) were calculated, using bootstrap (2000 bootstrap replicates). The prevalence of *Rickettsia* (percentage of fleas infected with *Rickettsia*) was calculated based on the PCR results. We used generalized linear models (GLM) with binomial distribution and logit function to identify the variables that may explain *Rickettsia* prevalence in fleas. The explanatory variables analyzed were bioclimatic regions (hyper-arid; arid; semi-arid; sub-humid; and hyper-humid), location type (city; village and natural area) and season (summer and winter). First, we built a model that included all bioclimatic regions and then we built models for each bioclimatic region. To assess the relationship between the prevalence and sample size, a Spearman correlation analysis was performed. The Chi-square test or Fisher's exact test (if an expected cell count was < 5) was used to evaluate the differences in the prevalence of *Rickettsia* among species of flea. A *P*-value < 0.05 was considered statistically significant. The data were analyzed using JMP software® (SAS Institute Inc., Cary NC, USA).

Nucleotide sequence accession numbers

Rickettsia sequences generated in this study were deposited in the NCBI GenBank database under the following accession numbers: MN630893-MN630962 (*gltA*); MN630963-MN630997 (*rpoB*) and MT834938-MT834942 (*sca5*).

Results

A total of 2272 fleas were collected from 13 micromammal species, with an overall prevalence of 46.7% ($n = 706$). The overall mean abundance was 1.5 fleas per host and the overall mean intensity was 3.2 fleas per parasitized host (Table 1). Excluding the species in which < 20 individuals were sampled, the micromammals that presented the highest prevalence of fleas were *Loxodontomys micropus* (Austral greater mouse, 87.5%) and *Octodon degus* (Fence degu, 78.3%), and the lowest prevalence was found in *R. rattus* (29.2%). The abundance and mean intensity were higher in *O. degus* (Table 1). The marsupial *Thylamys elegans* (Llaca mouse-opossum) had a prevalence of fleas of 51.4%. All of the flea species found in *T. elegans* corresponded to species that were also found in rodents (Table 3).

Of all collected fleas, 1315 flea specimens were analyzed, corresponding to 27 species from 15 genera and 8 families (Table 4). The most abundant flea species were *Sphinctopsylla ares* ($n = 211$) and *Neotyphloceras chilensis* ($n = 202$; Table 4). The rodents that presented the greatest flea richness were *Abrothrix olivacea* (olive grass mouse, 17 spp.), *R. rattus* (14 spp.), *A. hirta* (long-haired

Table 2 Primer sequences and annealing temperatures used to detect *Rickettsia* spp.

Target gene	Primer name	Nucleotide sequence (5′–3′)	Annealing T (℃)	Product length (bp)
ProgltA (401)	CS-78_F	GCAAGTATCGGTGAGGATGTAAT	48[a]	401
	CS-323_R	GCTTCCTTAAAATTCAATAAATCAGGAT		
gltA (830)	CS-239_F	GCTCTTCTCATCCTATGGCTATTAT	48[a]	830
	CS-1069_R	CAGGGTCTTCGTGCATTTCTT		
rpoB (395)	RirpoB_F	CCGACTCATTACGGTCGCATTTGT	55.5	395
	RirpoB_R	CCCATCAAAGCACGGTTAGCATCA		
sca5 (862)	120.M59F	CCGCAGGGTTGGTAACTGC	50[b]	862
	120.807R	CCTTTTAGATTACCGCCTAA		

[a] Labruna et al. [22]

[b] Roux & Raoult [23]

Abbreviations: F, forward; R, reverse; T, temperature

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 6 of 21

grass mouse, 11 spp.), and *Oligoryzomys longicaudatus* (long-tailed pygmy rice rat, 11 spp.; Table 3). Natural areas were where the largest number of flea species ($n = 25$) and specimens were collected ($n = 784$), followed by villages (18 species, 349 specimens) and cities (18 species, 181 specimens). *Agastopsylla boxi, Ctenoparia jordani, C. topali, Ectinorus cocyti* and *Plocopsylla lewisi* were exclusive to natural areas (national parks and national reserves). Conversely, *Xenopsylla cheopis* was only found in one city (Iquique). *Neotyphloceras chilensis* and *S. ares* were the dominant species in natural areas (*N. chilensis* ($n = 119$); *S. ares* ($n = 151$)), and villages (*N. chilensis* ($n = 83$); *S. ares* ($n = 50$)), while *Nosopsyllus fasciatus* ($n = 37$), and *C. inopinata* ($n = 25$) were the most frequently collected in cities. *Leptopsylla segnis, N. fasciatus*, and *X. cheopis* are synanthropic rodent fleas [26], and were more abundant in cities than in villages and natural areas.

Rickettsiae prevalence on fleas

Fifteen flea species were found to be *Rickettsia*-positive for the short fragment (401 bp) of the *gltA* gene, 9 for the long fragment (830 bp) of the *gltA* gene, 10 for the *rpoB* gene, and 4 for the *sca5* gene (Table 4). The highest prevalence (13.2%) was detected with the *gltA* 401-bp gene, followed by the *rpoB* (5.9%), *gltA* 830-bp (5.0%) and *sca5* (0.5%) genes (Table 4). Among the flea species in which more than 20 individuals were analyzed, the prevalence varied between 0–35.1%. The *Neotyphloceras* spp. had the highest prevalence of *Rickettsia* (*gltA* 401-bp = 29.4%, *gltA* 830-bp = 9.56%, and *rpoB* = 11.25%; Table 4). The four fragments (*gltA* 401-bp, *gltA* 830-bp, *rpoB* and *sca5*) showed significant differences in the prevalence of detected *Rickettsia* ($\chi^2 = 193.207$, $df = 3$, $P < 0.001$), exception for *gltA* 830-bp and *rpoB*, which did not show significant differences ($\chi^2 = 1.934$, $df = 1$, $P = 0.164$). No association was found between the number of fleas analyzed and the prevalence of *Rickettsia* detected for any of the genes analyzed (*rpoB*: $\rho = 0.4267$, $P = 0.12$; *gltA*: $\rho = 0.3757$, $P = 0.18$; *sca5*: $\rho = 0.3272$, $P = 0.35$).

According to the GLM analysis, the prevalence of *Rickettsia* infection was significantly higher in the semi-arid region (27.8%). In addition, the overall prevalence was significantly higher in the winter (20.6%) than in the summer (5.3%). The prevalence of *Rickettsia* was higher in natural areas (15.9%), and cities exhibited a marginally significant lower prevalence (4.97%) compared to the other two location types (village: 11.2%; Table 5). Comparisons between bioclimatic regions showed that in the arid region, the prevalence of *Rickettsia* was higher in the natural areas and in the winter. While in the semi-arid region, the highest prevalence occurred in the winter (73.7%), and the highest prevalence of *Rickettsia* was detected in the natural areas (77.8%), differentiating from

the cities (14.0%). In the sub-humid region, there was no effect of the factors on the prevalence of *Rickettsia*, whereas in the hyper-humid region, we detected *Rickettsia* (5.49%) only in the natural areas.

BLAST analysis and phylogenetic inference

A total of 167 sequences of *gltA* 401-bp ($n = 68$), *gltA* 830-bp ($n = 40$), *rpoB* ($n = 54$) and *sca5* ($n = 5$) genes were analyzed (Table 6). For *gltA* 401-bp, out of the 68 sequences, 28 isolated from *Delostichus phyllotis* ($n = 1$), *L. segnis* ($n = 1$), *N. crassispina* ($n = 1$), *N. pardinasi* ($n = 3$), *Neotyphloceras* spp. ($n = 7$), *N. fasciatus* ($n = 3$), *Plocopsylla* sp. ($n = 2$), *S. ares* ($n = 3$), *T. rhombus* ($n = 1$) and *Tetrapsyllus tantillus* ($n = 6$) were 100% identical to *Rickettsia* sp. (GenBank: KY705378) obtained from the tick *Amblyomma parvitarsum*. Another 19 *gltA* sequences (401-bp) detected in *Neotyphloceras* spp. ($n = 16$), *Chiliopsylla allophyla* ($n = 2$) and *C. inopinata* ($n = 1$) were closely related to *Rickettsia* sp. MEAM1 (99%; GenBank: CP016305) isolated from whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) ($n = 16$) and *Rickettsia* sp. Gr15 (GenBank: KP675966) detected in the tick *Hyalomma marginatum* ($n = 3$). Twenty-one sequences amplified from *Neotyphloceras* spp. ($n = 1$), *S. ares* ($n = 13$) and *T. rhombus* ($n = 6$) showed 97–98% identity with *Rickettsia* sp. (GenBank: U59712) isolated from *Adelia bipunctata* (Coleoptera: Coccinellidae). One sequence amplified from *S. ares* showed 93% similarity with uncultured *Rickettsia* sp. (GenBank: KY433588) detected in a tick.

Two sequences of *gltA* 830-bp segments showed high identity (99%) to "*Candidatus* Rickettsia senegalensis" (GenBank: KU499847) previously identified in a cat flea (*C. felis*). Forty sequences obtained from *S. ares* ($n = 12$), *T. rhombus* ($n = 6$), *Neotyphloceras* spp. ($n = 19$) and *C. inopinata* ($n = 1$) shared 97–98% identity with *Rickettsia* spp. (GenBank: KF646706; KY799066; U76908; and AJ269522) isolated from the insects *Nesidiocoris tenuis* (Heteroptera: Miridae), *Mansonia uniformis* (Diptera: Culicidae), *Empoasca papayae* (Hemiptera: Cicadellidae) and *Adalia decempunctata* (Coleoptera: Coccinellidae).

Seventeen amplified *rpoB* sequences in *Neotyphloceras* spp. shared 93–100% similarity with *Rickettsia* sp. MEAM1 (GenBank: CP016305) isolated from *B. tabaci*. Another 24 sequences derived from *C. allophyla* ($n = 2$), *C. inopinata* ($n = 1$), *Neotyphloceras* spp. ($n = 1$), *S. ares* ($n = 14$) and *T. rhombus* ($n = 6$) showed between 91% and 100% homology with *Rickettsia* sp. (GenBank: JF966777) of *Synosternus pallidus* (Siphonaptera: Pulicidae). Nine amplified sequences from *Neotyphloceras* spp. ($n = 9$) were 94–96% similar to *Rickettsia* sp. (GenBank: KX300157) isolated from a bat (*Myotis emarginatus*). Finally, 4 sequences isolated from *Neotyphloceras* spp.

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 7 of 21

Table 3 Flea species identified for each micromammal species collected in this study

Family/species of micromammal	Family of flea	Species of flea
Cricetidae		
Abrothrix hirta	Hystricopsyllidae	*Chiliopsylla allophyla* (Rothschild, 1915)
		Ctenoparia inopinata (Rothschild, 1909)
		Ctenoparia topalli (Smit, 1963)
		Ctenoparia jordani (Smit, 1955)
	Ctenophthalmidae	*Neotyphloceras crassispina* (Rothschild, 1914)
		Neotyphloceras pardinasi (Sanchez & Lareschi, 2014)
		Neotyphloceras spp.
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Tetrapsyllus amplus* (Jordan & Rothschild, 1923)
		Tetrapsyllus tantillus (Jordan & Rothschild, 1923)
		Tetrapsyllus rhombus (Smit, 1955)
Abrothrix lanosus	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
Abrothrix longipilis	Ctenophthalmidae	*Neotyphloceras chilensis* (Lewis, 1976)
	Rhopalopsyllidae	*Tetrapsyllus corfidii* (Rothschild, 1904)
	Pulicidae	*Hectopsylla* spp.
Abrothrix olivacea	Hystricopsyllidae	*Ctenoparia inopinata* (Rothschild, 1909)
		Ctenoparia jordani (Smit, 1955)
		Ctenoparia topalli (Smit, 1963)
	Ctenophthalmidae	*Neotyphloceras crassispina* (Rothschild, 1914)
		Neotyphloceras chilensis (Lewis, 1976)
		Neotyphloceras pardinasi (Sánchez & Lareschi, 2014)
		Agastopsylla boxi (Jordan & Rothschild, 1923)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Ectinorus cocyti* (Rothschild, 1904)
		Tetrapsyllus amplus (Jordan & Rothschild, 1923)
		*Tetrapsyllus tantillus (*Jordan & Rothschild, 1923)
		Tetrapsyllus rhombus (Smit, 1955)
		Tetrapsyllus corfidii (Rothschild, 1904)
		Listronius spp.
	Pulicidae	*Hectopsylla* spp.
	Leptopsyllidae	*Leptopsylla segnis* (Schönherr, 1811)
Oligoryzomys longicaudatus	Hystricopsyllidae	*Ctenoparia inopinata* (Rothschild, 1909)
		Ctenoparia topalli (Smit, 1963)
	Ctenophthalmidae	*Neotyphloceras chilensis* (Lewis, 1976)
		Neotyphloceras crassispina (Rothschild, 1914)
		Neotyphloceras pardinasi (Sánchez & Lareschi, 2014)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Ectinorus chilensis* (Lewis, 1976)
		Tetrapsyllus amplus (Jordan & Rothschild, 1923)
		Tetrapsyllus rhombus (Smit, 1955)
	Leptopsyllidae	*Leptopsylla segnis* (Schönherr, 1811)

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 8 of 21

Table 3 (continued)

Family/species of micromammal	Family of flea	Species of flea
Phyllotis darwini	Ctenophthalmidae	*Neotyphloceras chilensis* (Lewis, 1976)
		Neotyphloceras crassispina (Rothschild, 1914)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Delostichus* spp.
		Delostichus phyllotis (Johnson, 1957)
		Delostichus smiti (Jameson & Fulk, 1977)
		Tetrapsyllus rhombus (Smit, 1955)
		Tetrapsyllus tantillus (Jordan & Rothschild, 1923)
	Pulicidae	*Hectopsylla* spp.
	Tungidae	*Tunga* spp.
Loxodontomys micropus	Ctenophthalmidae	*Neotyphloceras* spp.
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
Octodontinidae		
Octodon bridgesi	Rhopalopsyllidae	*Delostichus phyllotis* (Johnson, 1957)
		Tetrapsyllus spp.
Octodon degus	Ctenophthalmidae	*Neotyphloceras* spp.
		Neotyphloceras chilensis (Lewis, 1976)
	Rhopalopsyllidae	*Delostichus* spp.
		Delostichus coxalis (Rothschild, 1909)
		Delostichus degus (Beaucournu, Moreno & González, 2011)
		Delostichus phyllotis (Johnson, 1957)
		Delostichus smiti (Jameson & Fulk, 1977)
		Ectinorus chilensis (Lewis, 1976)
		Tetrapsylllus corfidii (Rothschild, 1904)
		Tetrapsyllus tantillus (Jordan & Rothschild, 1923)
Abrocomidae		
Abrocoma bennetti	Ctenophthalmidae	*Neotyphloceras* spp.
		Neotyphloceras chilensis (Lewis, 1976)
	Rhopalopsyllidae	*Delostichus* spp.
		Delostichus coxalis (Rothschild, 1909)
		Delostichus phyllotis (Johnson, 1957)
		Delostichus smiti (Jameson & Fulk, 1977)
		Ectinorus chilensis (Lewis, 1976)
		Tetrapsyllus corfidii (Rothschild, 1904)
Muridae		

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 9 of 21

Table 3 (continued)

Family/species of micromammal	Family of flea	Species of flea
Rattus rattus	Hystricopsyllidae	*Ctenoparia inopinata* (Rothschild, 1909)
		Ctenoparia jordani (Smit, 1955)
	Ctenophthalmidae	*Neotyphloceras* spp.
		Neotyphloceras chilensis (Lewis, 1976)
		Neotyphloceras pardinasi (Sánchez & Lareschi, 2014)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
		Plocopsylla spp.
		Plocopsylla wolffsohni (Rothschild, 1909)
	Rhopalopsyllidae	*Delostichus coxalis* (Rothschild, 1909)
		Delostichus smiti (Jameson & Fulk, 1977)
		Tetrapsyllus rhombus (Smit, 1955)
	Leptopsyllidae	*Leptopsylla segnis* (Schönherr, 1811)
	Pulicidae	*Xenopsylla cheopis* (Rothschild, 1903)
		Hectopsylla spp.
Mus musculus	Leptopsyllidae	*Leptopsylla segnis* (Schönherr, 1811)
Order Didelphimorphia		
Didelphidae		
Thylamys elegans	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Ctenophthalmidae	*Neotyphloceras* spp.
		Neotyphloceras chilensis (Lewis, 1976)
		Neotyphloceras crassispina (Rothschild, 1914)
	Rhopalopsyllidae	*Delostichus smiti* (Jameson & Fulk, 1977)
		Tetrapsyllus tantillus (Jordan & Rothschild, 1923)

(*n* = 3) and *T. rhombus* (*n* = 1) showed lower homology with *Rickettsia* sp. (94%, GenBank: KX300203) isolated from a bat (*Eptesicus serotinus*).

Three *sca5* fragments isolated from *C. allophyla* (*n* = 2) and *C. inopinata* (*n* = 1) showed homology with *R. felis* (94%; GenBank: GQ385243), and 2 fragments detected from *S. ares* showed low identity to *R. hoogstraalii* (GenBank: EF629536) (Table 6).

The phylogenetic tree shows two well-differentiated clades with 100% nodal support (Fig. 2). Clade R1 was formed by sequences obtained from *Neotyphloceras* fleas collected in Las Chinchillas NR (31°30′36″S, 71°05′15″W), Canela Baja (31°23′54″S, 71°27′27″W), and Fray Jorge NP (30°23′S, 71°23′W). *Rickettsia bellii* (Gen-Bank: DQ146481) was positioned on a basal branch in this group. The clade R2 was subdivided into two subclades: R2a and R2b. R2a, with 93% nodal support, is related to sequences obtained from *T. rhombus* and *S. ares* collected in Los Queules NR, Cobquecura, and Coyhaique NR, comprising a larger area of distribution

(latitude: − 35° to − 45°S) than clade R1. Subclade R2b was formed by sequences obtained from *C. inopinata* and *C. allophyla* collected in Los Queules NR and Non-guén NR, respectively. The newly generated sequences were positioned closely to *R. hoogstraalii* (GenBank: FJ767737) isolated from *Haemaphysalis sulcata* (tick) in Croatia [27], *R. asembonensis* detected in *C. felis* from Peru (GenBank: KY650697) [28] and *R. felis* isolated from *C. felis* in Brazil (GenBank: JN375498) [29].

Discussion

To the best of our knowledge, we have provided for the first time evidence for the presence of *Rickettsia* DNA in 15 flea species identified on wild micromammals and synanthropic rodents in Chile. The prevalence of *Rickettsia* spp. infections in fleas varied between species of flea, bioclimatic regions, seasons and location type. We found a higher prevalence in winter, the semi-arid region and natural areas.

Moreno-Salas *et al. Parasites Vectors* (2020) 13:523

Page 10 of 21

Table 4 *Rickettsia* prevalence detected on fleas for each gene used in the different flea species analyzed

Family and species of flea	No. of fleas analyzed	No. of fleas positive for gene fragment (Prevalence in %)			
		gltA 401 bp	*gltA* 830 bp	*rpoB* 395 bp	*sca5* 862 bp
Hystricopsyllidae					
Chiliopsylla allophyla	7	2 (28.6)	2 (28.6)	2 (28.6)	2 (28.6)
Ctenoparia spp.	20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ctenoparia inopinata	85	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.2)
Ctenoparia topali	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ctenoparia jordani	5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ctenophthalmidae					
Agastopsylla boxi	3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Neotyphloceras spp.	128	40 (31.3)	7 (5.5)	10 (7.8)	0 (0.0)
Neotyphloceras crassispina	35	2 (5.7)	0 (0.0)	2 (5.7)	0 (0.0)
Neotyphloceras chilensis	202	71 (35.1)	29 (14.4)	29 (14.4)	0 (0.0)
Neotyphloceras pardinasi	43	7 (16.3)	3 (7.0)	5 (11.6)	0 (0.0)
Ceratophyllidae					
Nosopsyllus fasciatus	52	7 (13.5)	1 (1.9)	2 (3.8)	0 (0.0)
Stephanocircidae					
Sphinctopsylla ares	211	20 (9.5)	16 (7.6)	19 (9.0)	2 (0.9)
Plocopsylla spp.	4	2 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
Plocopsylla wolffsohni	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Plocopsylla lewisi	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Rhopalopsyllidae					
Delostichus spp.	12	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Delostichus degus	22	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Delostichus coxalis	53	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Delostichus phyllotis	7	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)
Delostichus smiti	85	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ectinorus spp.	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ectinorus cocyti	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ectinorus chilensis	12	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)
Tetrapsyllus spp.	11	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tetrapsyllus amplus	17	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tetrapsyllus tantillus	93	10 (10.8)	1 (1.1)	1 (1.1)	0 (0.0)
Tetrapsyllus corfidii	16	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tetrapsyllus rhombus	74	8 (10.8)	6 (8.1)	7 (9.5)	1 (1.4)
Listronius spp.	3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tungidae					
Tunga spp.	4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pulicidae					
Hectopsylla spp.	30	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)
Xenopsylla cheopis	11	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Leptopsyllidae					
Leptopsylla segnis	63	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)
Total	1315	174 (13.2)	66 (5.0)	78 (5.9)	6 (0.5)

The fleas were characterized as being highly host-opportunistic, occupying various host species [7]. This is confirmed by our study, since of the 27 flea species collected, 19 parasitized more than one species of micromammal. We also highlight the high flea species richness recorded in *R. rattus*, where 10 of the 14 species identified in this rodent correspond to the flea species identified on native rodents. This rodent was mainly

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 11 of 21

Table 5 Generalized linear models (GLM) of *Rickettsia* prevalence

Modell	Model performance			Model component				
	L-R χ^2	df	Prob > χ^2	Source of variation	Estimate	SE	L-R χ^2	*P*-value
All bioclimatic regions	102.61	7	< 0.0001*	Intercept	2.51	0.33	60.16	< 0.0001*
				Season (winter)	− 0.83	0.12	49.71	< 0.0001*
				Bioclimatic region (arid)	− 0.29	0.34	0.76	0.3840
				Bioclimatic region (hyper-arid)	1.00	1.24	0.65	0.4205
				Bioclimatic region (hyper-humid)	− 0.18	0.46	0.14	0.7001
				Bioclimatic region (semi-arid)	− 1.40	0.37	14.07	0.0002*
				Location type (natural area)	− 0.42	0.15	8.02	0.0046*
				Location type (city)	0.45	0.23	3.57	0.0588#
Arid	62.80	3	< 0.0001*	Intercept	2.31	0.20	314.21	< 0.0001*
				Season (winter)	− 0.87	0.16	44.07	< 0.0001*
				Location type (natural area)	− 0.56	0.19	10.98	0.0009*
				Location type (city)	0.50	0.31	3.40	0.0652
Semi-arid	65.52	3	< 0.0001*	Intercept	0.81	0.42	3.89	0.0484*
				Season (winter)	− 2.27	0.455	56.73	< 0.0001*
				Location type (natural area)	0.62	0.48	1.73	0.1880
				Location type (city)	− 0.99	0.79	1.35	0.2445
Sub-humid	4.45	3	0.2167	Intercept	2.96	0.33	266.75	< 0.0001*
				Season (winter)	0.36	0.22	2.66	0.1026
				Location type (natural area)	− 0.37	0.36	1.65	0.1992
				Location type (city)	0.69	0.58	2.36	0.1241
Hyper-humid	5.08	2	0.0788	Intercept	3.51	0.69	128.38	< 0.0001*
				Location type (natural area)	− 1.08	0.73	5.09	0.0240*
				Location type (city)	0.38	1.09	0.00	1.0000

Abbreviations: L-R, likelihood ratio; *df*, degrees of freedom; SE, standard error; *P* ≤ 0.05, # marginally significant

captured in urban areas; however, we also found it in rural and natural areas, this occurs mainly because these rodents have an omnivore diet and plasticity in their behavior, characteristics that allow them to inhabit a great diversity of environments, adapting successfully to urban, rural and wild environments [30, 31]. *Rickettsia*-positive fleas parasitizing *R. rattus* in these three areas indicate that this species could play a key role in spreading the disease from wild to urban environments [16, 32]. Conversely, we also observed that wild species enter human-occupied environments since they provide shelter and food. *Abrothrix olivacea* was the most frequently captured wild species in urban and rural areas and had the highest flea richness and the highest number of *Rickettsia*-positive fleas. This species has been described to have a "random walk" type of dispersal behavior, so it can easily go from wild to domestic environments [33]. These findings are important because these rodent species could act as "bridge hosts" and aid in the spread of the disease [32, 34]. On the other hand, in natural areas, the rodent species most frequently captured was *A. hirta*; this species, like *A. olivacea*, had a high prevalence of *Rickettsia*-positive fleas. This rodent decreased

its presence in areas with human intervention, which is consistent with the findings reported by Monteverde & Hadora [33], who described that this rodent preferably moves within the wild environment. Rodent populations can act as "source populations" and may be involved in the direct transmission of the pathogen to the target population [34].

The prevalence of *Rickettsia* spp. infections detected in our study was variable (0–35%), and associated with the identity of the flea species, season, type of locality and bioclimatic area. However, similar differences have been reported in other studies. For example, Radzijevskaja et al. [35] reported different prevalence related to the flea species analyzed (range: 0–43%). Also, Kuo et al. [36] carried out an extensive sampling analyzing the presence of *Rickettsia* in six species of flea, reporting 0–12.1% of prevalence in the different species of flea analyzed. Furthermore, flea infestations in this study were generally higher during the winter; however, this did not occur in all bioclimatic areas. Other studies have found similar results, attributing this variation to the differences in the seasonal reproductive cycles of the different species of flea [37], which are unknown in most of the species

Table 6 Similarity percentage for obtained sequences with BLAST analyses

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence on GenBank)							
				gltA 401 bp		gltA 830 bp		rpoB 395 bp		sca5 862 bp	
Flea species	Total sequences amplified per flea	Host	Collection site	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive
Chiliopsylla allophyla	2	*Abrothrix hirta*	Nonguén NR	*Rickettsia* sp. Gr15 (99%; KP675966)	2	*"Candidatus* Rickettsia senegalensis" (99%; KU499847)	2	*Rickettsia* sp. (100%; JF966777)	2	*Rickettsia felis* (94%; GQ385243)	2
Ctenoparia inopinata	1	*Abrothrix hirta*	Los Queules NR	*Rickettsia* sp. Gr15 (97%; KP675966)	1	*Rickettsia* sp. (99%; KY799066)	1	*Rickettsia* sp. (97%; JF966777)	1	*Rickettsia felis* (94%; GQ385243)	1
Delostichus phyllotis	1	*Octodon degus*	La Campana NP	*Rickettsia* sp. (100%; KY705378)	1	na		na		na	
Leptopsylla segnis	1	*Rattus rattus*	Lolol	*Rickettsia* sp. (100%; KY705378)	1	na		na		na	
Neotyphloceras chilensis	4	*Phyllotis darwini*	Las Chinchillas NR	*Rickettsia* sp. MEAM1 (99%; CP016305)	4	*Rickettsia* sp. (97%; KF646706)	4	*Rickettsia* sp. MEAM1 (97–98%; CP016305)	4	na	
	2	*Abrothrix olivacea*	Las Chinchillas NR	*Rickettsia* sp. MEAM1 (98–99%; CP016305)	2	*Rickettsia* sp. (97%; KF646706)	2	*Rickettsia* sp. MEAM1 (98%; CP016305)	2	na	
	1	*Abrothrix olivacea*	Fray Jorge NP	*Rickettsia* sp. MEAM1 (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia* sp. MEAM1 (98%; CP016305)	1	na	
	3	*Abrothrix olivacea*	Canela Baja	*Rickettsia* sp. MEAM1 (99%; CP016305)	3	*Rickettsia* sp. (97%; U76908)	3	*Rickettsia* sp. MEAM1 (98%; CP016305)	3	na	
	1	*Octodon degus*	Canela Baja	*Rickettsia* sp. MEAM1 (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia* sp. MEAM1 (98%; CP016305)	1	na	
	2	*Phyllotis darwini*	Canela Baja	*Rickettsia* sp. MEAM1 (99%; CP016305)	2	*Rickettsia* sp. (97%; U76908)	2	*Rickettsia* sp. MEAM1 (98-100%; CP016305)	2	na	
	1	*Thylamys elegans*	Canela Baja	*Rickettsia* sp. MEAM1 (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia* sp. MEAM1 (98%; CP016305)	1	na	

Table 6 (continued)

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence on GenBank)							
				gltA 401 bp		gltA 830 bp		rpoB 395 bp		sca5 862 bp	
Flea species	Total sequences amplified per flea	Host	Collection site	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive	*Rickettsia* spp.	No. positive
Neotyphloceras crassispina	1	*Oligoryzomys longicaudatus*	Laguna Torca NR	*Rickettsia* sp. (100%; KY705378)	1	na		na		na	
	1	*Oligoryzomys longicaudatus*	Cobquecura	na		na		*Rickettsia* sp. (94%; KX300203)	1	na	
	1	*Abrothrix hirta*	Cobquecura	na		na		*Rickettsia* sp. (94%; KX300203)	1	na	
Neotyphloceras pardinasi	2	*Abrothrix hirta*	Cobquecura	na		na		*Rickettsia* sp. (94–96%; KX300157)	2	na	
	1	*Abrothrix olivacea*	Cobquecura	na		na		*Rickettsia* sp. (94%; KX300157)	1	na	
	1	*Rattus rattus*	La Campana NP	*Rickettsia* sp. (100%; KY705378)	1	na		na		na	
	1	*Rattus rattus*	Quirihue	na		na		*Rickettsia* sp. (94%; KX300203)	1	na	
	2	*Rattus rattus*	Laguna Torca NR	*Rickettsia* sp. (99–100%; KY705378)	2	na		na		na	
	1	*Rattus rattus*	Canela Baja	*Rickettsia* sp. (100%; KY705378)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia* sp. MEAM1 (93%; CP016305)	1	na	

Table 6 (continued)

| | | | | Identification by gene sequence (% similarity with the corresponding sequence on GenBank) | | | | | | | |
| | | | | gltA 401 bp | | gltA 830 bp | | rpoB 395 bp | | sca5 862 bp | |
Flea species	Total sequences amplified per flea	Host	Collection site	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive
Neotyphloceras spp.	1	Oligoryzomys longicaudatus	Cobquecura	na		na		Rickettsia sp. (95%; KX300157)	1	na	
	1	Abrothrix hirta	Laguna Torca NR	Rickettsia sp. (100%; KY705378)	1	na		na		na	
	3	Abrothrix olivacea	Laguna Torca NR	Rickettsia sp. (100%; KY705378)	3	na		na		na	
	1	Oligoryzomys longicaudatus	Laguna Torca NR	Rickettsia sp. (99%; KY705378)	1	na		na		na	
	2	Thylamys elegans	Laguna Torca NR	Rickettsia sp. (100%; KY705378)	2	na		na		na	
	2	Abrothrix olivacea	Canela Baja	Rickettsia sp. MEAM1 (99%; CP016305)	2	Rickettsia sp. (97%; U76908)	2	Rickettsia sp. MEAM1 (98%; CP016305)	2	na	
	1	Abrothrix olivacea	Fray Jorge NP	Rickettsia sp. MEAM1 (99%; CP016305)	1	Rickettsia sp. (97%; U76908)	1	na		na	
	1	Abrothrix hirta	Los Queules NR	Rickettsia sp. (97%; U59712)	1	na		Rickettsia sp. (91%; JF966777)	1	na	
	1	Abrothrix olivacea	Los Queules NR	na		na		Rickettsia sp. (94%; KX300157)	1	na	
	1	Thylamys elegans	Fray Jorge NP	na		Rickettsia sp. (97%; U76908)	1	na		na	

Table 6 (continued)

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence on GenBank)								
				gltA 401 bp		gltA 830 bp		rpoB 395 bp		sca5 862 bp		
Flea species	Total sequences amplified per flea	Host	Collection site	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	
Nosopsyllus fasciatus	2	Rattus rattus	Lolol	Rickettsia sp. (100%; KY705378)	2	na		na		na		
	1	Rattus rattus	Santa Cruz	Rickettsia sp. (100%; KY705378)	1	na		na		na		
Plocopsylla spp.	1	Rattus rattus	La Campana NP	Rickettsia sp. (100%; KY705378)	1	na		na		na		
	1	Rattus rattus	Til Til	Rickettsia sp. (100%; KY705378)	1	na		na		na		

Moreno-Salas *et al. Parasites Vectors* (2020) 13:523

Page 16 of 21

Table 6 (continued)

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence on GenBank)							
				gltA 401 bp		gltA 830 bp		rpoB 395 bp		sca5 862 bp	
Flea species	Total sequences amplified per flea	Host	Collection site	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive
Sphinctopsylla ares	6	*Abrothrix hirta*	Los Queules NR	*Rickettsia sp.* (97%; U59712)	6	*Rickettsia sp.* (98%; AJ269522)	5	*Rickettsia sp.* (91%; JF966777)	5	na	
	2	*Abrothrix hirta*	Cobquecura	*Rickettsia sp.* (98%; U59712)	2	*Rickettsia sp.* (98%; AJ269522)	1	*Rickettsia sp.* (91%; JF966777)	2	na	
	1	*Abrothrix hirta*	Coyhaique NR	*Rickettsia sp.* (98%; U59712)	1	*Rickettsia sp.* (98%; AJ269522)	1	*Rickettsia sp.* (93%; JF966777)	1	na	
	3	*Abrothrix olivacea*	Coyhaique NR	*Rickettsia sp.* (97%; U59712)	3	*Rickettsia sp.* (98%; AJ269522)	3	*Rickettsia sp.* (93%; JF966777)	3	na	
	2	*Abrothrix olivacea*	Los Queules NR	*Rickettsia sp.* (98%; U59712)	1	*Rickettsia sp.* (96%; KY799066)	2	*Rickettsia sp.* (91–93%; JF966777)	3	*Rickettsia hoogstraalii* (88%; EF629536)	2
	2	*Abrothrix olivacea*	Cobquecura	na		na		*Rickettsia sp.* (95%; KX300157)	2	na	
	1	*Abrothrix olivacea*	Cobquecura	Rickettsia sp. (93%; KY433588)	1	na		*Rickettsia sp.* (95%; KX300157)	1	na	
	1	*Oligoryzomys longicaudatus*	Cobquecura	na		na		*Rickettsia sp.* (95%; KX300157)	1	na	
	2	*Rattus rattus*	Laguna Torca NR	*Rickettsia sp.* (100%; KY705378)	2	na		na		na	
	1	*Thylamys elegans*	Laguna Torca NR	*Rickettsia sp.* (100%; KY705378)	1	na		na		na	

Table 6 (continued)

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence on GenBank)							
				gltA 401 bp		gltA 830 bp		rpoB 395 bp		sca5 862 bp	
Flea species	Total sequences amplified per flea	Host	Collection site	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive	Rickettsia spp.	No. positive
Tetrapsyllus rhombus	1	Abrothrix hirta	Los Queules NR	Rickettsia sp. (98%; U59712)	1	Rickettsia sp. (98%; AJ269522)	1	Rickettsia sp. (91%; JF966777)	1	na	
	1	Abrothrix hirta	Cobquecura	Rickettsia sp. (97%; U59712)	1	Rickettsia sp. (98%; AJ269522)	1	Rickettsia sp. (91%; JF966777)	1	na	
	3	Abrothrix olivacea	Los Queules NR	Rickettsia sp. (97%; U59712)	3	Rickettsia sp. (98%; AJ269522)	3	Rickettsia sp. (91%; JF966777)	3	na	
	1	Abrothrix olivacea	Los Queules NR	na		na		Rickettsia sp. (94%; KX300203)	1	na	
	1	Abrothrix olivacea	Coyhaique NR	Rickettsia sp. (97%; U59712)	1	Rickettsia sp. (98%; AJ269522)	1	Rickettsia sp. (93%; JF966777)	1	na	
	1	Rattus rattus	La Campana NP	Rickettsia sp. (100%; KY705378)	1	na		na		na	
Tetrapsyllus tantillus	4	Abrothrix olivacea	Lolol	Rickettsia sp. (100%; KY705378)	4	na		na		na	
	1	Abrothrix olivacea	Til Til	Rickettsia sp. (100%; KY705378)	1	na		na		na	
	1	Octodon degus	La Campana NP	Rickettsia sp. (100%; KY705378)	1	na		na		na	

Note: The table shows genes, GenBank accession numbers, similarity percent (%), flea species positive for *Rickettsia*, micromammal flea hosts, and locations where fleas were collected

Abbreviations: NR, National Reserve; NP, National Park; na, no amplification

found in this study. On the other hand, the higher prevalence of *Rickettsia* in fleas detected in natural areas can be explained by the greater diversity of species of micromammals and, therefore, of fleas. Thus, the differences in the prevalence of infection in the different species of flea, localities, seasons and bioclimatic zones found in our study, reveal the importance of the composition of the community, both fleas, and hosts, in determining the prevalence of *Rickettsia* in fleas, and therefore in the risk of infection in areas with different human disturbance.

In this study, we found two well-differentiated clades with a high degree of support. Clade R1 is formed by sequences obtained from fleas of the genus *Neotyphloceras*, collected from rodents *Phyllotis darwini*, *A. olivacea*, *O. degus*, *R. rattus*, and the marsupial *T. elegans* from central-north Chile (latitude: − 30° to − 31°S). This clade is related to *R. bellii* and is described as an ancestral group of *Rickettsia* [38], and which exhibits some specificity concerning its host [39]. This supports our results, where only bacteria detected in *Neotyphloceras* were found in this clade. *Rickettsia bellii* is endosymbiont of hard (Ixodidae) and soft (Argasidae) ticks throughout the

American continent [39]. It has been classified as non-pathogenic for animals and humans [40], although seropositive samples have been found in dog blood in Brazil; however, the pathogenic effect is unknown [41]. Experimentally, this bacterium grows easily in mammalian cells. In experimental inoculations in guinea pig and rabbit, it produces, depending on the inoculated dose received, from a mild inflammatory reaction to necrotic scabs a typical symptomatology of other pathogenic rickettsiae [29]. Furthermore, it is capable of producing antibodies in experimental infections in the big-eared opossum *Didelphis aurita*, but without rickettsemia [42]. These results indicate that some flea species present in wild and synanthropic micromammals could carry a new ancestral genotype of *Rickettsia*, just like those reported by Song et al. [43] in China from fleas of wild rodents.

The R2 clade was divided into two large groups, R2a and R2b. R2a grouped all of the sequences detected in fleas being extracted from two species of flea, *S. ares* (Stephanocircidae) and *T. rhombus* (Rhopalopsyllidae), which were obtained from villages and natural environments through wide latitudinal distribution (latitude of

Fig. 2 Phylogenetic tree of *gltA* 830-bp gene of *Rickettsia*. The values on each node show the Bayesian probability of each clade. The accession number for each sequence is indicated. Flea species and locality are indicated for the sequences generated in this study. The principal clades are labelled R1, R2a and R2b

Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 19 of 21

− 35° to − 45°S). This corresponds to the wide distribution of the hosts of infected fleas (*A. hirta* and *A. olivacea*). Conversely, R2b was formed by sequences obtained from *C. allophyla* and *C. inopinata* belonging to the same family (Hystricopsylidae); both species of flea were collected in wild rodents (*A. hirta* and *A. olivacea*) from wild areas (Los Queules NR and Nonguén NR) in the south-central zone of Chile. These sequences are closely related to *R. hoogstraalii*, *R. asembonensis* and *R. felis*, all of which are members of the spotted fever group rickettsiae (SFG) [28, 29, 38]. The SFG consists of > 30 species that can be found worldwide, most of them with pathogenic effects on humans [44]. Our analysis showed a close relationship with *R. hoogstraalii*, a widely distributed bacterium that is still unknown for its pathogenicity in humans. This bacterium has been detected in both hard ticks (*H. punctata*, *H. sulcate* and *H. parva*) and soft ticks (*Ornithodoros moubata*, *Carios capensis*, *C. sawaii* and *Argas persicus*) present in domestic animals, bird nests, vegetation, and human dwellings [3, 45–47]. A similar situation occurs with *R. asembonensis*. It also has a wide distribution worldwide, having been reported in North America and South America, Asia, the Middle East and Europe [48], although it is associated with a greater number of ectoparasites, including fleas, ticks, and mites of domestic and peridomestic animals (*C. canis*, *C. felis*, *X. cheopis*, *Pulex irritans*, *Amblyomma ovale*, *Rhipicephalus sanguineus*, *R. microplus* and *Ornithonysus bacoti*) [49–53]. It has also been detected in monkey blood in Malaysia [54] and in dog blood in South Africa [55]. Although these bacteria live in parasitic arthropods close to humans and are closely associated with *R. felis*, there is no evidence yet of possible infection or pathogenicity [48]. On the other hand, *R. felis* is an emergent, widely distributed, flea-borne human pathogen, and like *R. asembonensis* and *R. hoogstraalii*, is associated with domestic and peridomestic animals and their ectoparasites [56, 57]. The main vector is *C. felis*, although mosquitoes (*Anopheles gambiae*) have also been detected as competent vectors [58]. Unlike *R. asembonensis* and *R. hoogstraalii*, this bacterium is of known pathogenicity causing fever, fatigue, nausea, muscle aches, back pain, headaches, macular rash, joint pain and eschar [49]. Although the BLAST analysis showed a low percentage of similarity with *R. felis* (*sca5* 94%), the phylogenetic analysis shows a close relationship with *Rickettsia* detected in *C. allophyla* in south-central Chile. Until now, in Chile, only *R. felis* has been registered in *C. felis* [12].

Conclusions
To the best of our knowledge, our study reports, for the first time in Chile, the presence of *Rickettsia* in different species of parasitic fleas of wild micromammals and invasive rodents found in both natural and human environments. Moreover, there is evidence of at least two clades of *Rickettsia* associated with fleas. These data increase the knowledge of possible *Rickettsia* vectors/reservoirs in Chile. However, greater efforts should be made to monitor and determine the degree of pathogenicity of the detected rickettsiae.

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Moreno-Salas *et al. Parasites Vectors*   (2020) 13:523

Page 20 of 21

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