


# Genetic diversity of *Hepatozoon* spp. in rodents from Chile

## Diversidade genética de *Hepatozoon* spp. em roedores do Chile

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**How to cite:** Alabí AS, Monti G, Otth C, Sepulveda-García P, Perles L, Machado RZ, et al. Genetic diversity of *Hepatozoon* spp. in rodents from Chile. *Braz J Vet Parasitol* 2021; 30(4): e012721. <https://doi.org/10.1590/S1984-29612021082>

### Abstract

This study aimed to investigate the genetic diversity of *Hepatozoon* spp. in rodents from Valdivia, Chile. A total of 74 rodents (synanthropic n=38; wild n=36) were trapped in Valdivia. We performed conventional PCR assays for Apicomplexa organisms targeting two overlapping 18S rDNA gene fragments (600 bp and 900 bp) followed by sequencing of selected amplicons. *Hepatozoon* spp. occurrence was 82.43% (61/74). Twelve sequences obtained from the 600 bp and ten from the 900 bp 18S rDNA fragments were identified as *Hepatozoon* sp. Six sequences obtained from 18S rDNA-based overlapping PCR protocols were used for concatenated (1,400 bp) phylogenetic, haplotype and distance analyses. *Hepatozoon* spp. 18S rDNA concatenated sequences from the present study were detected in *Oligoryzomys longicaudatus*, *Rattus norvegicus*, *Mus musculus*, and *Abrothrix longipilis* grouped with *Hepatozoon* species earlier described in rodents and reptiles from Chile and Brazil. Nucleotide polymorphism of the six 18S rDNA sequences (1,400 bp) from this study, and other Chilean sequences from rodents and rodent's ticks, showed high diversity with a total of nine Chilean haplotypes. Three haplotypes from Valdivia were identified for the first time in this study, suggesting the circulation of novel haplotypes in rodents from southern Chile.

**Keywords:** Apicomplexan, hepatozoonosis, rodentia, PCR, phylogenetics, South America.

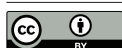
### Resumo

Este estudo teve como objetivo investigar a diversidade genética de *Hepatozoon* spp. em roedores de Valdivia, Chile. Um total de 74 roedores (sinantrópicos n=38; selvagens n=36) foram capturados. PCR convencional foi realizada para organismos Apicomplexa, visando dois fragmentos sobrepostos do gene 18S rDNA (600 bp e 900 bp), seguida pelo sequenciamento de amplicons selecionados. A ocorrência de *Hepatozoon* spp. foi de 82,43% (61/74). Doze sequências obtidas dos fragmentos de 18S rDNA de 600 pb e dez dos fragmentos de 18S rDNA de 900 pb foram identificadas como *Hepatozoon* sp. Seis sequências obtidas, a partir de protocolos de PCR sobrepostos, foram usadas para análises filogenéticas (1.400 bp), de haplótipos e de distância. Sequências concatenadas 18S rDNA do presente estudo foram detectadas em *Oligoryzomys longicaudatus*, *Rattus norvegicus*, *Mus musculus* e *Abrothrix longipilis* e agrupadas com *Hepatozoon* descrito em roedores e répteis do Chile e do Brasil. A análise de polimorfismos das seis sequências deste estudo, junto com outras sequências chilenas de roedores e carrapatos de roedores, mostrou alta diversidade com um total de nove haplótipos no Chile. Três haplótipos detectados em Valdivia foram identificados pela primeira vez neste estudo, sugerindo que novos haplótipos circulam em roedores do sul do Chile.

**Palavras-chave:** Apicomplexa, hepatozoonose, rodentia, PCR, filogenia, América do Sul.

Received July 3, 2021. Accepted September 20, 2021.

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## Introduction

The genus *Hepatozoon* (Adeleorina; Hepatozoidae) comprises apicomplexan parasites that were first detected in India (Bentley, 1905). Since then, many species have been described, from various vertebrate hosts, such as mammals, birds, reptiles, and amphibians. Although not considered a primary zoonotic pathogen, zoonotic potential has been described, as *Hepatozoon* sp. was detected in Russian and Philippine patients (Craig, 2006; Lappin, 2010; Shuikina et al., 2004).

The life cycle of *Hepatozoon* spp. includes invertebrates (definitive hosts), represented by ticks, fleas, flies, mosquitoes, or lice, from which vector competence was proved, and vertebrates (intermediate hosts) (Modrý et al., 2017; Rubini et al., 2006; Lainson et al., 2003; Watkins & Nowell, 2003). The vertebrate host is usually infected by ingestion of a hematophagous arthropod, although infection can be acquired also by intrauterine transmission or by predation (Modrý et al., 2017).

Even though *Hepatozoon* spp. were detected in rodents from several geographical locations, such as Europe (Criado-Fornelio et al., 2006; Laakkonen et al., 2001; Rigó et al., 2016), Africa (Harris et al., 2017; Maia et al., 2014), North America (Johnson et al., 2007, 2008a), and South America (de Sousa et al., 2017; Demoner et al., 2016; Perles et al., 2019; Wolf et al., 2016), their role as intermediate hosts for carnivore-associated *Hepatozoon* species has only been confirmed in the USA (Johnson et al., 2008a). For instance, *Hepatozoon americanum* is transmitted due to the predation of infected rabbits (*Oryctolagus cuniculus*) and rodents [*S. hispidus*, *Mus musculus*, and *Rattus rattus*] (Johnson et al., 2008a, b, 2009).

In Chile, although the presence of *Hepatozoon* was confirmed in rodents, marsupials, and associated ectoparasites, little is known about the genetic diversity of this group of tick-borne pathogens. *Hepatozoon* sp. was detected in 54.5% (6/11) of Olive gray mouse (*Abrothrix olivaceus*) and 50% (2/4) of long-haired akodont (*Abrothrix sanborni*) sampled in Senda Darwin Biological Station and forest in Chiloé Island, southern Chile (Merino et al., 2009). Recently, *Hepatozoon* spp. was detected in *Ixodes* sp. and *Ornithodoros atacamensis* ticks collected from wild rodents (*Phyllotis darwini*) in national parks of Pan de Azúcar and Bosque Fray Jorge, in Northern Chile (Muñoz-Leal et al., 2019). Phylogenetic analysis of *Hepatozoon* sp. detected in ectoparasites from Chile showed its relatedness to *Hepatozoon* spp. detected in the marsupial Monito del monte (*Dromiciops gliroides*) and Olive gray mouse (*A. olivaceus*) from Chile (Merino et al., 2009).

This study aimed to investigate the genetic diversity of *Hepatozoon* spp. in wild and synanthropic rodents from the Valdivia province, southern Chile.

## Materials and methods

### Study site

The study was approved by the Universidad Austral de Chile (UACH) bioethics committee (Uach/1141070). The sampling for this study included four locations within the Valdivia province, Southern Chile: Corral (Arica Interior [39°53'18.806"S, 73°26'25.272"W]) and Huiro [39°57'52.0"S, 73°38'55.3"W]), Valdivia (39°47'21.48"S, 73°14'37.72"W), Reumén (39°59'54.96"S, 72°49'18.12"W), and Riñihue (39°46'25.32"S, 72°28'20.28"W) (Figure 1). The rodents were sampled by convenience for an unrelated study at UACH.

### Rodent trapping and sampling

The trapping occurred from November 2016 and November 2017, on dairy farms from the Valdivia province. Traps (20 cm~ 20 cm~ 60 cm Tomahawk cages) were baited with oatmeal and vanilla flavoring, placed in areas where rodents were usually seen and reviewed daily, during the morning for the period of four days. Any endangered, threatened, or protected species were immediately released.

The captured rodents were euthanized in the Pathology building of the Universidad Austral de Chile (UACH). Euthanasia was performed using a lethal dose, equivalent to 5 times the anesthetic dose (inhaled Isoflurane, followed by an intraperitoneal injection of a combination of Xylazine-Ketamine) (Hedenqvist & Hellebrekers, 2003). Each rodent's spleen was aseptically removed after euthanasia, stored and preserved in 70% Ethanol (Merck®, USA) at -20°C, until further analyses.



**Figure 1.** Rodent sampling sites within the Valdivia Province, Southern Chile.

The capture, management, and euthanasia of rodents followed the specifications of the American Society of Mammologists and the protocols and norms established by the funding agency (CONICYT, 2008). Biological protocols for dangerous material were used for carcasses disposal.

#### Distribution of sampled rodents

Seventy-four rodents were sampled: 35.13% (26/74) were trapped in Corral, 8.1% (6/74) in Valdivia, 22.97% (17/74) in Reumén, and 33.78% (25/74) in Riñihue. Four rodent genera were identified: 5.40% (4/74) were represented by house mouse (*Mus musculus*), 21.62% (16/74) by brown rat (*Rattus norvegicus*), 24.32% (18/74) by black rat (*Rattus rattus*), 17.56% (13/74) by long-haired grass mouse (*Abrothrix longipilis*), 10.81% (8/74) by grass mouse (*Abrothrix olivaceus*), 13.51% (10/74) by long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*), and 6.75% (5/74) that were not identified on a species level, being classified as *Abrothrix* spp. (once it was not possible to differentiate between *A. olivaceus* or *A. longipilis*).

#### DNA Extraction from Rodent Spleen and PCR for Mammals' endogenous gene

The frozen rodent spleens were thawed at room temperature and 15 mg portions were refrozen with liquid nitrogen and manually macerated with a plastic pestle. DNA extraction of the macerated spleen suspension was performed with the "Tissue DNA Kit" (E.Z.N.A. Omega BioTek®, Norcross, GA, USA), as per the manufacturer's instructions (100 µL elution). A spectrophotometer (NanoDrop ND-1000 Thermo Scientific®, Waltham, MA, USA) was used for measuring the DNA concentration and absorbance ratio (260/280nm). Nuclease-free water (Thermo Scientific®, USA) was used as a template to verify cross-contamination, every 20 extractions. DNA was stored at -20 °C before performing PCR assays.

To verify the presence of amplifiable DNA and check the integrity of the DNA template, the *irbp* ("interphotoreceptor retinoid-binding protein") endogenous mammalian gene was used (Ferreira et al., 2010).

## Molecular detection of apicomplexan organisms

Positive samples for the *irbp* gene were submitted to a conventional PCR protocol to amplify a fragment (600 bp) of the 18S rDNA of Apicomplexa organisms, as previously described (Vilcins et al., 2009). All PCR runs were performed with nuclease-free water (Thermo Scientific®, Waltham, MA, USA) as a negative control and Taq DNA polymerase (Life technologies®, Carlsbad, CA, USA) for amplification. *Hepatozoon caimani* DNA obtained from naturally infected crocodiles was used as a positive control (Bouer et al., 2017).

All the samples were further tested for a second 18S rDNA-based PCR protocol targeting a complimentary (900 bp) fragment of Apicomplexan organisms (Perkins & Keller, 2001), aiming at obtaining a larger portion of the 18S rDNA gene (1,400 bp) for phylogenetic and haplotype analyses.

Both 600 bp and 900 bp 18S rDNA amplicons showing high intensity bands in agarose gel electrophoresis were purified using "Silica Bead DNA Gel Extraction Kit" (Fermentas, São Paulo-SP), following the manufacturer's instructions, and sent to the Center of Biological Resources and Genomic Biology (CREBIO, Jaboticabal, SP, Brazil) for sequencing by Sanger's method with ABI PRISM 3700 DNA Analyzer (Applied Biosystems®, Foster city, CA, USA). Only sequences obtained for both overlapping 18S rDNA-based PCR protocols were used for concatenated (1,400 bp) phylogenetic and haplotype analyses.

## BLAST Analysis

Electropherograms were submitted to PhredPhrap analysis (Ewing et al., 1998), with the Phred quality score (peaks around each base call) established as higher than 20 (99% accuracy of the base call), to determine the nucleotide composition. We performed a BLAST analysis to evaluate the identity percentage of our sequences with those deposited in the GenBank database (Altschul et al., 1990; Benson et al., 2004).

The consensus sequences were obtained by aligning the sense and antisense sequences using PhredPhrap software. Consensus sequences were submitted to GenBank under the following accession numbers MK454902/MH594207; MK454899/MH216198; MK454898/MH216197; MK454895/MH216195; MK454896/MH216196; MK454901/MH216199.

## Phylogenetic analysis

Concatenated sequences (~1400 bp) were used for phylogenetic analysis. Initially, the best evolutionary model was selected by the program jModelTest2 (version 2.1.6) on 11 XSEDE (Santorium et al., 2014), under the Akaike Information Criterion (AIC) (Posada & Buckley, 2004). Maximum likelihood (ML) inference was performed with the IQ-TREE webserver (Trifinopoulos et al., 2016). The phylogenetic tree was edited with Treegraph 2.0.56-381 beta (Stöver & Müller, 2010) and cluster design was created with BioRender.com (Biorender, 2020).

## Haplotype analysis

Nucleotide polymorphism analysis of 18S rDNA concatenated sequences obtained in this study was performed using DnaSP v5 (Librado & Rozas, 2009). Haplotype diversity (Hd), number of haplotypes (n), and nucleotide diversity (Pi) were analyzed to investigate the genetic diversity among *Hepatozoon* sequences detected in the sampled rodents (using parameters: not considered missing/gaps and considered invariable sites). Haplotype networks were generated with PopArt (Clement et al., 2002; Leigh & Bryant, 2015). Additional haplotype analyses were performed by combining the sequences detected in this study and those previously detected in rodents and rodents' ticks from Chile (GenBank accession numbers: FJ719819, FJ719818, FJ719816, FJ719817, MH174343, MH174344, MH174345) (Merino et al., 2009; Muñoz-Leal et al., 2019). A haplotype network was constructed using the TCS Network and the PopArt software (PopART, 2020) (Clement et al., 2002; Leigh & Bryant, 2015).

## Splits network analysis

Splits tree v4.11.3 (Huson, 1998) was used to generate a phylogenetic distance network with sequences obtained from the present study and sequences from Genbank (Supplementary Table S1). Final trees and haplotype network design were created with Biorender.com (Biorender, 2020).

## Results

### Endogenous gene and Hepatozoon occurrence in sampled rodents

All 74 DNA samples tested positive for the *irbp* gene (Mean and Standard Deviation (SD) with a DNA concentration=159.60 ng/μL ± 212.09 ng/μL; mean and SD 260/280 ratio=2.12 ± 0.21).

An overall proportion of 82.4% (61/74) of the rodents were positive for *Hepatozoon* spp. Synanthropic and wild rodents presented *Hepatozoon* prevalence rates of 78.94% (30/38) and 86.11% (31/36), respectively. Within the synanthropic rodents, *M. musculus* showed an occurrence of 4.05% (3/74), *R. norvegicus* 17.56% (13/74) and *R. rattus* 18.91% (14/74) and in the wild rodents' group are included *A. longipilis* which showed an occurrence of 16.21% (12/74), *A. olivaceus* 10.81% (8/74), *Abrothrix* spp. 2.70% (2/74) and *O. longicaudatus* 12.16% (9/74).

### Sequenced samples, Blast Analysis and Genetic characterization

Twelve 18S rDNA amplicons obtained with the 600bp PCR protocol for Apicomplexan organisms (primers HepF300 and HepF900; 600 bp) presented strong band intensity and were sequenced. 18S rDNA *Hepatozoon* partial sequences obtained from six rodents in this study (MK454892 [*M. musculus*], MK454894 [*R. norvegicus*], MK454896 [*R. norvegicus*], MK454897 [*M. musculus*], MK454898 [*R. norvegicus*], MK454900 [*R. norvegicus*]) presented 99-100% identity with *Hepatozoon* sp. detected in *A. olivaceus* (FJ719818) rodents from Chile (Merino et al., 2009); two sequences (MK454901 [*A. longipilis*], MK454903 [*A. longipilis*]) presented 99-100% identity with *Hepatozoon* spp. detected in *A. sanborni* rodents from Chile (FJ719819, FJ719816) (Merino et al., 2009); two sequences (MK454893 [*R. norvegicus*], MK454899 [*R. norvegicus*]) presented 100% identity with *Hepatozoon* sp. detected in *Lytorhynchus diadema* reptiles (KX453636) from Oman (Maia et al., 2016b); one sequence (MK454895 [*M. musculus*]) was 100% identical to *Hepatozoon* sp. detected in the reptile *Hemidactylus mabouia* (KM234616) (Harris et al., 2015) from Brazil; and finally, one sequence (MK454902 [*O. longicaudatus*]) showed 100% identity with *Hepatozoon* sp. detected in *Thylamys macrurus* marsupial (KX776354) from Brazil (de Sousa et al., 2017).

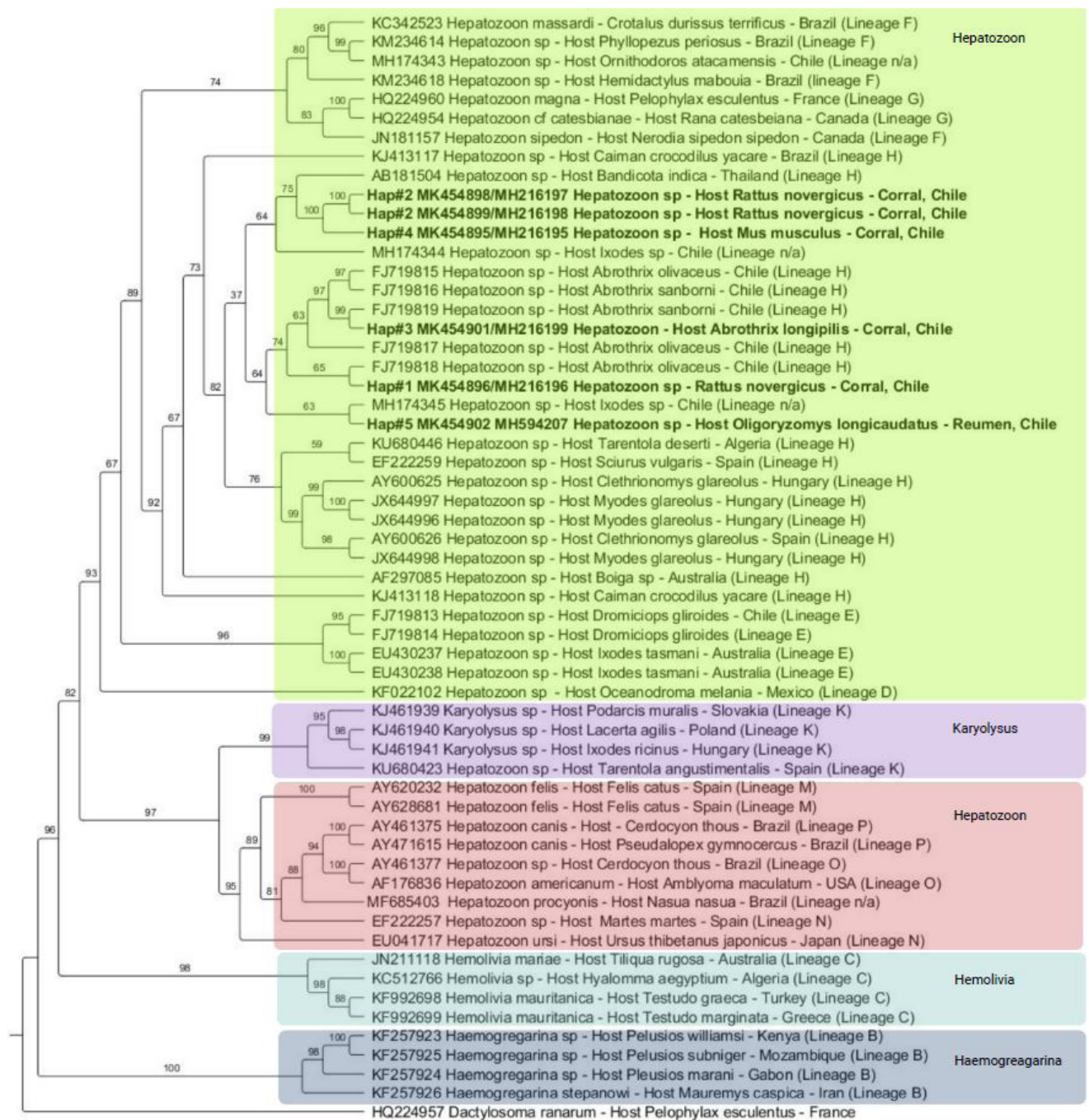
Ten amplicons obtained from the second Apicomplexan 18S rDNA-based PCR protocol (primers HEMO1 and HEMO2; 900 bp) presented high-intensity bands in agarose gel electrophoresis and were submitted to sequencing. *Hepatozoon* 18S rDNA partial sequences obtained from five rodents in this study (MH216195 [*M. musculus*], MH216196 [*R. norvegicus*], MH216199 [*A. longipilis*], MH594205 [*A. olivaceus*], MH594208 [*A. olivaceus*]) showed 100% identity with *Hepatozoon* sp. detected in *A. olivaceus* (FJ719818) and *A. sanborni* (FJ719819) rodents from Chile (Merino et al., 2009); three sequences (MH594204 [*O. longicaudatus*], MH594206 [*O. longicaudatus*], MH594207 [*O. longicaudatus*]) were 99% identical to *Hepatozoon* spp. detected in *Oecomys marmorae* (KX776332) rodents from Brazil (de Sousa et al., 2017); finally, two sequences (MH216197 [*R. norvegicus*], MH216198 [*R. norvegicus*]) presented 99% identity with *Hepatozoon* sp. detected in *Tarentola deserti* (KU680460) reptile from Morocco (Tomé et al., 2016).

Six sequences were obtained for both Apicomplexan 18S rDNA-based PCR protocols and used for concatenated (1,400 bp) phylogenetic, haplotype and distance analyses. These concatenated sequences were: MK454902/MH594207 detected in an *O. longicaudatus*; MK454899/MH216198, MK454898/MH216197, and MK454896/MH216196 detected in *R. norvegicus*; MK454895/MH216195 from *M. musculus*; and MK454901/MH216199 from *A. longipilis*.

### Phylogenetic analysis

According to the phylogenetic inference, five different clades were observed, two of them represented by *Hepatozoon* spp. (Figure 2). The major clade (green colored) contained different *Hepatozoon* species from avian, reptilian, amphibian, rodentia, arachnoidea and marsupialia representants, including sequences of the present study. The second and minor clade of *Hepatozoon* species (pink colored) was formed by different *Hepatozoon* spp. detected in carnivores from Canidae, Felidae, Ursidae, Procyonidae, Mustelidae families, and an Ixodidae tick. The other clades comprised *Hemolivia* (blue colored), *Karyolysus* (purple colored) and *Haemogregarina* (grey colored) parasites.

Concatenated sequences from this study were clustered in two different subclades with other *Hepatozoon* 18S rDNA sequences. The first subtree contained the concatenated sequences from Valdivia, Southern Chile (MK454899/MH216198; MK454898/MH216197 [Haplotype #2] and MK454895/MH216195 [Haplotype #4]), along with sequences from a bandicoot (*Bandicota indica*) in Thailand (AB181504) and Ixodid tick



**Figure 2.** Phylogenetic tree based on an alignment of concatenated *Hepatozoon* 18S rDNA sequences, using Maximum likelihood method and TVM+I+G4+F as an evolutionary model. Numbers at nodes correspond to bootstrap. *Hepatozoon* sp. sequences detected in the present study are in bold letters. *Dactylosoma ranarum* was used as outgroups. Squared colors identify each clade.

(*Ixodes* sp.) from Chile (MH174344). The second subtree contained some concatenated sequences from the present study (MK454896/MH216196 [Haplotype #1]) (MK454901/MH216199 [Haplotype #3]) (MK454902/MH594207 [Haplotype #5]) and other sequences from rodents from Chile (*A. olivaceus* [FJ719815, FJ719817 and FJ719818], *A. sanborni* [FJ719816 and FJ719819]) and an Ixodid tick (*Ixodes* sp.) from Chile (MH174345) (Figure 2).

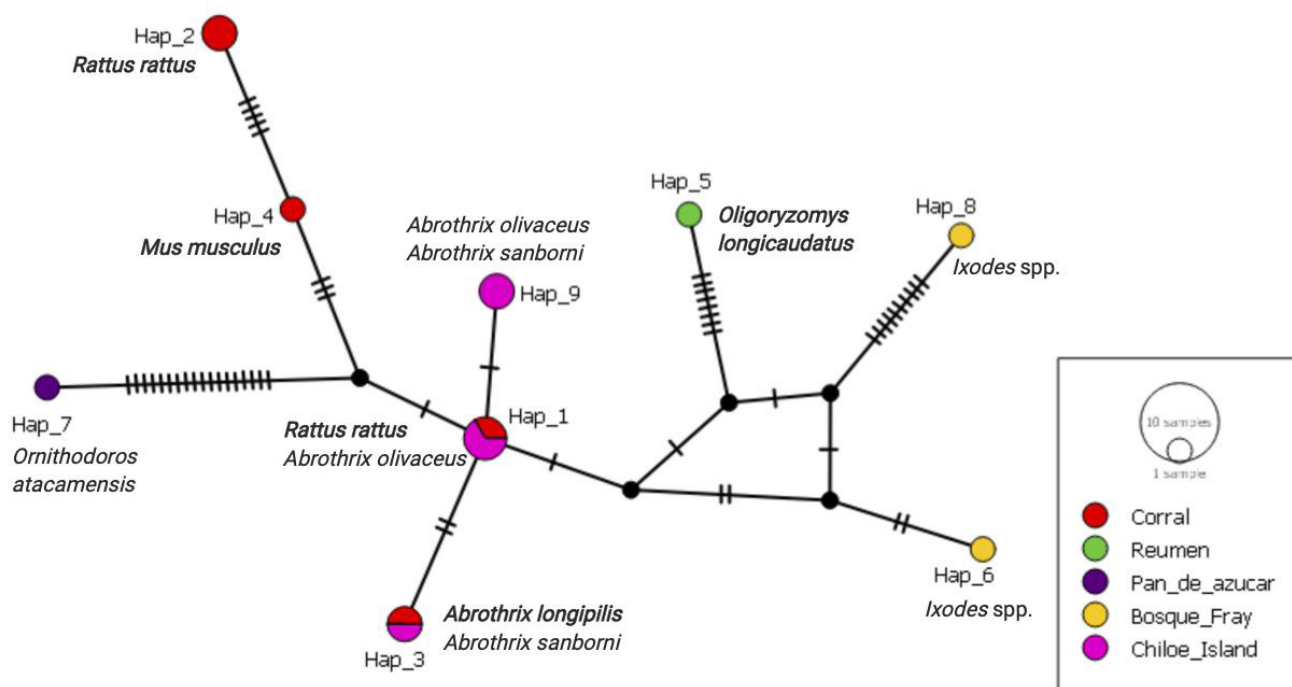
Both subclades containing the concatenated sequences from Chile shared a common ancestor. *Hepatozoon* 18S rDNA sequences from rodents in Valdivia were closely related to those previously detected in rodents and reptiles, while distant from carnivore related *Hepatozoon* species.

### Haplotype analysis

Nine different rodent-associated *Hepatozoon* 18S rDNA haplotypes were observed in Chile: while three were exclusively obtained in the Valdivia province (#2, #4, #5) and comprised novel haplotypes, two were shared between rodents from this study and previously detected sequences from rodents in southern Chile (#1, #3) (Merino et al., 2009). Finally, other four haplotypes were previously detected in rodents (#9) and rodent-associated ticks (#6, #7, #8) (Merino et al., 2009; Muñoz-Leal et al., 2019). The novel haplotypes from this study were observed in *R. rattus* and *M. musculus* in Corral and *O. longicaudatus* in Reumén.

Four haplotypes were found within the Corral locality (haplotypes #1, #2, #3 and #4): while haplotypes #1 and #2 were detected in *R. norvegicus*, haplotype #3 was identified in *A. longipilis*, and haplotype #4 in *M. musculus*. Additionally, Reumén showed only one haplotype (#5). For the other localities reported in previously published studies (northern Chile) one haplotype was observed in a rodent tick from Pan de Azúcar (northern Chile) (#7), two (haplotypes #6 and #8) were detected in rodents' ticks from National Park Bosque Fray Jorge (northern Chile) and three haplotypes were found in rodents from Chiloé (southern Chile) (haplotypes #1, #3 and #9). The only localities that shared haplotypes were Corral (this study, southern Chile) and Chiloé (haplotypes #1 and #3). Table S1 summarizes the polymorphism and genetic diversity of 18S rDNA sequences of *Hepatozoon* species detected in rodents from Valdivia. The haplotype network is presented (Figure 3).

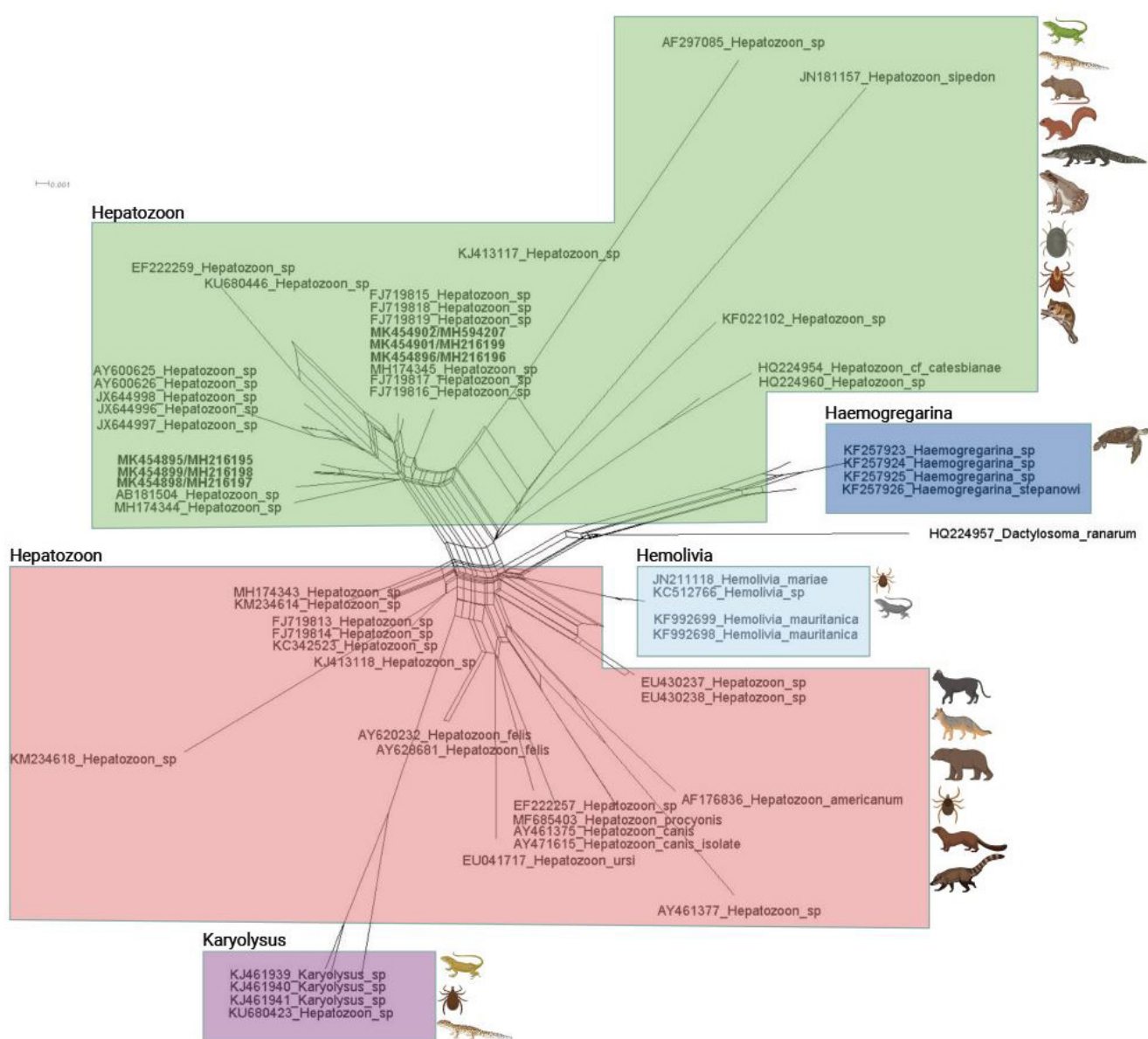
When analyzing the haplotype network (Figure 3) containing *Hepatozoon* species sequences from Chile, the nine observed haplotypes were unfrequently shared between sylvatic and synanthropic rodents or ticks. Consensus sequences (MK454902/MH594207) representing haplotype #5 were formed by *O. longicaudatus* in the present study and rodents from Brazil (KX776336, KX776354, KU667309) comprising strictly sylvatic rodents. Haplotypes #2 (MK454899/MH216198; MK454898/MH216197) from *R. norvegicus* and #4 (MK454895/ MH216195) from *M. musculus* consensus sequences were obtained only from synanthropic rodents and separated by only few mutational events. Haplotypes #3 (MK454901/MH216199) and #9 were detect strictly in *Abrothix* sylvatic rodents. Only one haplotype #1 (MK454896/MH216196) was shared between a synanthropic (*R. norvegicus*) and a sylvatic rodent species (*A. olivaceus*). Moreover, haplotypes from ticks *Ixodes* sp. (#6, #8) and *O. atacamensis* (#7) detected in a previous study were different from the rodents' ones.



**Figure 3.** *Hepatozoon* spp. 18S rDNA haplotype network with northern (ticks from Pan de Azucar and Bosque Fray) (Muñoz-Leal et al., 2019) and southern Chile (rodents from Chiloé Island) (Merino et al., 2009) sequences, including the concatenated sequences from the present study in rodents from the Valdivia province (Corral, Reumen). Each dash line represents a mutational event. Present study's rodents are in bold letters.

### Splits network distance analysis

The Splits tree comprised six concatenated *Hepatozoon* sequences detected in rodents from the Valdivia province, along with worldwide *Hepatozoon* spp., *Haemogregarina* spp., *Hemolivia* spp., and *Karyolysus* spp. sequences (Table S1). In accordance with the Phylogenetic tree, the Splits tree positioned samples from this study separated from carnivora and subdivided into two groups. The major group contained rodents from our study (MK454902/MH594207, MK4901/MH216199 and MK454896/MH216196) and other rodents from Chile (MH174345 MH174345, FJ719815, FJ719816, FJ719817, FJ719818 and FJ719819). The second group was comprised by sequences in rodents from this study (MK454895/MH216195, MK454899/MH216195 and MK454898/MH216197), a bandicoot from Thailand (AB181504) and an Ixodid tick from Chile (MH174344) (Figure 4).



**Figure 4.** SplitsTree analysis generated by Neighbor-net and uncorrected P distance of *Hepatozoon* spp. 18S rDNA sequences obtained from rodents sampled in the present study. Present study's sequences are in bold letters.

### Discussion

Previous studies described the presence of different Apicomplexan organisms in rodents worldwide, such as those from the genus *Hepatozoon* (Allen et al., 2011; Criado-Fornelio et al., 2006; de Sousa et al., 2017; Demoner et al., 2016; Harris et al., 2017; Johnson et al., 2008a; Laakkonen et al., 2001; Maia et al., 2014; Rigó et al., 2016; Wolf et al.,



2016), *Sarcocystis* (Kamani et al., 2018; Merino et al., 2008; Moustafa et al., 2017), *Eimeria* (Kvičerová et al., 2011; Lambert et al., 1988), *Cryptosporidium* (García-Livia et al., 2020; Ježková et al., 2021) and *Besnoitia* (Dubey et al., 2003).

*Hepatozoon* species are widely distributed geographically (Baneth et al., 2000; Latrofa et al., 2014), and have been detected by PCR screening in a broad variety of hosts from America, Africa, Europe, and Asia (Criado-Fornelio et al., 2006; Moustafa et al., 2017). Although *Hepatozoon* spp. were previously reported in rodents (*A. olivaceus* and *A. sanborni*) and in a marsupial (*D. gliroides*), both from Chiloé Island, southern Chile (Merino et al., 2009) as well as in ticks collected from rodents in northern Chile (Muñoz-Leal et al., 2019), little is known about their genetic diversity. To the best of the authors' knowledge, this is the first report of *Hepatozoon* spp. in rodents belonging to the following species: *M. musculus*, *R. norvegicus*, *R. rattus*, *A. longipilis*, and *O. longicaudatus* from Chile. Additionally, the nucleotide diversity and the haplotype structure of *Hepatozoon* species were evaluated for the first time in biological samples of rodents and rodents' ticks from Chile, using the 18S rDNA gene.

According to the phylogenetic inference of the 18S rDNA gene, *Hepatozoon* spp. sequences detected in rodents from the Valdivia province were grouped into two clades, and separated from carnivore *Hepatozoon* spp. by the genus *Karyolysus*, corroborating previous studies (Maia et al., 2016a; Karadjian et al., 2015). The clustering patterns observed in our results were similar to those described by Maia et al. (2016a) and Karadjian et al. (2015). As such, *Hepatozoon* sequences were separated between carnivores (lineages M-P) and rodents, reptiles, amphibian, ticks, marsupials and birds (lineages D-H). *Hepatozoon* spp. sequences from synanthropic and wild rodents from the present study were positioned with other *Hepatozoon* sequences from lineage H.

In South America, there are reports of *Hepatozoon* species in rodents from Brazil and Chile (Criado-Fornelio et al., 2006, 2009; de Sousa et al., 2017; Demoner et al., 2016; Gimenez et al., 2009; Maia et al., 2014; Merino et al., 2009; Perles et al., 2019; Wolf et al., 2016). 18S rDNA nucleotide sequences of *Hepatozoon* spp. obtained in this study showed high identity to those previously detected in rodents sampled in Chile (Merino et al., 2009) and Brazil (de Sousa et al., 2017).

The nucleotide polymorphism analysis of *Hepatozoon* concatenated 18S rDNA sequences were diverse with a high number of haplotypes (n=5) among the population of sampled rodents, with some of the haplotypes (n=3) only identified in the present study, suggesting that novel haplotypes occur in rodents from the Valdivia province, southern Chile. Haplotype diversity is influenced by multiple processes, such as mutation, recombination, and demography (Stumpf, 2004). The haplotype diversity of *Hepatozoon* spp. found in rodents in the present study [(Hd) = 0.933] was higher than the one described [(Hd) = 0.426] by Perles et al. (2019) in rodents from Brazil. The former study covered a much broader geographic area, whereas the Chilean samples were collected only within the Valdivia province (southern Chile). Other studies, based on 18S rDNA sequence data, found four 18S rDNA *Hepatozoon* haplotypes in capybaras (*Hydrochoerus hydrochaeris*) from northern Brazil (de Azevedo Gomes et al., 2018), and three *Hepatozoon* haplotypes in rodents (*Thrichomys fosteri*) from the Brazilian Pantanal (de Sousa et al., 2017).

The haplotype analysis network showed a possible haplotype affinity to certain rodent groups, disregarding the geographic location. For instance, the Corral locality presented a variety of haplotypes associated with *R. rattus* (synanthropic, haplotypes #1 and #2), *A. longipilis* (wild, haplotype #3), and *M. musculus* (synanthropic, haplotype #4); while, Reumén showed only one haplotype, found in *O. longicaudatus* (wild, haplotype #5). Different rodent groups (synanthropic versus sylvatic) and genera may harbor different haplotypes of *Hepatozoon* spp. However, *Hepatozoon* spp. are known to have low host specificity. Host preference for *Hepatozoon* haplotypes in rodents was previously described in Finland, Estonia, Russia, Poland, and Nigeria (Kamani et al., 2018; Karbowski et al., 2005; Laakkonen et al., 2001), and thus the structure of the rodent populations may play a role in the occurrence of certain *Hepatozoon* spp. haplotypes. Further molecular characterization based on fast evolving genes is required to confirm this hypothesis.

Interestingly, in our study Corral was the locality with the highest number of *Hepatozoon* spp. haplotypes (n=3) in rodents. Corral also shared haplotypes with the previous study in Chiloé island (#1 and #3) (Merino et al., 2009), albeit geographically distant. The distribution and sharing of some haplotypes might result from the versatility of synanthropic and wild rodents, the microclimate conditions, and the topography of each sampling site (Muñoz-Zanzi et al., 2014).

A higher number of haplotypes was observed from southern (n=6) compared to northern (n=3) Chile, and they did not share any *Hepatozoon* spp. haplotypes. This could be due to the distance and the biomes' specific characteristics from which rodents were sampled. While Pan de Azúcar (northern Chile) is characterized by coastal desert weather (Squeo et al., 1998), Chiloé (southern Chile) shares the same microclimate with Valdivia (southern

Chile), which is classified as a temperate rain forest (Carmona et al., 2010; Villagran, 1991; Villagrán et al., 2004), and also shares similar elevations to the coastal region, which varies from 0-700 meters (Carmona et al., 2010; Instituto Nacional de Estadísticas, 2007). Also, different *Hepatozoon* spp. host adaptability (vertebrate vs invertebrate) could be related to the divergence of haplotypes, as the northern Chile samples only included rodent ticks and the southern samples included rodents. The variability in the southern samples may be due to a variety of ticks involved or other arthropods that play a role as vectors and remain unknown at the time. To the best of our knowledge there are no studies of ticks associated to rodents in Southern Chile (Valdivia or Chiloé). Ticks previously described in rodents from other regions in Chile include *Ixodes* spp., *Ixodes abrocomae*, *Ixodes sigelos*, *Rhipicephalus sanguineus* and *Amblyomma tigrinum* (González-Acuña & Guglielmone, 2005; Landaeta-Aqueveque et al., 2021).

According to the Splits tree analysis, the *Hepatozoon* 18S rDNA sequences obtained from reptiles and rodents clustered together in a major clade. On the other hand, minor clades grouped *Hepatozoon* sequences from rodents and ixodid ticks reported in Chile by Muñoz-Leal et al. (2019). These findings are similar to the results reported by de Sousa et al. (2017), by Hamšíková et al. (2016) and Perles et al. (2019), and validated by Karadjian et al. (2015) and Maia et al. (2016a), confirming that *Hepatozoon* spp. from rodents were closely related to *Hepatozoon* spp. from reptiles, but distant from *Hepatozoon* spp. described in canids and felids. As previously reported with rodent-associated *Hepatozoon* from Brazil (Perles et al., 2019), *Hepatozoon* in rodents from Chile did not seem to participate in epidemiological cycles of *Hepatozoon* species infecting domestic and wild canids and felids. Those results suggest that rodents from Chile might play a role as intermediate hosts for *Hepatozoon* infections in reptiles and future studies should explore this hypothesis.

The results from Chile are preliminary and based on the 18S rDNA gene. Future studies in South America should explore mitochondrial genes for further *Hepatozoon* spp. diversity characterization, as recently described (Léveillé et al., 2019).

## Conclusions

The findings of this study revealed *Hepatozoon* spp. in synanthropic and wild rodents in the province of Valdivia. This is the first molecular detection of *Hepatozoon* in *M. musculus*, *R. norvegicus*, *R. rattus*, *A. longipilis* and *O. longicaudatus* rodents from Chile. The 18S rDNA sequences from this study were closely related to those previously detected in rodents and reptiles from Chile and Brazil, but distant from *Hepatozoon* spp. described in canids and felids. Different *Hepatozoon* haplotypes were observed in southern and northern Chile. Finally, *Hepatozoon* haplotypes from rodents sampled in Valdivia were genetically diverse, and novel haplotypes were described in rodents from southern Chile. The preliminary results from this study warrant for additional investigation on the genetic diversity of *Hepatozoon* spp., including in a broader population of rodents from Chile and the analysis of mitochondrial genes.

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### **Supplementary Material**

Supplementary material accompanies this paper.

Supplementary Table S1. Phylogenetic and Splitstree analyses sequences.

This material is available as part of the online article from <https://www.scielo.br/j/RBPV>