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Molecular detection of *Cryptosporidium parvum* in wild rodents (*Phyllotis darwini*) inhabiting protected and rural transitional areas in north-central Chile

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ABSTRACT

Wild rodents often harbor *Cryptosporidium* species that can be transmitted to multiple mammal hosts. In Chile, little is known about *Cryptosporidium* in wild rodents, and available studies have been focused on morphological findings with no molecular-based evidence. A longitudinal survey was conducted between 2021 and 2022 to investigate the occurrence of *Cryptosporidium* spp. in populations of the Darwin's leaf-eared mouse (*Phyllotis darwini*) living in protected and rural transitional areas in north-central Chile, using staining and molecular methods. A total of 247 fecal samples were collected and examined by the modified Ziehl–Neelsen (ZN) staining test, 54 of which were positive for *Cryptosporidium*-like oocysts. Molecular analyses were carried out by PCR of the partial 18S ribosomal RNA and 60 kDa glycoprotein (*gp60*) genes. *Cryptosporidium* infection was confirmed in 34 samples (13.7 %) based on the PCR amplification, and individual (i.e., sex, and body mass index) and ecological variables (i.e., type of site and season) were not statistically significant ($p > 0.05$). Using the nucleotide sequencing of the partial 18S rRNA gene, *Cryptosporidium parvum* was identified in nine isolates. Also, *C. parvum* subgenotype family *IIa* was determined in seven samples by the partial *gp60* gene, including the subtype *IIaA17G4R1* in two samples. This is the first molecular evidence of *Cryptosporidium parvum IIa* in *Phyllotis darwini* in Chile. These results indicate potential cross-species transmition between wild rodents and domestic-wild animals in north-central Chile. More research is needed to understand better the role of wild rodents in the transmission of *Cryptosporidium* spp. in Chile.

1. Introduction

Various animal taxa can act as reservoirs or carriers of *Cryptosporidium* spp., such as small mammals. Wild rodents, in particular, can play an important role in the maintenance and transmission of *Cryptosporidium* spp. given that they often exhibit high population densities and can adapt to different kinds of habitats due to high behavioural plasticity (Mills and Childs, 1998; Taghipour et al., 2020; Zhang et al., 2022).

Globally, the overall prevalence of *Cryptosporidium* spp. in rodents is estimated to be 20 %, and it reaches to 7 % in South America according to the few available studies (Ferraz Fehlberg et al., 2021; Taghipour et al., 2020; Zhang et al., 2022). In addition, rodents can harbor up to 25 *Cryptosporidium* species and 43 subgenotypes, some of which are of public health significance such as *C. parvum* Tyzzer 1912, *C. muris* Tyzzer 1907, and *C. ubiquitum* Fayer, Santin & Macarisin 2010 (Stensvold et al., 2024; Zhang et al., 2022). Other *Cryptosporidium* species identified specifically in rodents include *C. proliferans* Kváč et al., 2016 in moles; *C. alticolis* Hor et al., 2018 and vole genotypes I–VII in voles; and, *C. homai* Kueh et al. 2017 in guinea pigs (Hor et al., 2018; Kueh et al., 2017; Kváč et al., 2016; Zhang et al., 2022). Also, some human cases of cryptosporidiosis caused by rodent-adapted *Cryptosporidium* species (e.g., *C. mortiferum* Tůmová et al. 2023) have been detected (Stensvold et al., 2024; Tůmová et al., 2023).

Conventional staining methods are commonly used for the diagnosis of the *Cryptosporidium* genus based on the morphological features and staining properties (Taylor et al., 2016). In addition, molecular tools

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using the partial 18S ribosomal RNA (SSu-rRNA) and the glycoprotein 60 (*gp60*) genes have been frequently used in the identification of *Cryptosporidium* species, subgenotypes, and subtypes in rodents and other small mammals (Alves et al., 2003; García-Livia et al., 2020, 2022; Silva et al., 2013; Xiao et al., 1999).

Anthropogenic land use changes and climate can have different effects on *Cryptosporidium* infection rates in wild animals. Evidence indicates that infection rates of directly-transmitted parasites such as *Cryptosporidium* spp. may vary by the inherent host and environmental conditions in those altered habitats (e.g., agriculture, urbanization, and forestry) (Carrera-Játiva and Acosta-Jamett, 2023; Werner and Nunn, 2020). Also, climate variation has been reported as a driver of cryptosporidiosis (Ikiroma and Pollock, 2021; Mosier and Oberst, 2000). While *Cryptosporidium* oocysts have the capacity to survive in the environment and remain infective for at least 6 months in suitable conditions (Xiao et al., 2004), higher prevalence and incidence rates of *Cryptosporidium* spp. have been observed in warmer months and during rainy seasons in human and animals (Ikiroma and Pollock, 2021; Jagai et al., 2009).

Studies on *Cryptosporidium* infections in wild rodents are still limited in Chile. In particular, little is known about the presence and seasonal dynamics of *Cryptosporidium* spp. in wild rodent populations living in human-altered habitats (Infante et al., 2022), and no molecular information is available on *Cryptosporidium* species and subgenotypes in Chilean free-living rodents. Knowledge of *Cryptosporidium* species and subgenotypes in small wild mammals in Chile can contribute to a better understanding of the regional and global distribution, risks of transmission across species, including humans, and the impact of human-induced habitat changes regarding cryptosporidiosis.

The landscape of the semi-arid Mediterranean ecosystem in north-central Chile has been changing due to human-related activities (e.g., agriculture and urbanization) over the past 20 years, with implications for native and endemic fauna that still need to be addressed (Beltrami, 2021; Pavez et al., 2010). One of the most abundant native rodents inhabiting such area is the Darwin's leaf-eared mouse (*Phyllotis darwini* Waterhouse 1837) [Family: Cricetidae], which has exhibited fluctuating populations during recent years (Beltrami, 2021; Meserve et al., 2016). Thefore, the objective of the present study was to investigate the occurrence of *Cryptosporidium* in populations of *Phyllotis darwini* living in two areas with different kinds of anthropogenic impact (i.e., protected and rural transitional areas) in north-central Chile using staining and molecular methods in fecal samples. The authors hypothesized that if anthropogenic habitat alteration and climate variability promote transmission of directly transmitted parasites due to changes in host and environmental conditions, then a higher prevalence rate of *Cryptosporidium* sp. would occur in *P. darwini* inhabiting rural altered areas and in the winter season.

2. Materials and methods

2.1. Ethics statement

Animal capture and sampling were carried out under the approval and supervision of the Scientific Ethics Committee Resolution for the Use of Animals in Research of the Universidad Austral de Chile (Nº 430/2021), the Agricultural and Livestock Service of Chile, SAG, Chile (Exempt Resolution Nº 3245/2021), and the National Forest Corporation, CONAF, Chile (Letter Nº 26/2021).

2.2. Study population and design

A longitudinal cohort study was carried out to assess *Cryptosporidium* spp. in *P. darwini* living in two areas with different kinds of anthropogenic impact (i.e., protected and rural transition), in the Coquimbo Region, in north-central Chile, during five consecutive seasons (i.e., spring 2021 [September–December], summer 2022 [December–March], Autumn, 2022 [March–June], winter 2022 [June–September], and spring 2022 [September–December]) (Fig. 1).

The protected area is located within the Bosque Fray Jorge National Park (BFJNP: 9959 ha), which was legally constituted in 1941 and represents protected natural habitats with xeric and mesic vegetation such as thorn scrub, and scrub with cacti (CONAF. Corporación Nacional Forestal, 2024; Squeo et al., 2016). In counterpart, the rural transition encompasses an area located in the agri-pastoral domains of El Tangue Farm (ET: 45000 ha) adjacent to the BFJNP (27 km) and Tongoy city (~19 km). Lands of ET have been historically used for sheep farming and dairy production, and current economic activities also include agriculture, tourism, and real state (Sociedad Agrícola y Ganadera El Tangue Ltda, 2022). The vegetation in the rural transitional area is featured by agricultural plantations (e.g., *Olea europea* Linneo 1753*)* and native and exotic shrubs (e.g. *Atriplex nummularia* Linneo 1753) vegetation. In both areas, *P. darwini* is present with varying population densities (Beltrami, 2021; Meserve et al., 2011, 2016).

In each sampling season and site type, three or four rectangular grids (150 m × 135 m; 2 ha) were deployed, and 200 capture points were established by the methods for estimating population density for small mammals (Beltrami, 2021; Romairone et al., 2018; Royle et al., 2018). Briefly, each grid was formed by allocating 10 parallel rows (150 m) with a distance separation of 15 m. Capture points were placed at equal distances (15 m) along each row (i.e., 110 capture points in 10 rows). An additional capture point was located at the center of four capture points (i.e., 90 capture points). Selected grids were located >1 km apart from one another in relation to the average home range of *P. darwini* (i.e., 1154 m^2) to avoid individual replication between grids (Muñoz-Pedreros and Gill, 2009). See Fig. 1.

2.3. Host capture and sampling

Capture was carried out by setting up Sherman-like traps (dimensions = 300 x 100 × 110 mm) in the established capture points of the studied grids in each site type per season (i.e., a total of 200 traps were placed per grid). Traps were placed under vegetal material, baited with oat flakes and vanilla essence, and activated for 12 h overnight during 3–4 nights. Trapping effort ranged from 1800 to 3200 trap-night per site in each season. During the morning hours (7:00–11:59 a.m.), captured rodents were handled, sampled, and released at the specific capture point once processed. Rodent sample collection was conducted under sedation using a solution of Ketamine (0,044 mg/g BW) and Xylazine (0,006 mg/g BW), administered intramuscularly. Individuals were ear-tagged (National Band & Tag Company®, New Port, RI, US), measured morphometrically with a digital caliper (Uberman®, precision 0.01 mm), and weighed (Pesamatic Newton Series®, Model EJ1500, A&D Weighing, San Jose, CA, U.S.A.; ±0.1 gr SD). Rodents were classified according to sex and age (i.e., adults and subadults) considering that females were those with narrow anogenital distance, and adults comprised those with higher body weight according to sex (i.e., males = > 40 g; females = > 35 g) (Lima et al., 2001; Muñoz-Pedreros and Gill, 2009). Fresh fecal samples were taken directly from the anus and/or collected from the previously disinfected trap base and subsequently placed in sterile plastic vials with 1.5 ml ethanol (96%) to then be stored at room temperature until further analysis (Lalonde and Gajadhar, 2009).

2.4. Parasitological examination by staining method

Parasitological procedures were carried out at the Laboratorio de Parasitología, Instituto de Patología Animal, Universidad Austral de Chile. Fecal samples (0.01–0.05 g) were homogenized with distilled water, vortexed for 20 s, and filtered. The content was poured into a tube to be centrifuged at 250×*g* for 5 min, and the supernatant was discarded. An aliquot of the sediment (300 µl) was used to make a smear (2 cm × 1 cm). Subsequently, the smear was air-dried, fixed in 100% methanol for 5 min, and stained using the modified Ziehl–Neelsen (ZN) technique as

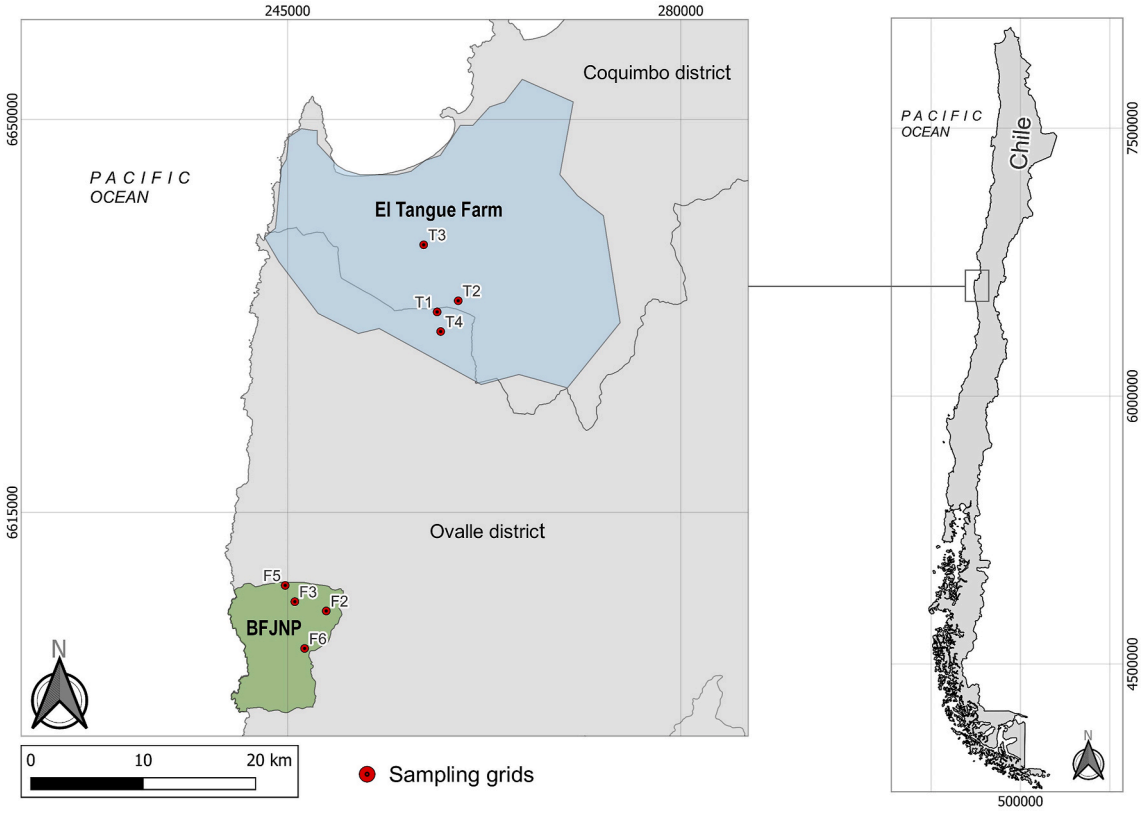

Fig. 1. Map of the Coquimbo region in Chile showing the two types of areas (i.e., Bosque Fray Jorge National Park - BFPNP [in green]; El Tangue Farm [in blue]) in which Darwin's leaf-eared mice (*Phyllotis darwini*) were sampled. In each area, 4 grids were established, and 200 capture points were allocated per grid. (UTM projection. Datum WGS84, Zone 19J).

described (Bukhari and Smith, 1995; Henriksen and Pohlenz, 1981; Taylor et al., 2016). All fecal smears were examined microscopically at 1000x magnification with oil-immersion using a microscope (Novel DN-117M, Ningbo Yongxin Optics Co., Ltd., 169, Ningbo, China). Oocysts were identified by the alcohol-resistant feature (i.e., bright pink color) and size (i.e., 3 × 8 μm) (Pinto et al., 2022), and all *Cryptosporidium*-compatible structures were photographically recorded with a microscope camera (Ningbo Yongxin Optics Co., Ltd., 169, Ningbo, China) and measured digitally (i.e., length and width) using the ToupTek ToupView® Software 2021 (ToupTek Photonics, Hangzhou, 310030, Zhejiang, P.R. China). In addition, all oocysts throughout the border of each fecal smear (a total of ~80 microscope fields; 1 field at 1000x magnification = 80 μm × 65 μm) were counted. Positive samples were stored at −80 °C to be used for molecular identification.

2.5. Nucleic acid extraction

Molecular procedures were carried out at the Laboratorio de Enfermedades Infecciosas, Instituto de Medicina Preventiva Veterinaria, Universidad Austral de Chile. Total genomic DNA (gDNA) was extracted from those fecal samples that exhibited *Cryptosporidium*-like oocysts using the QIAamp® Fast DNA Stool mini kits (Qiagen, Hilden, Germany). Initially, 300 μL InhibitEx buffer (QIAamp® Fast DNA Stool mini kit) and 0.3 g glass lysis beads (0.5 mm diameter) (BioSpec Products, Inc., Bartlesville, OK 74005, USA) were added to a microvial (1.5 ml) containing 200 μL of the stored fecal sample with concentrated oocysts. Later, samples were subjected to mechanical lysis using a Mini-beadbeater 24 grinder (BioSpec Products, Inc., Bartlesville, OK 74005, U.S.A.) in accordance with established protocols (Dougnac Opitz, 2015; Painean et al., 2022). Then, 20 μL of proteinase K (QIAamp® Fast DNA Stool mini kit) was added, and DNA extraction was completed as per the manufacturer's instructions. Finally, the extracted DNA was stored at −20 °C until further analysis.

2.6. Molecular detection and sequencing

Prior to genus-specific amplification, the presence of DNA inhibitors was assessed through a conventional Polymerase chain reaction (PCR) targeting the mammalian *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene (Birkenheuer et al., 2003). Subsequently, only those positive samples with *gapdh* amplicons of the expected size (i.e. 400 bp) were evaluated for the partial 18S ribosomal RNA and 60-kDa glycoprotein (*gp60*) genes for *Cryptosporidium* spp. using conventional and nested PCR protocols, respectively (Muñoz et al., 2011; Xiao et al., 2004). The primer sequences required for PCR amplification were obtained from previous studies (Alves et al., 2003; Muñoz et al., 2011; Santodomingo et al., 2022) and were synthesized by Integrated DNA Technologies, Inc., (Eugene, OR 97402. U.S.A). Primer and PCR thermal conditions used in this research are provided in ESM 1.

Each PCR reaction was performed using the SapphireAmp Fast PCR Master Mix (Cat. No. RR350A, Takara Bio Inc., Shiga, Japan) according to adapted protocols (Christensen, 2020; Takara Bio Inc., 2021). Briefly, all reactions were carried out in a final volume of 25 μl, containing 12.5 μl of the master mix, 0.5 μl of each primer (10 μM each), 3 μl of cDNA template (or 3 μl of primary PCR product for the secondary PCR in the nested PCR), and 8.5 μl of ultra-pure water. All PCR reactions were carried out during 40 cycles in a Axygen® MaxyGene II Thermal Cycler (Corning Incorporated Life Sciences, NY 14831 U.S.A.). Positive and negative controls were used in each PCR run. Positive controls included DNA of *C. parvum* subgenotype *IIaA15G2R1* from infected cattle (Painean et al., 2022). The negative control samples consisted of nuclease-free water.

Amplicon sizes were confirmed by agarose gel electrophoresis using a 1.5% (w/v) CSL-AG100 LE agarose gel (Cleaver Scientific, Rugby,

CV22 7DH, UK) in 50X TAE (Bioneer Corp., Daejeon 34302, Korea), and stained with SYBR™ Safe (Life Technologies Corp., Carlbad CA 92008 U. S.A). The 100bp DNA Ladder (New England Biolabs, Ipswich, MA 01938, U.S.A.) was used as a molecular size marker. Processed gels were transferred to a UV transillumination to be photographically recorded, and fragments of the expected size were excised to subsequently be purified using an E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Inc., Georgia 30071, U.S.A.) according to manufacturer's instructions. Purified products of conventional (i.e., *18S rRNA* gene) and nested (i.e., *gd60* gene) PCR reactions were then submitted for bi-directional sequencing to the AUSTRAL-*omics* Institute at the Universidad Austral de Chile (Valdivia, Chile), where they used the Applied Biosystems® Sanger Sequencing 3500 and 3500xL Genetic analyzers with the Sequencing Analysis Software v6.0 (Life Technologies Corp., Carlsbad, CA 92008, U. S.A.). The secondary primers (i.e., forward and reverse) of the nested PCR were used for the sequencing analysis of the *gp60* gene.

2.7. Molecular characterization of Cryptosporidium species and subtypes

Consensus sequences were assembled and edited manually from the forward and reverse reads of the partial *18S rRNA* gene using the Unipro UGENE v49.1 software (Unipro, Novosibirsk 630090, Russia) (Okonechnikov et al., 2012) with the references (Acces. number MK014785) and a mapping similarity >60%. The consensus sequences derived were then used to identify species by comparing to data in nucleotide databases using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed May 10, 2024). Later, published sequences, including those with the highest similarity, were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/; last accessed May 10, 2024), and multiple sequence alignments were carried out to determine the homology between published isolates and the consensus sequences using the MUSCLE (Edgar, 2004) function included in MEGA 11 software V.11.0.13 (Tamura et al., 2021). The best fitting model for the DNA/protein phylogeny was selected for each alignment based on the Bayesian information criterion, and a phylogenetic tree was constructed by the maximum likelihood (ML) algorithm, using the Tamura 3-parameter with discrete Gamma distribution (T92+ G) (5 categories) (Tamura and Nei, 1993) nucleotide substitution model in MEGA 11. Final tree were edited in Mega 11 using *Cryptosporidium muris* as out group.

For determination of the *Cryptosporidium* subgenotype family and subtypes, the sequencing reads (i.e., forward and reverse) of the partial *gp60* gene were assessed using the software CryptoGenotyper® (Yanta et al., 2021).

2.8. Statistical analyses

Prevalence and 95% confidence intervals (CI) were obtained following the Clopper-Pearson method (Clopper and Pearson, 1934). A semi-quantitative score of *Cryptosporidium* intensity was assessed by counting all oocysts present throughout the border of each smear, and the presence of 1–3 oocysts was classified as very low, 4–10 oocysts as low, 11–20 oocysts as intermediate, and >20 oocysts as high (Painean et al., 2022; Taylor et al., 2016).

The Scaled Mass Index (SMI) was calculated for adults and juveniles separately based on the morphometric information of rodents (i.e., body weight, and body length) and according to the established method (Peig and Green, 2009).

Generalized Linear Mixed models (GLMER) with binomial errors were applied to evaluate the infection probability in relation to ecological factors (i.e., sex, site type, season, and SMI) (Bates et al., 2015). Dependent variables consisted of the presence-absence (binomial) of *Cryptosporidium* infection confirmed by molecular analyses (*18S rRNA* and *gp60* genes). Fixed effects included sex, type of site (i.e., protected area, rural transition), trapping season (spring 21, summer 22, autumn 22, winter 22, spring 22), and host body condition (i.e., SMI data). Rodent ID was included as a random factor to account for the individual recaptures between seasons. The models followed a forward selection procedure, in which unconditional models of each fixed effect were first assessed, and only those variables associated with the outcome ($p < 0.05$) were included in the conditional model. No interaction and confounders were considered due to biological insignificance. The fit of models was assessed using the Akaike Information Criteria (AIC) index. Statistical significance was set at $p < 0.05$. The "lme4", "MASS", and "Broom Mixed" packages were used to calculate the information about fitted models using the software R (R Development CoreTeam, 2013) and RStudio (RStudio Team, 2022). The map of the sampling locations was constructed by the software QGIS 3.20 (QGIS, 2024).

3. Results

Two hundred forty-seven (247) fecal samples of *P. darwini* were analyzed from individuals captured from spring 2021 to spring 2022. Sample size of individuals according to sex, type of site, and season are shown in Table 1. Body weight of sampled rodents ranged from 12 to 68 g. (weight mean 43.1 ± standard error = 0.8, n = 241). The scaled body mass index ranged from 13.1 to 71.5 (43.81 ± 13.05, n = 240). During the period of this research, 32 individuals were recaptured in two or more sampling seasons.

3.1. Parasitological examination

Of 247 fecal samples of *P. darwini*, 54 were positive for *Cryptosporidium*-like oocysts in the modified Ziehl–Neelsen test. Based on the semi-quantitative score, most of the samples samples (n = 42) exhibited a very low intensity with 1–3 oocysts per sample. Nine samples were classified as low-intensity with 4–10 oocysts per sample. Only three samples showed intermediate to high intensity with 13–28 oocysts per sample. Overall size dimensions of *Cryptosporidium* oocysts were 4.16 μm length (Standard error = 0.09, n = 153) and 3.3 μm width (±0.07).

3.2. Molecular detection of Cryptosporidium

DNA was extracted from the 54 fecal samples exhibiting *Cryptosporidium* oocyst-compatible structures. The successful DNA extraction was proven in 43 out of the 54 samples based on the positive PCR reactions with products of amplicons of the expected size for the *gapdh* gene (i.e., 400 bp) (ESM 2).

For the *18S rRNA* gene (Muñoz et al., 2011), a ~500-base-pair amplicon specific to *Cryptosporidium* spp. was amplified in 27 out of the 43 samples (ESM 3). For the *gp60* gene (Alves et al., 2003), an ~800-base-pair amplicon was amplified in 23 out of the 43 fecal samples (ESM 4). A total of 34 fecal samples were found to be positive for

Table 1
Sample size and prevalence (CI 95 %) of *Cryptosporidium* sp. infection in *Phyllotis darwini* in north-central Chile based on the PCR amplification on the *18S rRNA* (n = 17) and *gp60* (n = 23) genes according to host and environmental factors. Overall prevalence 13.7 % (95% CI = 9.7–18.7) [34/247].

Variable	N	PCR Positive	Prevalence % (CI 95 %)
Sex			
Female	89	14	15.7 (8.8–24.9)
Male	138	17	12.3 (7.3–18.9)
Missing	20		
Type of site			
Protected area	127	17	13.4 (7.9–20.6)
Rural transition	120	17	14.1 (8.4–21.7)
Season			
Spring 2021	51	10	19.6 (9.8–33.1)
Summer 2022	56	4	7.1 (1.9–17.3)
Autumn 2022	62	5	8.0 (2.6–17.8)
Winter 2022	52	7	13.5 (5.6–25.8)
Spring 2022	26	8	30.8 (14.3–51.8)

Cryptosporidium sp. by PCR analyses with products of amplicons of the expected size for either the *18S rRNA* and *gp60* genes. Therefore, the occurrence of *Cryptosporidium* infection in *Phyllotys darwini* in north-central Chile was 13.7 % (34/247; 95% CI = 8.7–17.3 %). A summary of parasitological and molecular results are shown in ESM5.

3.3. Epidemiological associations

The prevalence of *Cryptosporidium* sp. infection in *P. darwini* in north-central Chile based on molecular analyses and associated factors are shown in Table 1. *Cryptosporidium* infection in *P. darwini* did not exhibit statistical associations with either individual (i.e., sex, and SMI) or ecological variables (i.e., type of site and season) in the GLMER models ($p > 0.05$).

3.4. Molecular characterization of Cryptosporidium species and subtypes

Twenty-one (21) PCR products with the highest band intensity in the agarose gel were submitted to Sanger sequencing for the partial *18S rRNA* gene. After sequencing, only nine isolates presented homogeneous reads in both directions (i.e., forward and reverse), and the other 12 samples were not adequate for further analysis (ESM 5). After the assemblage and edition of the forward and reverse reads, nine consensus sequences (fragments between 397 and 512 bp) were finally obtained. The BLAST showed the highest homology (between 99% and 100% identity) with various *Cryptosporidium parvum* isolates.

Maximum likelihood phylogenetic analysis based on an alignment of 466 bp (nucleotide positions 665–1297) of the *18S rRNA* gene showed that evaluated consensus sequences of *Cryptosporidium* found *P. darwini* in the present study were clustered together within a monophyletic group containing isolates of *Cryptosporidium parvum* from several host species including ruminants (China), humans (Netherlands, Spain), felines (China), a horse *Equus caballus* (Iraq), and river water sample (Chile) (Fig. 2).

For subtyping, 16 nested PCR products with the highest band intensity in the agarose gel were submitted to Sanger sequencing of partial *gp60* gene. After sequencing, seven isolates exhibited both forward and reverse nucleotide sequencing reads. The other nine samples were not appropriate for further analysis. Using the CryptoGenotyper® software (Yanta et al., 2021), *C. parvum* subgenotype family *IIa* was determined in seven samples, including the subtype *IIaA17G4R1* (100% identity) in two samples from the protected and rural areas (deposited in GenBank under the accession numbers: PQ084644, PQ084647).

4. Discussion

The occurrence of *Cryptosporidium* sp. in fecal samples of *P. darwini* from north-central Chile was 13.7% (34/247) by the molecular analyses of the partial *18S rRNA and gp60* gene markers. The prevalence rate in the current study was higher than those described in a rodent community (4.7 %; 29/614) in the Maule region in central Chile using a staining method (Infante et al., 2022). In other regional research, prevalence rates of *Cryptosporidium* spp. in wild rodents varied between 1.47 % (2/136) in cricetid rodents (i.e., *Rhipidomys mastacalis* Lund 1841 and *Hylaeamys laticeps* Lund 1840) in Brazil by molecular analysis and 50.4% (69/137) in the brown rat (*Rattus norvegicus* Berkenhout 1769) in

Fig. 2. Phylogram representing analysis of the *18 rRNA* region. The evolutionary history was inferred with maximum likelihood method and the Tamura 3-parameter (T92) model with a discrete Gamma distribution (5 categories (+G)). Analysis contains sequences uploaded from GenBank (with *Cryptosporidium* species, host, country, and accessions numbers in brackets) and those obtained in the present study are shown in triangles (with *ID* isolate, host, site of sampling and country, and accessions numbers in brackets). Bootstrap values are represented as per cent of internal branches (1000 replicates), and values lower than 50 are hidden. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Cryptosporidium muris* was used to root the tree.

Argentina using microscopy (Ferraz Fehlberg et al., 2021; Hancke and Suárez, 2020). According to a meta-analysis, the pooled global prevalence in rodents ranged from 13 % to 20 % (Taghipour et al., 2020). Differences in prevalence rates in *P. darwini* in north-central Chile compared to other rodent populations in Chile and South America can be attributed to inherent ecological conditions, variable sample size, host features (e.g., population densities), climate variability, and the use of different diagnostic methods (Taghipour et al., 2020; Zhang et al., 2022).

Due to the impacts of anthropogenic disturbance on hosts and the environment, we expected to find higher occurrences of *Cryptosporidium* infection in *P. darwini* inhabiting the rural transition compared to those in the protected area in north-central Chile. However, there was no statistical difference in the prevalence of *Cryptosporidium* according to site type. A comparable nonspecific effect was observed in a rodent community living in native forests and exotic Monterey pine (*Pinus radiata* D.Don, 1836) plantations in central Chile, in which habitat alteration was not related to *Cryptosporidium* spp. infection (Infante et al., 2022). *P. darwini* living in protected and human-altered areas in north-central Chile may be facing host and environmental factors that maintain similar patterns of *Cryptosporidium* sp. transmission. For example, different reservoir hosts (e.g., native or introduced) may be available in the protected and rural areas that favour transmission (Suzán et al., 2012).

Also, we expected to find a higher occurrence of *Cryptosporidium* infection in *P. darwini* during the winter season since higher rates have been reported in warm and wet conditions (Jagai et al., 2009). However, no statistical differences of infection rates were observed. In a previous work in a rodent community in central Chile, a higher prevalence rate of *Cryptosporidium* spp. was reported during the winter season, and a lower rate was observed during spring (Infante et al., 2022). The semiarid Mediterranean climate in north-central Chile is characterized by sporadic precipitation during the cooler months (.i.e., March to September [autumn - winter]) and drought may extend during the spring (September–November) and summer (December–March) seasons (Armesto et al., 2007; Novoa and López, 2001). *Cryptosporidium* infection in *P. darwini* in the assessed areas may have not been significantly influenced by the seasonality since wet conditions are limited even during the winter season. Likewise, individual features of *P. darwini* in north-central Chile (i.e., sex and scaled body mass index) were not statistically associated with *Cryptosporidium* sp. infection. Similar finding was also reported in a study in a rodent community in central Chile (Infante et al., 2022). In a meta-analysis of global *Cryptosporidium* in rodents, no significant difference between males and females was also reported (Taghipour et al., 2020). Thus, *P. darwini* individuals of both sexes, and different body compositions may be equally exposed to *Cryptosporidium* infection.

Cryptosporidium parvum was detected in fecal samples in *P. darwini* in north-central Chile by the partial nucleotide bi-directional sequencing of the *18S rRNA* and *gp60* genes (see ESM 5). *C. parvum* is a low-host specific protozoa of mammals that is known to infect ruminants and humans as major hosts as well as rodents as minor hosts (Xiao et al., 2004; Zhang et al., 2022). In this context, *C. parvum* is considered the most prevalent species in rodents globally (Taghipour et al., 2020; Zhang et al., 2022). In Brazil, *Cryptosporidium parvum* was described infecting the wild rodent *Rhipidomys mastacalis* (Ferraz Fehlberg et al., 2021). In Chile, *C. parvum* has been reported in cattle and humans in different locations (e.g., Valparaiso, Metropolitan, Los Rios regions) with prevalence rates that ranged between 1% and 50% in cattle and ~6% in humans (Díaz-Lee et al., 2011; Mercado et al., 2015; Neira-Otero et al., 2005; Painean et al., 2022). Moreover, *C. parvum* was detected in Cholga mussels (*Aulacomya atra* Molina, 1782) in the Concepcion Bay in BioBío region, Chile, which account for its environmental presence (Suarez et al., 2024). In addition, *Cryptosporidium parvum* subgenotype family *IIa* was determined in *P. darwini* fecal samples according to the nucleotide sequences for the partial *gp60* gene (n = 7), including *C. parvum* subtype *IIaA17G4R1* in two samples. *Cryptosporidium parvum IIa* and *IId* are considered as the most frequent zoonotic *Cryptosporidium gp60* subgenotypes globally with host species that included ruminants, humans, and rodents (Nader et al., 2019). In central Chile, *Cryptosporidium parvum IIaA17G4R1* was previously detected in a fecal sample of a calf (Mercado et al., 2015) as well as there are reports of the same subtype (i.e., *C. parvum IIaA17G4R1*) in humans in Australia (Waldron et al., 2011).

It is plausible that domestic or wild animals (e.g., ruminants) harbouring *C. parvum IIa* are acting as reservoir hosts to *P. darwini* populations in protected and rural transitional areas in north-central Chile. In this sense, no information is available on *Cryptosporidium* species wild ruminants in Chile such as in the native Chilean guanaco *Lama guanicoe* Müller 1776. In Perú, *C. parvum* (1%; 2/274) was detected in free-living alpacas (*Vicugna pacos* Linnaeus, 1758) (Gómez-Couso et al., 2012). Evidence in the present work shows that *C. parvum IIa* is common in populations of *P. darwini* in protected and rural areas in north-central Chile, and several domestic and wild host species, including *P. darwini*, may take part in its maintenance and transmission cycles in the region. To the best authors' knowledge, this is the first molecular-based evidence of *Cryptosporidium parvum IIa* in small wild mammals in the country. More studies are required to understand better the role of wild rodents in the transmission of *Cryptosporidium parvum IIa* among wildlife, domestic animals, and human populations in various ecosystems in Chile in the context of human-induced habitat change.

The multi-stage diagnostic approach taken in this research with parasitological and molecular examination allowed to verify the presence of oocysts in fecal samples and optimize the use of laboratory resources. However, the selection of the modified ZN staining as a screening test for *Cryptosporidium* has some limitations. The ZN staining method can exhibit low sensitivity (68.3–81.8 %) and specificity (96.5–100%) in comparison to other tests (e.g., immunofluorescence microscopy IFM) (Chalmers et al., 2011; Checkley et al., 2015). This may have led to mis-identification of *Cryptosporidium*-like structures in some samples since the specificity of the test for animal feces may differ according to the occurrence of the acid-fast objects in the correct size range within the fecal matrix, as previously reported (Chang'a et al., 2011). To address this issue, statistical analyses in the present work were based on the results of the molecular analyses. In future research, the use of the established gold standard tests (i.e., the real-time PCR with oocyst detection by IFM tests) in all fecal samples are recommended (Chalmers et al., 2011). Also, most fecal samples (n = 42) of *P. darwini* exhibited low intensity (i.e., 1–3 oocysts per sample) based on the semi-quantitative score in the ZN test. While spurious findings due to transit oocysts are possible, it has been established that the lower detection limits of the ZN staining and PCR methods for *Cryptosporidium* oocysts in cattle feces were 22,813 oocysts per ml and 11,406 oocysts per ml, respectively. Findings in *P. darwini* are likely to be due to subclinical infection as evinced by no statistical relationship with SMI. Additional histopathological studies may be required to assess the actual levels of infection in *P. darwini* in north-central Chile. Finally, in this research some samples exhibited PCR products with low band intensity in the agarose gel and low quality of electropherograms, which limited the subtype determination in certain samples. The collection and assessment of higher amounts of individual fecal samples (>0.05 g) and the use of novel sequencing techniques (e.g., in-vitro cultivation in parasite systems or sorted single-cell genomic sequencing) may be required in future research (Baptista et al., 2021).

5. Conclusion

The zoonotic *Cryptosporidium parvum* subtype *IIaA17G4R1* was found in fecal samples of *Phyllotis darwini* inhabiting protected and rural transitional areas in north-central Chile. The occurrence of *Cryptosporidium* infection did not differ according to individual (i., sex, SMI) and environmentar variables (i.e., site and season). These results indicate

potential cross-species transmission between wild rodents and domestic-wild animals in north-central Chile. More research is needed to understand better the role of wild rodents in the transmission of cryptosporidiosis in Chile in the context of human-induced habitat changes and the risks of (emerging) zoonoses for public health.

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CRediT authorship contribution statement

Patricio D. Carrera-Játiva: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gerardo Acosta-Jamett:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Pamela Muñoz:** Writing – review & editing, Validation, Resources, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gerardo Acosta-Jamett reports financial support was provided by ANID Fondecyt Regular 2021 N. 1211190. Patricio D. Carrera-Jativa reports financial support was provided by ANID Programa de Becas Doctorado Nacional N. 21200220. Patricio D. Carrera-Jativa reports financial support was provided by WWF Russell E. Train Fellowship. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100971.

References

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype analysis of *Cryptosporidium* isolates from humans , cattle, and Zoo ruminants in Portugal. J. Clin. Microbiol. 41, 2744–2747. https://doi.org/10.1128/JCM.41.6.2744.

Armesto, J.J., Arroyo, K., Mary, T., Hinojosa, L.F., 2007. The Mediterranean environment of Central Chile. In: Velben, T.T., Young, K.R., Orme, A.R. (Eds.), The Physical Geography of South America. Oxford University Press, New York, pp. 184–199.

Baptista, R.P., Cooper, G.W., Kissinger, J.C., 2021. Challenges for *Cryptosporidium* population studies. Genes 12, 894.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-effects models using lme4. J. Stat. Software 67, 1–48.

Beltrami, M.E., 2021. Ecology, Parasitism and Physiological Patterns of Wild Small Mammals through an Anthropogenic Landscape Gradient in a Semi-arid Ecosystem of Chile. Universidad Austral de Chile. Doctoral Thesis.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a Seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J. Clin. Microbiol. 41, 4172–4177. https://doi.org/10.1128/JCM.41.9.4172.

Bukhari, Z., Smith, H., 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. J. Clin. Microbiol. 33, 2592–2595.

Carrera-Játiva, P.D., Acosta-Jamett, G., 2023. Influence of habitat alteration on the structure of helminth communities in small mammals: a systematic review and critical appraisal of theory and current evidence. Parasitol. Res. 122, 1053–1070. https://doi.org/10.1007/s00436-023-07804-8.

Chalmers, R.M., Campbell, B.M., Crouch, N., Charlett, A., Davies, A.P., 2011. Comparison of diagnostic sensitivity and specificity of seven *Cryptosporidium* assays used in the UK. J. Med. Microbiol. 60, 1598–1604. https://doi.org/10.1099/jmm.0.034181-0.

Chang'a, J.S., Robertson, L.J., Mtambo, M.M.A., Mdegela, R.H., Løken, T., Reksen, O., 2011. Unexpected results from large-scale cryptosporidiosis screening study in calves in Tanzania. Ann. Trop. Med. Parasitol. 105, 513–519. https://doi.org/10.1179/2047773211Y.0000000007.

Checkley, W., Jr, A.C.W., Jaganath, D., Arrowood, M.J., Chalmers, R.M., Chen, X., Fayer, R., Griffi, K., Guerrant, R.L., Hedstrom, L., Huston, C.D., Kotloff, K.L., Kang, G., Mead, J.R., Miller, M., Jr, W.A.P., Priest, J.W., Roos, D.S., Striepen, B., Thompson, R.C.A., Ward, H.D., Voorhis, W.A. Van, Xiao, L., Zhu, G., Houpt, E.R., 2015. A review of the global burden , novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. Lancet Infect. Dis. 15, 85–94. https://doi.org/10.1016/S1473-3099(14)70772-8.

Christensen, K., 2020. SapphireAmp PCR Master Mix – CHEM 584 V.1. protocols.Io. https://doi.org/10.17504/protocols.io.bmc7k2zn.

Clopper, C.J., Pearson, E.S., 1934. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 26, 404–413.

CONAF. Corporación Nacional Forestal, 2024. Parque Nacional Bosque Fray Jorge [WWW Document]. URL. https://www.conaf.cl/parques/parque-nacional-bosque-fray-jorge/, 3.18.24.

Díaz-Lee, A., Mercado, R., Onuoha, E.O., Ozaki, L.S., Muñoz, P., Muñoz, V., Martínez, F. J., Fredes, F., 2011. *Cryptosporidium parvum* in diarrheic calves detected by microscopy and identified by immunochromatographic and molecular methods. Vet. Parasitol. 176, 139–144. https://doi.org/10.1016/j.vetpar.2010.11.001.

Dougnac Opitz, C.A., 2015. Búsqueda de evidencia de transmisión interespecie de *Cryptosporidium* y *Salmonella* entre Pingüinos de Magallanes (Spheniscus magellanicus) y seres humanos. Doctoral Thesis. Universidad de Chile.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. https://doi.org/10.1093/nar/gkh340.

Ferraz Fehlberg, H., Ribeiro, C.M., de Alcântara Brito Junior, P., Oliveira, B.C.M., dos Santos, C.A., del Valle Alvarez, M.R., Harvey, T.V., Albuquerque, G.R., 2021. Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in small wild mammals in northeastern Brazil. PLoS One 16, 1–11. https://doi.org/10.1371/journal.pone.0256199.

García-Livia, K., Fernández-Álvarez, Á., Feliu, C., Miquel, J., Quilichini, Y., Foronda, P., 2022. *Cryptosporidium* spp. in wild murids (rodentia) from corsica, France. Parasitol. Res. 121, 345–354. https://doi.org/10.1007/s00436-021-07369-4.

García-Livia, K., Martín-Alonso, A., Foronda, P., 2020. Diversity of *Cryptosporidium* spp. in wild rodents from the canary islands, Spain. Parasites Vectors 13, 1–9. https://doi.org/10.1186/s13071-020-04330-9.

Gómez-Couso, H., Ortega-Mora, L.M., Aguado-Martínez, A., Rosadio-Alcántara, R., Maturrano-Hernández, L., Luna-Espinoza, L., Zanabria-Huisa, V., Pedraza-Díaz, S., 2012. Presence and molecular characterisation of *Giardia* and *Cryptosporidium* in alpacas (*Vicugna pacos*) from Peru. Vet. Parasitol. 187, 414–420. https://doi.org/10.1016/j.vetpar.2012.01.025.

Hancke, D., Suárez, O.V., 2020. Co-occurrence of and risk factors for *Cryptosporidium* and *Giardia* in brown rats from Buenos Aires, Argentina. Zoonoses Public Health 67, 903–912. https://doi.org/10.1111/zph.12777.

Henriksen, S.A., Pohlenz, J.F.L., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet. Scand. 22, 594.

Hor, M., Č, Š., Holubová, N., Sak, B., Kv, D., Hlásková, L., Kone, R., Clark, M., Giddings, C., Mcevoy, J., Kvá, M., 2018. Diversity of *Cryptosporidium* in common voles and description of *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n. (Apicomplexa: Cryptosporidiidae). Parasitology 146, 220–233.

Ikiroma, I.A., Pollock, K.G., 2021. Influence of weather and climate on cryptosporidiosis — a review. Zoonoses Public Health 68, 285–298. https://doi.org/10.1111/zph.12785.

Infante, J., Riquelme, M., Huerta, N., Oettinger, S., Fredes, F., Simonetti, A., Rubio, V., 2022. *Cryptosporidium* spp. and *Giardia* spp. in wild rodents: using occupancy models to estimate drivers of occurrence and prevalence in native forest and exotic *Pinus radiata* plantations from Central Chile. Acta Trop. 235, 106635 https://doi.org/10.1016/j.actatropica.2022.106635.

Jagai, J.S., Castronovo, D.A., Monchak, J., Naumova, E.N., 2009. Seasonality of cryptosporidiosis: a meta-analysis approach. Environ. Res. 109, 465–478. https://doi.org/10.1016/j.envres.2009.02.008.

Kueh, S., Austen, J., Lawson, M., Callahan, L., Jardine, J., Ryan, U., 2017. *Cryptosporidium homai* n. sp. (Apicomplexa: Cryptosporidiidae) from the Guinea pig (*Cavia porcellus*). Vet. Parasitol. 245, 92–101. https://doi.org/10.1016/j.vetpar.2017.08.014.

Kváč, M., Havrdová, N., Hlásková, L., Daňková, T., Kanděra, J., Ježková, J., Vítovec, J., Sak, B., Ortega, Y., Xiao, L., Modrý, D., Jesudoss Chelladurai, J.R.J., Prantlová, V., McEvoy, John, 2016. *Cryptosporidium proliferans* n . sp . (Apicomplexa: Cryptosporidiidae): molecular and biological evidence of cryptic species within Gastric *Cryptosporidium* of mammals. PLoS One 11, e0147090. https://doi.org/10.1371/journal.pone.0147090.

Lalonde, L.F., Gajadhar, A.A., 2009. Effect of storage media, temperature, and time on preservation of *Cryptosporidium parvum* oocysts for PCR analysis. Vet. Parasitol. 160, 185–189. https://doi.org/10.1016/j.vetpar.2008.11.022.

Lima, M., Julliard, R., Stenseth, N.C.H.R., Jaksic, F.M., 2001. Demographic dynamics of a neotropical small rodent (*Phyllotis darwini*): feedback structure , predation and climatic factors. J. Anim. Ecol. 70, 761–775.

Mercado, R., Peña, S., Ozaki, L.S., Fredes, F., Godoy, J., 2015. Multiple *Cryptosporidium parvum* subtypes detected in a unique isolate of a Chilean neonatal calf with diarrhea. Parasitol. Res. 114, 1985–1988. https://doi.org/10.1007/s00436-015-4364-8.

Meserve, P.L., Kelt, D.A., Gutiérrez, J.R., Previtali, M.A., Milstead, W.B., 2016. Biotic interactions and community dynamics in the semiarid thorn scrub of Bosque Fray Jorge National Park, north-central Chile: a paradigm revisited. J. Arid Environ. 126, 81–88. https://doi.org/10.1016/j.jaridenv.2015.08.016.

Meserve, P.L., Kelt, D.A., Previtali, M.A., Milstead, W.B., Gutirrez, J.R., 2011. Global climate change and small mammal populations in north-central Chile. J. Mammal. 92 (1), 1223–1235. https://doi.org/10.1644/10-MAMM-S-267.

Mills, J.N., Childs, J.E., 1998. Ecologic studies of rodent reservoirs: their relevance for human health. Emerg. Infect. Dis. 4, 529–537. https://doi.org/10.3201/eid0404.980403.

Mosier, D.A., Oberst, R.D., 2000. Cryptosporidiosis: a global challenge. Ann. N. Y. Acad. Sci. 916, 102–111.

Muñoz-Pedreros, A., Gill, C., 2009. Order Rodentia. In: Muñoz Pedreros, A., Yáñez Valenzuela, J. (Eds.), Mamiferos de Chile. CEA Ediciones, Santiago, Chile, pp. 93–157.

Muñoz, P., Diaz-Lee, A., Fredes, F., Mercado, R., Ozaki, L.S., 2011. Detección de *Cryptosporidium* spp. en terneras de lecherías de la Región Metropolitana mediante Ziehl Neelsen y confirmada por inmunocromatografía y ensayo molecular. Arch. Med. Vet. 43, 111–116.

Nader, J.L., Mathers, T.C., Ward, B.J., Pachebat, J.A., Swain, M.T., Robinson, G., Chalmers, R.M., Hunter, P.R., Oosterhout, C. Van, Tyler, K.M., 2019. Evolutionary genomics of anthroponosis in *Cryptosporidium*. Nat. Microbiol. 4, 826–836. https://doi.org/10.1038/s41564-019-0377-x.

Neira-Otero, P., Muñoz-Saldías, N., Sanchez-Moreno, M., Rosales-Lombardo, M.J., 2005. Molecular characterization of *Cryptosporidium* species and genotypes in Chile. Parasitol. Res. 97, 63–67. https://doi.org/10.1007/s00436-005-1391-x.

Novoa, J.E., López, D., 2001. IV Región: el escenario geográfico físico. In: Squeo, F.A., Arancio, G., Gutiérrez, J.R. (Eds.), Libro Rojo de La Flora Nativa y de Los Sitios Prioritarios Para Su Conservación: Región de Coquimbo. Ediciones Universidad de La Serena, La Serena, Chile, pp. 13–26.

Okonechnikov, K., Golosova, O., Fursov, M., the UGENE team, 2012. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28, 1166–1167. https://doi.org/10.1093/bioinformatics/bts091.

Painean, J., Raffo, E., Mercado, R., Peña, S., Muñoz, P., 2022. Detección y caracterización molecular de *Cryptosporidium* spp. en terneros de lechería de la provincia de Valdivia, Chile. Rev. MVZ Córdoba 27, e2197. https://doi.org/10.21897/RMVZ.2197.

Pavez, E., Lobos, G.A., Jaksic, F.M., 2010. Cambios de largo plazo en el paisaje y los ensambles de micromamíferos y rapaces en Chile central. Rev. Chil. Hist. Nat. 83, 99–111.

Peig, J., Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118, 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x.

Pinto, P., Ribeiro, C.A., Kváč, M., Anastasios, D.T., 2022. Cryptosporidium. In: de Souza, W. (Ed.), Lifecycles of Pathogenic Protists in Humans. Springer, pp. 338–339.

QGIS, 2024. Qgis. A free and open Source Geographic information system [WWW Document]. QGIS Doc. URL. https://qgis.org/en/site/about/index.html.

R Development CoreTeam, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

Romairone, J., Jiménez, J., Luque-Larena, J.J., Mougeot, F., 2018. Spatial capture-recapture design and modelling for the study of small mammals. PLoS One 13, e0198766.

Royle, J.A., Fuller, A.K., Sutherland, C., 2018. Unifying population and landscape ecology with spatial capture–recapture. Ecography 41, 444–456. https://doi.org/10.1111/ecog.03170.

RStudio Team, 2022. RStudio: Integrated Development for R. RStudio, PBC [WWW Document]. URL. http://www.rstudio.com/, 11.28.22.

Santodomingo, A.M., Thomas, R.S., Quintero-Galvis, J.F., Echeverry-Berrio, D., Silva-de la Fuente, C., Salas-Moreno, L., Muñoz-Leal, S., 2022. Apicomplexans in small mammals from Chile, with the first report of the *Babesia microti* group in South American rodents. Parasitol. Res. 121, 1009–1020. https://doi.org/10.1007/s00436-022-07452-4.

Silva, S.O.S., Richtzenhain, L.J., Barros, I.N., Gomes, A.M.M.C., Silva, A.V., Kozerski, N. D., Araújo, J.B. De, Keid, L.B., Soares, R.M., 2013. A new set of primers directed to 18S rRNA gene for molecular identification of *Cryptosporidium* spp. and their performance in the detection and differentiation of oocysts shed by synanthropic rodents. Exp. Parasitol. 135, 551–557. https://doi.org/10.1016/j.exppara.2013.09.003.

Sociedad Agrícola y Ganadera El Tangue Ltda, 2022. Hacienda El Tangue 1927 [WWW Document]. Historia Santiago. URL. https://www.eltangue.cl/, 6.28.22.

Squeo, F.A., Loayza, A.P., López, R.P., Gutiérrez, J.R., 2016. Vegetation of Bosque Fray Jorge National park and its surrounding matrix in the Coastal Desert of north-central Chile. J. Arid Environ. 126, 12–22. https://doi.org/10.1016/j.jaridenv.2015.10.013.

Stensvold, C.R., Larsen, T.G., Grüttner, J., Nielsen, L., Engberg, J., Lebbad, M., 2024. Rodent-adapted *Cryptosporidium* infection in humans: seven new cases and review of the literature. One Heal 100682. https://doi.org/10.1016/j.onehlt.2024.100682.

Suarez, P., Vallejos-Almirall, A., Fernández, I., Gonzalez-Chavarria, I., Alonso, J.L., Vidal, G., 2024. Identification of *Cryptosporidium parvum* and *Blastocystis hominis* subtype ST3 in Cholga mussel and treated sewage: Preliminary evidence of fecal contamination in harvesting area. Food Waterborne Parasitol 34, e00214. https://doi.org/10.1016/j.fawpar.2023.e00214.

Suzán, G., Esponda, F., Carrasco-Hernández, R., Aguirre, A., 2012. Habitat fragmentation and infectious Disease ecology. In: Aguirre, A., Ostfeld, R.S., Daszak, P. (Eds.), New Directions in Conservation Medicine. Applied Cases of Ecological Health. Oxford University Press, Inc., New York, pp. 135–150.

Taghipour, A., Olfatifar, M., Foroutan, M., Bahadory, S., Malih, N., Norouzi, M., 2020. Global prevalence of *Cryptosporidium* infection in rodents: a systematic review and meta-analysis. Prev. Vet. Med. 182, 105119 https://doi.org/10.1016/j.prevetmed.2020.105119.

Takara Bio Inc., 2021. SapphireAmp® Fast PCR Master Mix [WWW Document]. URL. https://www.takarabio.com/documents/UserManual/RR350A/RR350A_DS.pdf.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023.

Tamura, K., Stecher, G., Kumar, S., 2021. Mega11: molecular evolutionary Genetics analysis Version 11. Mol. Biol. Evol. 38, 3022–3027. https://doi.org/10.1093/molbev/msab120,3022-3027.

Taylor, M.A., Coop, R.L., Wall, R.L., 2016. Veterinary Parasitology, fourth ed. John Wiley & Sons, West Sussex, UK.

Tůmová, L., Ježková, J., Prediger, J., Holubová, N., Sak, B., Konečný, R., Květoňová, D., Hlásková, L., Rost, M., Mcevoy, J., Xiao, L., Santín, M., Kváč, M., 2023. *Cryptosporidium mortiferum* n. sp.(Apicomplexa: Cryptosporidiidae), the species causing lethal cryptosporidiosis in Eurasian red squirrels (*Sciurus vulgaris*). Parasites Vectors 16, 235. https://doi.org/10.1186/s13071-023-05844-8.

Waldron, L.S., Dimeski, B., Beggs, P.J., Ferrari, B.C., Power, M.L., 2011. Molecular epidemiology , spatiotemporal analysis , and ecology of sporadic human cryptosporidiosis in Australia. Appl. Environ. Microbiol. 77, 7757–7765. https://doi.org/10.1128/AEM.00615-11.

Werner, C.S., Nunn, C.L., 2020. Effect of urban habitat use on parasitism in mammals: a meta-analysis. Proc. R. Soc. B Biol. Sci. 287, 20200397 https://doi.org/10.1098/rspb.2020.0397rspb20200397.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small- subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578–1583. https://doi.org/10.1128/aem.65.4.1578-1583.1999.

Xiao, L., Fayer, R., Ryan, U.M., Upton, S.J., 2004. Cryptosporidium taxonomy: recent advances and implications for public health. Clin. Microbiol. Rev. 17, 72–97. https://doi.org/10.1128/CMR.17.1.72.

Yanta, C.A., Bessonov, K., Robinson, G., Troell, K., Guy, R.A., 2021. CryptoGenotyper: a new bioinformatics tool for rapid *Cryptosporidium* identification. Food Waterborne Parasitol 23, e00115. https://doi.org/10.1016/j.fawpar.2021.e00115.

Zhang, K., Fu, Y., Li, J., Zhang, L., 2022. Public health and ecological significance of rodents in *Cryptosporidium* infections. One Heal 14, 100364. https://doi.org/10.1016/j.onehlt.2021.100364.