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

Lucila Moreno Salas (lumoreno@udec.cl)
Universidad de Concepcion Facultad de Ciencias Naturales y Oceanograficas https://orcid.org/0000-0003-3159-4916
Mario Espinoza Carniglia
Centro de Estudios Parasitologicos y de Vectores
Nicol Lizama Schmeisser
Universidad de Concepcion Facultad de Ciencias Naturales y Oceanograficas
Luis Gonzalo Torres Fuentes
Universidad Austral
María Carolina Silva de la Fuente
Universidad de Concepcion - Campus Chillan
Marcela Lareschi
Centro de Estudios Parasitologicos y de Vectores
Daniel González Acuña
Universidad de Concepcion - Campus Chillan

Research

Keywords: pathogen, bacteria, vectors, fleas, rodents, marsupials

Posted Date: August 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-35524/v2

Version of Record: A version of this preprint was published at Parasites & Vectors on October 17th, 2020. See the published version at https://doi.org/10.1186/s13071-020-04388-5.

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 1,315 fleas collected from 1,512 micromammals in 29 locations, with different human population densities in Chile. A generalized linear models (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 1,315) 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 infections in fleas varied between species of fleas. The prevalence of *Rickettsia* among flea species ranged from 0%–35.1%. Areas of lower human density have the highest prevalence of *Rickettsia*. The phylogenetic tree shows two well-differentiated clades. *Rickettsia belli* is positioned as basal in a clade. Another clade is subdivided into two subclades, and are related to *Rickettsia* of typhus group.

Conclusions

To our knowledge, this is the first report of the occurrence and molecular characterization of *Rickettsia* spp. in 15 species of fleas 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.

Introduction

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 whole-genome sequencing analysis, two groups are recognized within the *Rickettsia* genus: 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 “classical” spotted fever group that includes several species transmitted by mites and hard ticks, of which the most important are *R. rickettsi* 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], scarce studies have detected *Rickettsia* in fleas [12–15], which have focused on the molecular detection of pathogens in fleas of domestics mammals, identifying *Rickettsia felis* from cat and dog fleas (*Ctenocephalides felis* and *Ctenocephalides canis*) in central (Metropolitan region) and southern Chile (Valdivia) [12–14]. Recently, *Candidatus* Rickettsia asembonensis, *Candidatus* Rickettsia senegalensis, and *Rickettsia 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.

Materials And Methods

Sample localities and micromammal-trapping procedures

A total of 1,512 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, 8 natural area) of the 29 sampled, covering 10 regions in Chile and five bioclimatic regions (hyper-arid, arid, semiarid, sub-humid and hyper-humid), between -20.2167 lat. to -53.1667 lat. (Figure 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: 1) City: urban entity that has >5,000 inhabitants; 2) village: urban entity with a population ranging from 2,001–5,000 inhabitants, or between 1,001 and 2,000 people, where less than 50% of the population that declares having worked, is engaged in primary activities (e.g. livestock, agriculture, fishing) [18], and 3) natural area, without human settlement, corresponding to natural park (N.P; unaltered areas of natural and biological diversity), and national reserves (N.R; areas protecting wildlife populations or natural resources).

Micromammals were captured using Sherman trap (23 × 7.5 × 9 cm, Sherman Co., USA) and wire-mesh traps (30 x 10 x 11 cm; Forma Ltd., 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 2 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 meters 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 mean infection intensity (the number of fleas collected from all species/number of infested hosts), the mean abundance of infection (the number of collected fleas from all species/number of total hosts), and prevalence (the proportion of infected hosts) were calculated. The rodents were identified following Iriarte (2007). Rodents 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]. All procedures were approved by the Universidad de Concepción

bioethics committee, Servicio Agrícola y Ganadero (SAG R.E: 8968-2015, 1657.2016, 73-2016, 23-2017), and Corporación Nacional Forestal (CONAF N°018-2015).

DNA extraction and PCR amplification

For DNA extraction, five fleas per host were selected, and when the number of fleas per host was less than five, all the fleas were analyzed. Finally, DNA extraction was performed from 1,315 fleas. Each flea was washed and cut between the third and fourth abdominal tergite with a scalpel. DNA was extracted from individual fleas using a commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The incubation time was 5 hours; 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 access number: AF076436; Table 1). The amplification conditions were as follows: 5 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 30 seconds of annealing temperature (see Table 1), 30 seconds at 72°C, followed by a final extension of 5 minutes at 72°C. The reactions were performed with Green Master Mix 2X 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).

Phylogenetic and BLAST analyses

All DNA sequences were edited and aligned using the Codon Code Aligner (CodonCode Corporation, Centerville, MA, USA). All sequences of this study were compared with those available in 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 an outgroup. We used the GTR+G substitution model to reconstruct the tree and 10,000,000 bootstrap trials. The GenBank sequence accession numbers used to reconstruct the tree are detailed in Figure 2.

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 and Lareschi [25]. Voucher specimens (slides) were catalogued in the Museo de Zoología at Universidad de Concepción (MZUC-UCCC, Chile).

Statistical analysis

The prevalence (percentage of micromammals parasitized with fleas) and abundance mean (mean number of fleas per micromammals) in species of micromammals was calculated with total of samples of fleas collected (n=2,272), and confidence intervals (95% CI) were calculated, using bootstrap (2,000 bootstrap replicates). The prevalence of *Rickettsia* (percentage of fleas infected with *Rickettsia*) was calculated based on 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/semiarid/sub-humid/ hyper-humid), location type (city/village/natural area) and season (summer/winter). First, we built a model that included all bioclimatic region 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-squared 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 fleas. A *P*-value <0.05 was considered statistically significant. The data were analyzed using JMP software®

Nucleotide sequence accession numbers

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

Results

A total of 2,272 fleas were collected from 13 micromammal species, with a total prevalence of 46.6% (n=706) parasitized micromammals. There was a mean abundance of 1.5 fleas per host and 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, 1,315 flea specimens were analyzed, corresponding to 27 species from 15 genera and eight families (Table 3). 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* (Olivaceous field mouse, 13 spp.), *R. rattus* (12 spp.), *Abrothrix hirta* (Long-haired grass mouse, 11 spp.), and *Oligoryzomys longicaudatus* (Long-tailed rice mice, 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 (species n=18, specimen n=349) and cities (species n=18, specimen n=181). *Agastopsylla boxi*, *Ctenoparia jordani*, *Ctenoparia topali*, *Ectinorus cocyti*, and *Plocopsylla lewisi* were exclusive to natural areas. 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, and *S. ares* n=151), and villages (*N. chilensis* n=83, and *S. ares* n=50), while in cities, *Nosopsyllus fasciatus* (n=37), and *Ctenoparia inopinata* (n=25) were the most frequently collected. *Leptopsylla segnis*, *N. fasciatus*, and *X. cheopis* are synanthropic rodent fleas [26], and were more abundant in cities than in villages and natural areas.

Rickettsia prevalence on fleas

Fifteen flea species were found to be *Rickettsia*-positive for the *gltA* 401-bp gene (short fragment), nine with *gltA* 830-bp (long fragment), ten with *rpoB*, and four with *sca5* (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 >20 individuals were analyzed, the prevalence varied between 0% and 35.1%. The *Neotyphloceras* species 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-squared statistic=193.207, df=3; *P*=0.000), with the exception of *gltA* 830-bp and *rpoB*, which did not show significant differences (Chi-squared statistic=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*: Spearman ρ=0.4267; *P*=0.12; *gltA*: Spearman ρ=0.3757; *P*=0.18; *sca5*: Spearman ρ=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%). Also, the overall prevalence was significantly higher in the winter (20.6%) than in the summer (5.3%). Although 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 natural areas and the winter. While in the semi-arid region, the highest prevalence occurred in winter (73.68%), and the highest prevalence of *Rickettsia* was detected in natural areas (77.78%), differentiating from the cities (14.0%). In the sub-humid region, in the sub-humid region, there was no effect of the factors on the prevalence of *Rickettsia*, while in the hyper-humid region, we detect *Rickettsia* (5.49%) only in 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 these 68 sequences, 28 isolated from *Delostichus phyllotis* (n = 1), *L. segnis* (n = 1), *N. crassipina* (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 acc. no. KY705378) obtained from tick *Amblyomma parvitarsum*. Another 19 *gltA* sequences (401-bp) detected in *Neotyphloceras* spp. (n = 16), *Chiliopsylla allophyla* (n = 2), *C. inopinata* (n = 1) were closely related to *Rickettsia* sp. MEAM1 (99%; GenBank acc. no CP016305) isolated from whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) (n = 16) and *Rickettsia* sp. Gr15 (GenBank acc. no KP675966) detected in a 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 acc. no U59712) isolated from *Adelia bipunctata* (Coleoptera: Coccinellidae). One amplified sequence of *S. ares* showed 93% similarity with uncultured *Rickettsia* sp. (GenBank acc. no KY433588) detected in a tick.

Two sequence analyses of amplified *gltA* 830-bp segments showed high identity (99%) to *Candidatus* Rickettsia senegalensis (GenBank acc. no 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 acc. no KF646706, KY799066, U76908, 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. (GenBank acc. no 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 acc. no JF966777) of *Synosternus pallidus* (Siphonaptera: Pulicidae). Nine amplified sequences from *Neotyphloceras* spp. (n = 9) were 94-96% similar to *Rickettsia* sp. (GenBank acc. no KX300157) isolated from a bat *Myotis emarginatus.* Finally, four sequences isolated from *Neotyphloceras* spp. (n=3) and *T. rhombus* (n = 1) showed lower homology with *Rickettsia* sp. (94%, GenBank acc. no KX300203) isolated from a bat *Eptesicus serotinus.*

Three *sca5* fragment isolated from *C. allophyla* (n = 2) and *C. inopinata* (n = 1) showed homology with *Rickettsia felis* (94%; GenBank acc. no GQ385243), and two fragment detected from *S. ares* showed low identity to *R. hoogstraalii* (GenBank acc. no EF629536) (Table 6).

The phylogenetic tree shows two well-differentiated clades with 100% support in the node (Figure 2). Clade R1 is formed by sequences obtained from *Neotyphloceras* fleas collected in Las Chinchillas N. R. (-31° lat S), Canela Baja (-31° lat S), and Fray Jorge N. P. (-30°lat S). *R. belli* (GenBank acc. no: *glt*A DQ146481) is positioned on a basal branch in this group. The clade R2 is subdivided into two subclades: R2a and R2b. R2a, with 93% support in the node, is related to sequences obtained from *T. rhombus* and *S. ares* collected in Los Queules N. R., Cobquecura, and Coyhaique N. R., comprising a larger area of distribution (-35° to -45° lat S) than clade R1. Subclade R2b is formed by a member of typhus group, and sequences obtained from *C. inopinata* and *C. allophyla* were collected in Los Queules N. R. and Nonguén N. R. The obtained sequences were positioned closely to *R. hoogstralii* (GenBank acc. no FJ767737) isolated from *Haemaphysalis sulcata* (tick) in Croatia [27]; *R. asembonensis* detected in *C. felis* from Peru (*gltA* KY650697) [28] and *R. felis* isolated from *C. felis* in Brazil (GenBank acc. no JN375498) [29].

Discussion

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

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 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, 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 and 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 is 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. report different prevalence related to the flea species analyzed (range: 0 to 43%) [35]. Also, Kuo et al. [36] carried out an extensive sampling analyzing the presence of *Rickettsia* in six species of fleas, reporting 0% to 12.1% of prevalence in the different species of fleas 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 fleas [37], which are unknown in most of the species 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 micro-mammals and, therefore, of fleas. Thus, the differences in the prevalence of infection in the different species of fleas, localities, seasons and bioclimatic zones found in our study, reveals 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 *Neotyphloceras* genus, collected from rodents *Phyllotis darwini*, *A. olivacea*, *O. degus*, *R. rattus*, and the marsupial *T. elegans* from central-north Chile (-30° to -31° lat. 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. *R. belli* 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 – typical symptomatology of other pathogenic rickettsiae [29]. Furthermore, it is capable of producing antibodies in experimental infections in opossum *Didelphis aurita*, but without ricketsemia [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 the 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 as being extracted from two species of fleas, *S. ares* (Stephanocircidae) and *Tetrapsyllus rhombus* (Rhopalopsyllidae), which were obtained from villages and natural environments through wide latitudinal distribution (-35° to -45° lat. 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* that belong to the same family (Hystricopsylidae); both species of fleas were collected in wild rodents (*A. hirta* and *A. olivacea*) from wild areas (Los Queules N. R. and Nonguén N. R.) in the south-central zone of Chile. These sequences are closely related to *R. hoogstrali*, *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. hoogstrali*, a widely distributed bacterium that is still unknown for its pathogenicity in humans. This bacterium has been detected in hard ticks (*Haemaphysalis punctata*, *Haemaphysalis sulcate*, and *Haemaphysalis 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. asemboensis*. 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*, *Rhipicephalus 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. asemboensis* and *R. hoogstrali*, 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. asemboensis* and *R. hoogstrali*, 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 shows 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* was registered in *C. felis* [12].

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.

Declarations

Ethics approval and consent to participate

All procedures were approved by the Universidad de Concepción bioethics committee, Servicio Agrícola y Ganadero (SAG R.E: 8968-2015, 1657.2016, 73-2016, 23-2017), and Corporación Nacional Forestal (CONAF N°018-2015).

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: Not applicable.

Funding

This work was supported by the National Fund for Scientific and Technological Development [FONDECYT 11150875, 1130948 and 1170972].

Authors' contributions

Lucila Moreno Salas, Mario Espinoza-Carniglia, Nicol Lizama-Schmeisser, Luis Gonzalo Torres Fuentes, Maria Carolina Silva-de La Fuente and Daniel González-Acuña participate on rodent sampling, identifying rodent species and flea's extraction. Lucila Moreno Salas, Nicol Lizama-Schmeisser and Marcela Lareschi made fleas identification on laboratory. Mario Espinoza-Carniglia performed DNA extraction and PCR analyses. All authors read and approved the final manuscript.

Acknowledgements

English-language editing of this manuscript was provided by Journal Prep Services.

References

1. Walker DH. Rickettsia. Int Encycl Public Heal. Elsevier Inc.; 2016. p. 370–7.
2. Schumacher L, Snellgrove A, Levin ML. Effect of Rickettsia rickettsii (Rickettsiales: Rickettsiaceae) infection on the biological parameters and survival of its tick vector - Dermacentor variabilis (Acari: Ixodidae). J Med Entomol. 2016;53:172–6.
3. Blanton LS, Walker DH. Flea-Borne Rickettsioses and Rickettsiae. Am. J. Trop. Med. Hyg. American Society of Tropical Medicine and Hygiene; 2017. p. 53–6.
4. Shpynov SN, Fournier PE, Pozdnichenko NN, Gumenuk AS, Skiba AA. New approaches in the systematics of rickettsiae. New Microbes New Infect. 2018;23:93–102.
5. Perlman SJ, Hunter MS, Zchori-Fein E. The emerging diversity of Rickettsia. Proc R Soc. 2006;273:2097–106.
6. Weinert LA, Werren JH, Aebi A, Stone GN, Jiggins FM. Evolution and diversity of *Rickettsia* bacteria. BMC Biol. 2009;7.
7. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D. Fleas and flea-borne diseases. Int J Infect Dis. 2010;14:e667–76.
8. Feng AYT, Himsworth CG. The secret life of the city rat: A review of the ecology of urban Norway and black rats (*Rattus norvegicus* and *Rattus rattus*). Urban Ecosyst. 2014;17:149–62.
9. Himsworth CG, Parsons KL, Jardine C, Patrick DM. Rats, cities, people, and pathogens: A systematic review and narrative synthesis of literature regarding the ecology of rat-associated zoonoses in urban centers. Vector-Borne Zoonotic Dis. 2013. p. 349–59.
10. Beaucournu JC, Moreno L, González-Acuña D. Le genre Dasypsyllus Baker 1905 : description d'une espèce nouvelle et essai de mise au point sur ce genre (Siphonaptera : Ceratophyllidae). Ann la Soc Entomol. 2016;51:470–6.
11. Beaucournu JC, Moreno L, González-Acuña D. Fleas (Insecta-Siphonaptera) of Chile: A review. Zootaxa. 2014. p. 151–203.
12. Labruna MB, Ogrzewalska M, Moraes-Filho J, Lepe P, Gallegos JL, López J. *Rickettsia felis* in Chile [1]. Emerg. Infect. Dis. Centers for Disease Control and Prevention (CDC); 2007. p. 1794–5.
13. Poo-Muñoz DA, Elizondo Patrone MC, Escobar LE, Astorga F, Bermúdez S, Martinez-Valdebenito CP, et al. Fleas and Ticks in Carnivores From a Domestic–Wildlife Interface: Implications for Public Health and Wildlife. J Med Entomol. 2016;53:1–11.
14. Müller A, Rodríguez E, Walker R, Bittencourt P, Pérez-Macchi S, Ricardo Gonçalves L, et al. Occurrence and genetic diversity of *Bartonella* spp. (Rhizobiales: Bartonellaceae) and *Rickettsia* spp. (Rickettsiales: Rickettsiaceae) in Cat Fleas (Siphonaptera: Pulicidae) From Chile. J Med Entomol. 2013;142:1627–32.
15. Cevidanes A, Cataldo S Di, Vera F, Lillo P, Millán J. Molecular detection of vector-borne pathogens in rural dogs and associated *Ctenocephalides felis* fleas (Siphonaptera: Pulicidae) in Easter Island (Chile). J Med Entomol. 2018;55(6):1659–1663
16. Moreno L, Espinoza-Carniglia M, Schmeisser NL, Torres LG, Silva-De La Fuente MC, Lareschi M, et al. Fleas of black rats (*Rattus rattus*) as reservoir host of *Bartonella* spp. In Chile. PeerJ.;2019. 7:e7371
17. Meerburg BG, Singleton GR, Kijlstra A. Rodent-borne diseases and their risks for public health Rodent-borne diseases and their risks for public health. Crit. Rev. Microbiol. 2009. p. 221–70.
18. Instituto Nacional de Estadisticas (Chile). Ciudades, pueblos, aldeas y caseríos [Internet]. 2005. Available from: http://www.censo2017.cl/servicio-de-mapas/descargas/mapas/Urbano-Rural-
19. Mills JN, Yates TL, Childs JE, Parmenter RR, Ksiazek TG, Rollin PE, et al. Guidelines for Working with Rodents Potentially Infected with Hantavirus. J Mammal. 1995;76:716.
20. Carpenter J, Marion C. Exotic Animal Formulary - 5th Edition. Elsevier © Saunders Press; 2017 [cited 2020 Aug 7]. Available from: https://www.elsevier.com/books/exotic-animal-formulary/carpenter/978-0-323-44450-7

21. Iriarte A. Los mamíferos de Chile. 2008;417.
22. Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, et al. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the State of São Paulo, Brazil, where Brazilian Spotted Fever is endemic. J Clin Microbiol. 2004;42:90–8.
23. Roux V, Raoult D. Phylogenetic analysis of members of the genus Rickettsia using the gene encoding the outer-membrane protein rOmpB (ompB). Int J Syst Evol Microbiol. 2000;50 Pt 4:1449–55.
24. Johnson P. A classification of the Siphonaptera of South America, with descriptions of new species. Washington: Entomological Society of Washington; 1957.
25. Sanchez J, Lareschi M. Two new species of Neotyphloceras (Siphonaptera: Ctenophthalmidae) from Argentinean Patagonia. Zootaxa. 2014;3784:159–70.
26. Lewis RE. Résumé of the Siphonaptera (Insecta) of the World. J Med Entomol. 1998;35:377–89.
27. Duh D, Punda-Polic V, Avsic-Zupanc T, Bouyer D, Walker DH, Popov VL, et al. *Rickettsia hoogstraalii* sp. nov., isolated from hard-and soft-bodied ticks. Int J Syst Evol Microbiol. 2010;60:977–84.
28. Loyola S, Flores-Mendoza C, Torre A, Kocher C, Melendrez M, Luce-Fedrow A, et al. *Rickettsia asembonensis* characterization by multilocus sequence typing of complete genes, Peru. Emerg. Infect. Dis. Centers for Disease Control and Prevention (CDC); 2018. p. 931–3.
29. Ogata H, La Scola B, Audic S, Renesto P, Blanc G, Robert C, et al. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genet. 2006;2:733–44.
30. Medina-Pinto RA, Torres-Castro MA, Medina-Pinto RA, Bolio-González ME, Rodríguez-Vivas RI. Natural cysticercus fasciolaris infection in rodents from a rural area in Yucatan, Mexico. Vet Mex. 2019;6:1–10.
31. Whisson DA, Quinn JH, Collins KC. Home range and movements of roof rats (*Rattus rattus*) in an old-growth riparian forest, california [Internet]. Available from: www.prbo.org/CPIF/Riparian/Riparian.html
32. Ahmed S, Dávila JD, Allen A, Tacoli And C, Fèvre EM. 4 4 3 Does urbanization make emergence of zoonosis more likely? Evidence, myths and gaps 866124E AU Environment & Urbanization. 2019;31:443–60.
33. Monteverde, Hadora. Movimientos de roedores intra- e inter-ambiente y riesgo de exposición al Hantavirus “Andes” en Patagonia norte, Argentina. Ecol Austral. 2017;27:279–89.
34. Caron A, Cappelle J, Cumming GS, De Garine-Wichatitsky C prion gene polymorphisms are associated with decreased incidence of classical scrapie in goat herds in the UKM, Gaidet N. Bridge hosts, a missing link for disease ecology in multi-host systems. Vet Res. 2015;46:83.
35. Radzijevskaja J, Kaminskiene E, Lipatova I, Mardosaite-Busaitiene D, Balčiauskas L, Stanko M, et al. Prevalence and diversity of *Rickettsia s*pecies in ectoparasites collected from small rodents in Lithuania. Parasites and Vectors. 2018;11:375.
36. Kuo Cc, Huang Jl, Lin Te, Wang Hc. Detection of *Rickettsia* spp. and host and habitat associations of fleas (Siphonaptera) in eastern Taiwan. Med Vet Entomol. 2012;26:341–50.
37. Kim H-C, Yang Y-C, Chong S-T, Ko S-J, Lee S-E, Klein TA, et al. Detection of *Rickettsia typhi* and seasonal prevalence of fleas collected from small mammals in the republic of Korea. J wild dis. 2009;46(1):165-72
38. Merhej V, Raoult D. Rickettsial evolution in the light of comparative genomics. Biol. Rev. 2011. p. 379–405.
39. Krawczak FS, Labruna MB, Hecht JA, Paddock CD, Karpathy SE. Genotypic Characterization of Rickettsia bellii Reveals Distinct Lineages in the United States and South America. Biomed Res Int. Hindawi Limited; 2018;2018.
40. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, et al. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev [Internet]. 2013 [cited 2019 Oct 23];26:657–702. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24092850
41. Fortes FS, Silveira I, Moraes-Filho J, Leite RV, Bonacim JE, Biondo AW, et al. Seroprevalence of *Rickettsia bellii* and *Rickettsia felis* in dogs, São José dos Pinhais, State of Paraná, Brazil. Rev Bras Parasitol Veterinária. FapUNIFESP (SciELO); 2010;19:222–7.

42. Horta MC, Sabatini GS, Moraes-Filho J, Ogrzewalska M, Canal RB, Pacheco RC, et al. Experimental infection of the opossum *Didelphis aurita* by *Rickettsia felis, Rickettsia bellii*, and *Rickettsia parkeri* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense* and *Amblyomma dubitatum*. Vector Borne Zoonotic Dis. 2010;10:959–67.
43. Song S, Chen C, Yang M, Zhao S, Wang B, Hornok S, et al. Diversity of *Rickettsia* species in border regions of northwestern China. Parasites and Vectors. BioMed Central Ltd.; 2018;11.
44. Robinson MT, Satjanadumrong J, Hughes T, Stenos J, Blacksell SD. Diagnosis of spotted fever group Rickettsia infections: the Asian perspective. Epidemiol Infect. NLM (Medline); 2019;147:e286.
45. Karasartova D, Gureser AS, Gokce T, Celebi B, Yapar D, Keskin A, et al. Bacterial and protozoal pathogens found in ticks collected from humans in Corum province of Turkey. PLoS Negl Trop Dis. 2018;12.
46. Brinkmann A, Hekimoğlu O, Dinçer E, Hagedorn P, Nitsche A, Ergünay K. A cross-sectional screening by next-generation sequencing reveals *Rickettsia, Coxiella, Francisella, Borrelia, Babesia, Theileria* and *Hemolivia* species in ticks from Anatolia. Parasites and Vectors. 2019;12:26.
47. Chisu V, Leulmi H, Masala G, Piredda M, Foxi C, Parola P. Detection of *Rickettsia hoogstraalii, Rickettsia helvetica, Rickettsia massiliae, Rickettsia slovaca* and *Rickettsia aeschlimannii* in ticks from Sardinia, Italy. Ticks Tick Borne Dis. 2017;8:347–52.
48. Maina AN, Jiang J, Luce-Fedrow A, St. John HK, Farris CM, Richards AL. Worldwide Presence and features of flea-borne *Rickettsia asembonensis*. Front Vet Sci. 2019;5:334.
49. Jiang J, Maina AN, Knobel DL, Cleaveland S, Laudisoit A, Wamburu K, et al. Molecular detection of *Rickettsia felis* and *Candidatus* Rickettsia Asemboensis in fleas from human habitats, Asembo, Kenya. Vector Borne Zoonotic Dis. 2013;13:550–8.
50. Luce-Fedrow A, Maina AN, Otiang E, Ade F, Omulo S, Ogola E, et al. Isolation of *Candidatus* Rickettsia asemboensis from Ctenocephalides Fleas. Vector-Borne Zoonotic Dis. 2015;15:268–77.
51. Kocher C, Morrison AC, Leguia M, Loyola S, Castillo RM, Galvez HA, et al. Rickettsial Disease in the Peruvian Amazon Basin. PLoS Negl Trop Dis. 2016;10(7): e0004843.
52. Troyo A, Moreira-Soto RD, Calderon-Arguedas Ó, Mata-Somarribas C, Ortiz-Tello J, Barbieri ARM, et al. Detection of rickettsiae in fleas and ticks from areas of Costa Rica with history of spotted fever group rickettsioses. Ticks Tick Borne Dis. Elsevier GmbH; 2016;7:1128–34.
53. Dall'Agnol B, Souza U, Webster A, Weck B, Stenzel B, Labruna M, et al. "*Candidatus* Rickettsia asemboensis" in *Rhipicephalus sanguineus* ticks, Brazil. Acta Trop. 2017;167:18–20.
54. Tay ST, Koh FX, Kho KL, Sitam FT. Rickettsial infections in monkeys, Malaysia. Emerg. Infect. Dis. 2015. p. 545–7.
55. Kolo AO, Sibeko-Matjila KP, Maina AN, Richards AL, Knobel DL, Matjila PT. Molecular Detection of zoonotic Rickettsiae and *Anaplasma* spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. Vector-Borne Zoonotic Dis. 2016;16:245–52.
56. Stevenson HL, Labruna MB, Montenieri JA, Kosoy MY, Gage KL, Walker DH. Detection of Rickettsia felis in a New World Flea Species, *Anomiopsyllus nudata* (Siphonaptera: Ctenophthalmidae). J Med Entomol. 2005;42:163–7.
57. Panti-May JA, Torres-Castro M, Hernández-Betancourt S, Dzul-Rosado K, Zavala-Castro J, López-Avila K, et al. Detection of *Rickettsia felis* in wild mammals from three municipalities in Yucatan, Mexico. Ecohealth. 2015;12:523–7.
58. Dieme C, Bechah Y, Socolovschi C, Audoly G, Berenger JM, Faye O, et al. Transmission potential of rickettsia felis infection by Anopheles gambiae mosquitoes. Proc Natl Acad Sci U S A. National Academy of Sciences; 2015;112:8088–93.

Tables

Table 1. Micromammal species captured from 29 locations in Chile. The total number of rodents captured for each species, number of parasitized rodents, prevalence of rodents parasitized by fleas, total number of fleas collected, mean abundance, and mean intensity are indicated.

Family and species of micromammals	no. micromammals collected	no. micromammals with fleas	% Prevalence of fleas (95 % CI)	Number of fleas collected	Mean abundance of fleas (95% CI)	Mean intensity of fleas (95% CI)
Order Didelphimorphia						
Didelphidae						
Thylamys elegans	35	18	51.4 (0.34-0.69)	54	1.54 (0.83-2.97)	3.0 (1.83-5.22)
Order Rodentia						
Cricetidae						
Abrothrix hirta	319	191	59.9 (0.55-0.65)	643	2.0 (1.73-2.32)	3.4 (2.98-3.76)
Abrothrix lanosus	1	1	100.0	1	1.0	1.0
Abrothrix longipilis	5	4	80.0 (0.34-0.98)	9	1.8 (0.60-2.80)	2.3 (1.25-3.25)
Abrothrix olivacea	434	206	47.5 (0.43-0.52)	518	1.2 (1.03-1.37)	2.5 (2.27-2.80)
Chelemys macronyx	1	0	0.0	0	0.0	-
Irenomys tarsalis	1	0	0.0	0	0.0	-
Loxodontomys micropus	24	21	87.5 (0.69-0.96)	66	2.8 (1.96-3.79)	3.1 (2.38-4.24)
Oligoryzomys longicaudatus	229	81	35.4 (0.29-0.41)	162	0.7 (0.55-0.88)	2.0 (1.72-2.36)
Phyllotis darwini	120	49	40.8 (0.32-0.50)	133	1.1 (0.82-1.42)	2.7 (2.24-3.20)
Phyllotis limatus	2	0	0.0	0	0.0	
Reithrodon physodes	5	2	40.0 (0.05-0.85)	6	1.2 (0.00-3.20)	3.0 (1.00-3.00)
Octodontidae						
Octodon bridgesi	1	1	100.0	2	2.0	2.0
Octodon degus	69	54	78.3 (0.67-0.87)	387	5.6 (4.20-7.78)	7.2 (5.56-9.93)
Abrocomidae						
Abrocoma bennetti	3	3	100.0	77	25.7	25.7
Muridae						
Mus musculus	11	2	18.2 (0.02-0.52)	0	0.18 (0.00-0.36)	1 (0.00-0.00)
Rattus norvegicus	2	0	0.0	0	0.0	
Rattus rattus	250	73	29.2 (0.27-0.35)	214	0.9 (0.64-1.14)	2.9 (2.40-3.70)
TOTAL	1,512	706	46.7 (0.44-0.49)	2,272	1.5 (1.38-1.66)	3.2 (2.99-3.59)

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

Target gene, and product lenght (bp)	Annealing temperature (°C)	Primer name	Nucleotide sequence (5'–3')
gltA (401)	48°C*	CS-78_F	GCA AGT ATC GGT GAG GAT GTA AT
		CS-323_R	GCT TCC TTA AAA TTC AAT AAA TCA GGA T
gltA (830)	48°C*	CS-239_F	GCT CTT CTC ATC CTA TGG CTA TTA T
		CS-1069_R	CAG GGT CTT CGT GCA TTT CTT
rpoB (395)	55.5°C	RirpoB_F	CCG ACT CAT TAC GGT CGC ATT TGT
		RirpoB_R	CCC ATC AAA GCA CGG TTA GCA TCA
sca5 (862)	50°C**	120.M59F	CCG CAG GGT TGG TAA CTG C
		120.807R	CCT TTT AGA TTA CCG CCT AA

*Labruna et al. (2004); **Roux and Raoult (2000).

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

Family /species of micromammals	Family of flea	Species of flea
Cricetidae		
Abrothrix hirta	Hystricopsyllidae	*Chiliopsylla allophyla* (Rothschild, 1915)
		Ctenoparia inopinata (Rothschild, 1909)
		Ctenoparia topalIi (Smit, 1963)
		Ctenoparia jordani (Smit, 1955)
	Ctenophthalmidae	*Neotyphloceras crassispina* (Rothschild, 1914)
		Neotyphloceras pardinasi (Sanchez and Lareschi, 2014)
		Neotyphloceras (spp.)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Tetrapsyllus amplus* (Jordan and Rothschild, 1923)
		Tetrapsyllus tantillus (Jordan and 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 topalIi (Smit, 1963)
	Ctenophthalmidae	*Neotyphloceras crassispina* (Rothschild, 1914)
		Neotyphloceras chilensis (Lewis, 1976)
		Neotyphloceras pardinasi (Sánchez and Lareschi, 2014)
		Agastopsylla boxi (Jordan and Rothschild, 1923)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Ectinorus cocyti* (Rothschild, 1904)
		Tetrapsyllus amplus (Jordan and Rothschild, 1923)
		Tetrapsyllus tantillus (Jordan and 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 topalIi (Smit, 1963)
	Ctenophthalmidae	*Neotyphloceras chilensis* (Lewis, 1976)
		Neotyphloceras crassispina (Rothschild, 1914)
		Neotyphloceras pardinasi (Sánchez and Lareschi, 2014)
	Ceratophyllidae	*Nosopsyllus fasciatus* (Bosc d'Antic, 1800)
	Stephanocircidae	*Sphinctopsylla ares* (Rothschild, 1911)
	Rhopalopsyllidae	*Ectinorus chilensis* (Lewis, 1976)
		Tetrapsyllus amplus (Jordan and Rothschild, 1923)
		Tetrapsyllus rhombus (Smit, 1955)
	Leptopsyllidae	*Leptopsylla segnis* (Schönherr, 1811)
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 and Fulk, 1977)
		Tetrapsyllus rhombus (Smit, 1955)
		Tetrapsyllus tantillus (Jordan and 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 and González, 2011)
		Delostichus phyllotis (Johnson, 1957)

		Delostichus smiti (Jameson and Fulk, 1977)
		Ectinorus chilensis (Lewis, 1976)
		Tetrapsylllus corfidii (Rothschild, 1904)
		Tetrapsyllus tantillus (Jordan and 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 and Fulk, 1977)
		Ectinorus chilensis (Lewis, 1976)
		Tetrapsyllus corfidii (Rothschild, 1904)
Muridae		
Rattus rattus	Hystricopsyllidae	*Ctenoparia inopinata* (Rothschild, 1909)
		Ctenoparia jordani (Smit, 1955)
	Ctenophthalmidae	*Neotyphloceras* (spp.)
		Neotyphloceras chilensis (Lewis, 1976)
		Neotyphloceras pardinasi (Sánchez and 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 and 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 and Fulk, 1977)
		Tetrapsyllus tantillus (Jordan and Rothschild, 1923)

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

Family and species of fleas	Number of fleas analyzed	N° of fleas positive for gene fragment (% prevalence)			
		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	1,315	174 (13.2)	66 (5.0)	78 (5.9)	6 (0.5)

Table 5. Generalized Linear Models (GLM) of *Rickettsia* prevalence.

	Model performance			Model component				
Modell	L-R ChiSquare	DF	Prob>ChiSquare	Source of variation	Estimate	Std Error	L-R-ChiSquare	P
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[y]
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

L-R: Likelihood ratio; DF: degrees of freedom; std error: standard error; *p≤0.05; [y]Marginally significant.

Table 6. Similarity percentage for obtained sequences with BLAST analyses. There are showed genes, GenBank accession numbers, similarity percent (%), flea species positive for *Rickettsia*, micromammal flea hosts, and locations where fleas were collected.

Flea collection				Identification by gene sequence (% similarity with the corresponding sequence available in GenBank)							
				gltA (401-bp)		gltA (830-bp)		rpoB		ompB	
Species of fleas collected	Total sequences amplified per fleas	Host	Collection sites	Species of *Rickettsia*	No. positive	Species of *Rickettsia*	No. positive	Species of *Rickettsia*	No. positive	Species of *Rickettsia*	No. positive
Chiliopsylla allophyla	2	*Abrothrix hirta*	Nonguén N.R.	*Rickettsia* sp.Gr15 (99% ; KP675966)	2	*Rickettsia senegalensis*(99%; KU499847)	2	*Rickettsia*sp. (100%; JF966777)	2	*Rickettsia felis*(94%; GQ385243)	2
Ctenoparia inopinata	1	*Abrothrix hirta*	Los Queules N.R.	*Rickettsia* sp. (97% ; KP675966)	1	*Rickettsia* sp. (99%; KY799066)	1	*Rickettsia*sp. (97%; JF966777)	1	*Rickettsia felis*(94%; GQ385243)	1
Delosticus phyllotis	1	*Octodon degus*	La Campana N.P.	*Rickettsia* sp. (100%; KY705378)	1	NA	0	NA			
Leptopsylla segnis	1	*Rattus rattus*	Lolol	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA	
Neotyphloceras chilensis	4	*Phyllotis darwini*	Las Chinchillas N.R.	*Rickettsia* sp. (99%; CP016305)	4	*Rickettsia* sp. (97%; KF646706)	4	*Rickettsia*sp. (97-98%; CP016305)	4	NA	
	2	*Abrothrix olivacea*	Las Chinchillas N.R.	*Rickettsia* sp. (98%-99%; CP016305)	2	*Rickettsia* sp. (97%; KF646706)	2	*Rickettsia*sp. (98%; CP016305)	2	NA	
	1	*Abrothrix olivacea*	Fray Jorge N.P.	*Rickettsia* sp. (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia*sp. (98%; CP016305)	1	NA	
	3	*Abrothrix olivacea*	Canela Baja	*Rickettsia* sp. (99%; CP016305)	2	*Rickettsia* sp. (97%; U76908)	3	*Rickettsia*sp. (98%; CP016305)	3	NA	
	1	*Octodon degus*	Canela Baja	*Rickettsia* sp. (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia*sp. (98%; CP016305)	1	NA	
	2	*Phyllotis darwini*	Canela Baja	*Rickettsia* sp. (99%; CP016305)	2	*Rickettsia* sp. (97%; U76908)	2	*Rickettsia* sp. (98-100%; CP016305)	2	NA	
	1	*Thylamys elegans*	Canela Baja	*Rickettsia* sp. (99%; CP016305)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia*sp. (98%; CP016305)	1	NA	
Neotyphloceras crassispina	1	*Oligoryzomys longicaudatus*	Laguna Torca N.R.	*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 N.P.	*Rickettsia* sp. (100%; KY705378)	1	NA		NA	0	NA	
	1	*Rattus rattus*	Quirihue	NA		NA		*Rickettsia*sp. (94%; KX300203)	1	NA	
	2	*Rattus rattus*	Laguna Torca N.R.	*Rickettsia* sp. (99%-100%; KY705378)	2	NA				NA	
	1	*Rattus rattus*	Canela Baja	*Rickettsia* sp. (100%; KY705378)	1	*Rickettsia* sp. (97%; U76908)	1	*Rickettsia*sp. (93%; CP016305)	1	NA	
Neotyphloceras spp.	1	*Oligoryzomys longicaudatus*	Cobquecura	NA		NA		*Rickettsia*sp. (95%; KX300157)	1	NA	
	1	*Abrothrix hirta*	Laguna Torca N.R.	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA	
	3	*Abrothrix olivacea*	Laguna Torca N.R.	*Rickettsia* sp. (100%; KY705378)	3	NA		NA		NA	

	1	*Oligoryzomys longicaudatus*	Laguna Torca N.R.	*Rickettsia* sp. (99%; KY705378)	1	NA		NA		NA		
	2	*Thylamys elegans*	Laguna Torca N.R.	*Rickettsia* sp. (100%; KY705378)	2	NA		NA		NA		
	2	*Abrothrix olivacea*	Canela Baja	*Rickettsia* sp. (99%; CP016305)	2	*Rickettsia* sp. (97%; U76908)	2	*Rickettsia*sp. (98%; CP016305)	2	NA		
	1	*Abrothrix olivacea*	Fray Jorge N.P.	*Rickettsia* sp. (99%; CP016305)		Rickettsia sp. (97%; U76908)	1	NA		NA		
	1	*Abrothrix hirta*	Los Queules N.R.	*Rickettsia* sp. (97%; U59712)	1	NA		*Rickettsia*sp. (91%; JF966777)	1	NA		
	1	*Abrothrix olivacea*	Los Queules N.R.	NA		NA		*Rickettsia*sp. (94%; KX300157)	1	NA		
	1	*Thylamys elegans*	Fray Jorge N.P.	NA		*Rickettsia* sp. (97%; U76908)	1	NA		NA		
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 sp.	1	*Rattus rattus*	La Campana N.P.	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA		
	1	*Rattus rattus*	Til Til	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA		
Sphinctopsylla ares	6	*Abrothrix hirta*	Los Queules N.R.	*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 N.R.	*Rickettsia* sp. (98%; U59712)	1	*Rickettsia* sp. (98%; AJ269522)	1	*Rickettsia*sp. (93%; JF966777)	1	NA		
	3	*Abrothrix olivacea*	Coyhaique N.R.	*Rickettsia* sp. (97%; U59712)	3	*Rickettsia* sp. (98%; AJ269522)	3	*Rickettsia*sp. (93%; JF966777)	3	NA		
	2	*Abrothrix olivacea*	Los Queules N.R.	*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				*Rickettsia*sp. (95%; KX300157)	2	NA		
	1	*Abrothrix olivacea*	Cobquecura	Rickettsia sp. (93%;KY433588)	1	NA		*Rickettsia*sp. (95%; KX300157)	1			
	1	*Oligoryzomys longicaudatus*	Cobquecura	NA		NA		*Rickettsia*sp. (95%; KX300157)	1	NA		
	2	*Rattus rattus*	Laguna Torca N.R.	*Rickettsia* sp. (100%; KY705378)	2	NA		NA		NA		
	1	*Thylamys elegans*	Laguna Torca N.R.	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA		
Tetrapsyllus rhombus	1	*Abrothrix hirta*	Los Queules N.R.	*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 N.R.	*Rickettsia* sp. (97%; U59712)	3	*Rickettsia* sp. (98%; AJ269522)	3	*Rickettsia*sp. (91%; JF966777),	3	NA		
	1	*Abrothrix olivacea*	Los Queules N.R.	na		NA		*Rickettsia*sp. (94%; KX300203)	1			
	1	*Abrothrix olivacea*	Coyhaique N.R.	*Rickettsia* sp. (97%; U59712)	1	*Rickettsia* sp. (98%; AJ269522)	1	*Rickettsia*sp. (93%; JF966777)	1	NA		
	1	*Rattus rattus*	La Campana	*Rickettsia* sp. (100%; KY705378)	1	NA		NA		NA		

			N.P.					
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 N.P.	*Rickettsia* sp. (100%; KY705378)	1	NA	NA	NA

N.R: National Reserve; N.P: National Park; NA: the gene does not amplify.

Figures

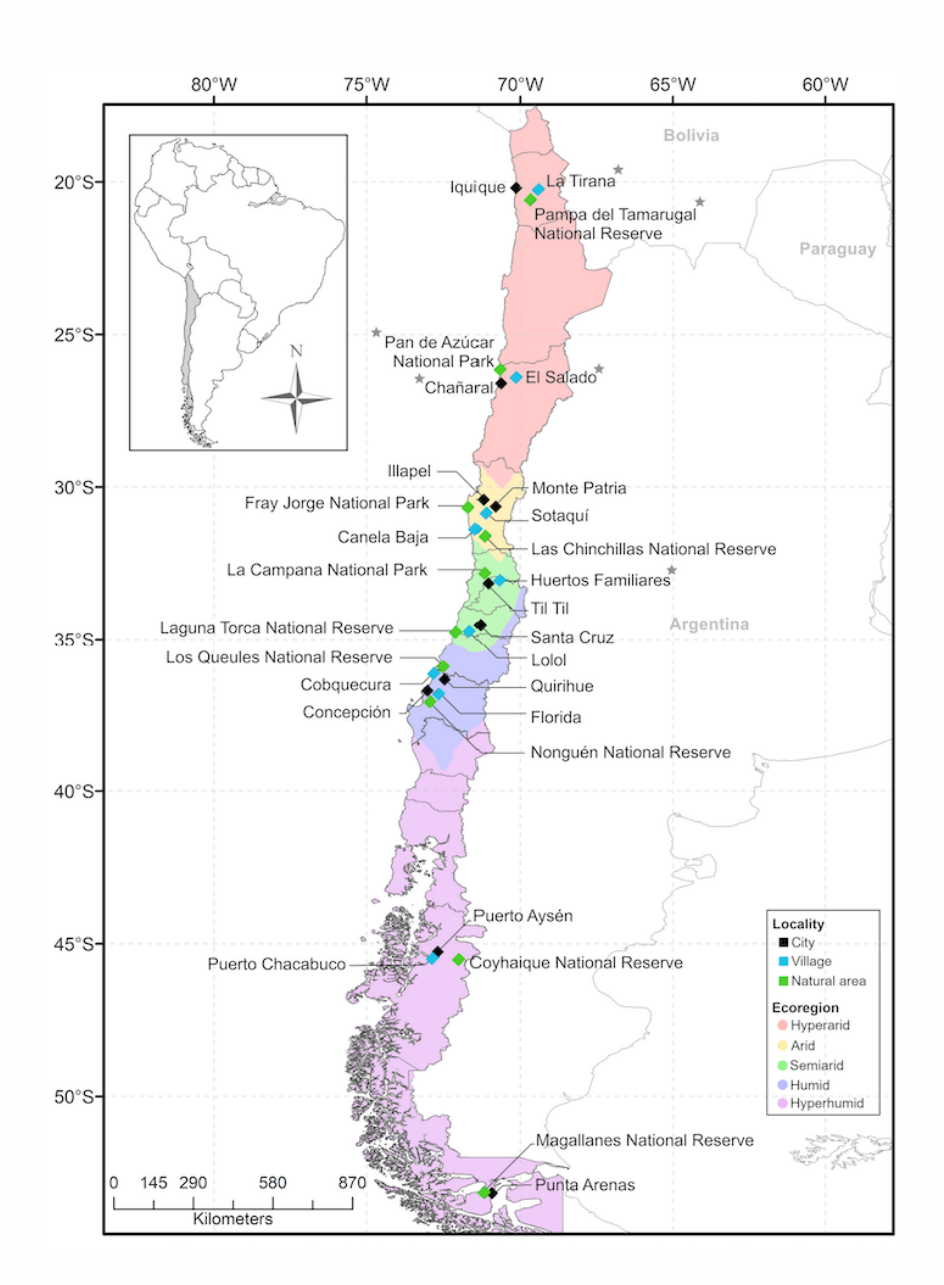

Figure 1

Study area. There are indicated the type of locality where the micromammals were collected. The stars indicate the locations where rodents were not captured.

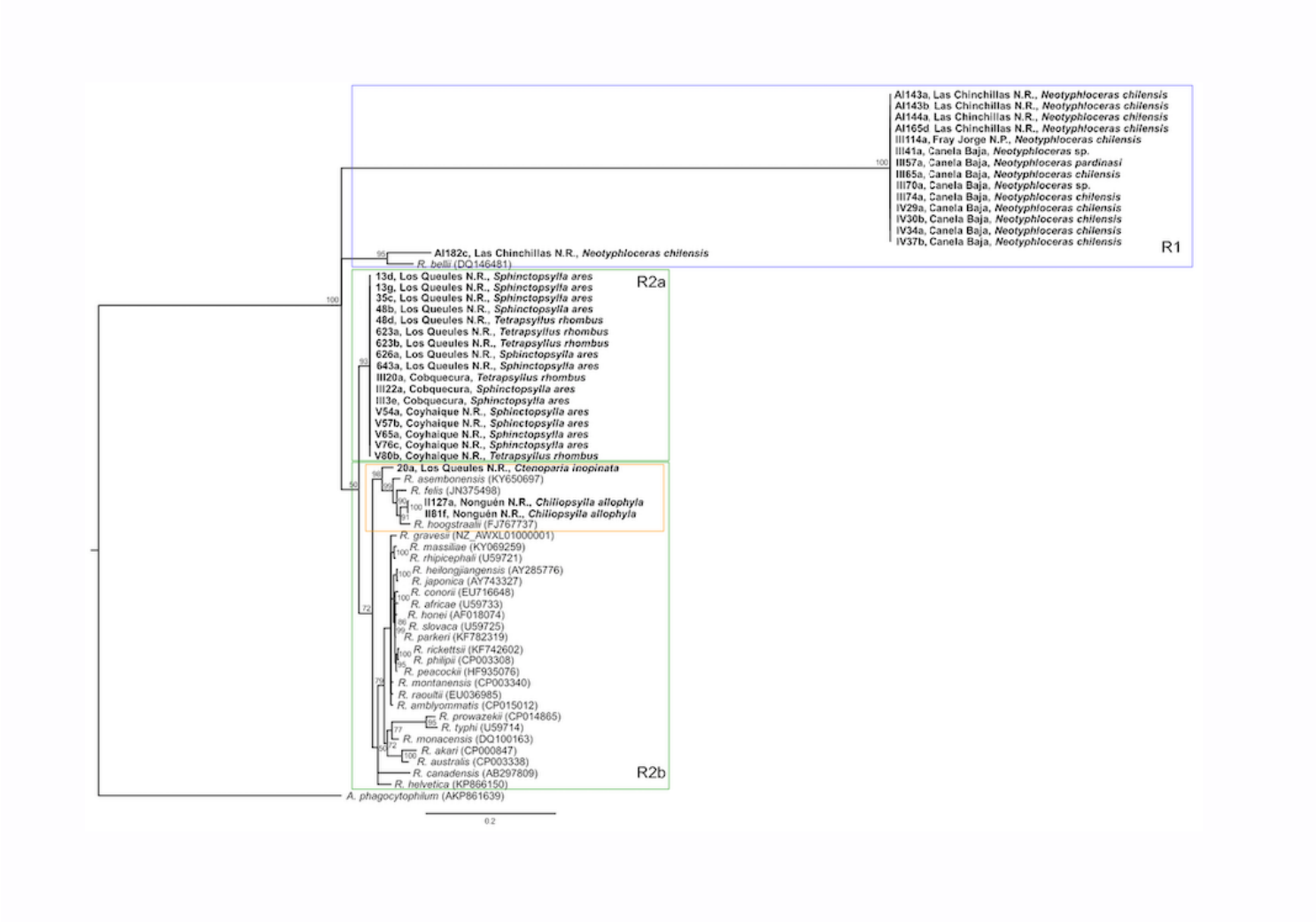

Figure 2

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 fragment is indicated below each Rickettsia species. Flea species and locality indicate the sequences determined in this study. Principal clades are showed as R1, R2a and R2b.