





Tetrathyridia in an endemic lizard from Chile: molecular evidence for South America

J. P. Correa¹ , F. Farías², E. San Juan², A. Yáñez-Meza², S. Muñoz-Leal³ ,
C. Botto-Mahan²  and P. Oyarzún-Ruiz⁴ 

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Corresponding author:

P. Oyarzún-Ruiz;
Email: pablooyarzunruiz@gmail.com

¹Facultad de Ciencias de la Naturaleza, Universidad San Sebastián, Concepción, Chile; ²Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile; ³Departamento de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad de Concepción, Chillán, Chile and ⁴Departamento de Patología y Medicina Preventiva, Facultad de Ciencias Veterinarias, Universidad de Concepción, Chillán, Chile

Abstract

Mesococestoides is a controversial tapeworm with significant lack of data related to systematics and life cycles. This helminth has an indirect life cycle with vertebrates, mostly carnivorous mammals, as definitive hosts. Theoretically, a coprophagous arthropod would be the first intermediate host, and herptiles, mammals, and birds, which prey on these insects, would represent the second intermediate hosts. However, recent evidence suggests that this life cycle would require only two hosts, with no arthropods involved. In the Neotropics, although there are records of mammals and reptiles as hosts for *Mesococestoides*, no molecular analyses have been performed. This work aimed to record an additional intermediate host and molecularly characterize the isolated larvae. Thus, 18 braided tree iguanas (*Liolaemus platei*) from Northern Chile were collected and dissected during 2019. One lizard was parasitized by three morphotypes of larvae compatible with tetrathyridia of *Mesococestoides*. To achieve its specific identity, a molecular approach was performed: 18S rRNA and 12S rRNA loci were amplified through cPCR. The inferred phylogenies confirmed the morphological diagnosis and stated that all morphotypes were conspecifics. The sequences for both loci formed a monophyletic clade with high nodal support, representing a sister taxon to *Mesococestoides* clade C. This study represents the first molecular characterization of any taxon of *Mesococestoides* from the Neotropics. Future surveys from potential definitive hosts would help to elucidate its life cycle. Furthermore, an integrative taxonomic approach is required in additional studies from the Neotropical region, which would contribute to a better understanding of the evolutionary relationships of this genus.

Introduction

Genus *Mesococestoides* is controversial mainly because its systematics and life cycles are not completely understood (Jesudoss Chelladurai & Brewer 2021). It is presumed to have a three-host life cycle, with carnivorous vertebrates, mostly mammals, as definitive hosts; meanwhile, coprophagous insects would act as first intermediate hosts, and small vertebrates, preying on these arthropods, as second intermediate hosts (Padgett *et al.* 2005). Thus, the life cycle of *Mesococestoides* would be complete once the definitive host preys upon the secondary intermediate host (Padgett & Boyce 2004). However, recently McAllister *et al.* (2018) suggested that there is a two-host life cycle based on the finding of pre-tetrathyridial larvae in the coelomic cavity of a ground skink (*Scincella lateralis*). Albeit there are myriad species recorded as intermediate hosts of *Mesococestoides* spp., its prevalence is frequently low (~7%) (Burse *et al.* 2012; Jesudoss Chelladurai & Brewer 2021). Moreover, this tapeworm has been recorded in domestic dogs and cats as definitive hosts, and as accidental hosts harboring tetrathyridia in the peritoneal cavity, causing the so-called peritoneal larval cestodiasis, which could lead to a fatal outcome (Jesudoss Chelladurai & Brewer 2021).

From the morphological point of view, the classification of *Mesococestoides* is controversial because measurements overlap, and morphological traits are similar in adult tapeworms of different species (Padgett *et al.* 2005; Skirnisson *et al.* 2016a). Currently, only five of 28 described taxa have compelling morphological and molecular evidence and are considered as valid species: *Mesococestoides litteratus*, *Mesococestoides lineatus*, *Mesococestoides melesi*, *Mesococestoides vogae* (syn. *Mesococestoides corti*), and *Mesococestoides canislagopodis* (McAllister *et al.* 2018; Berrilli & Simbula 2020; Jesudoss Chelladurai & Brewer 2021). All the above-mentioned species have been recorded exclusively in the Holarctic realm (Berrilli & Simbula 2020). Meanwhile, in the Neotropical realm there are records of adult tapeworms parasitizing wild and domestic canids such as *Mesococestoides variabilis* from Peru (Angulo-Tisoc *et al.* 2020), *Mesococestoides michaelsoni* from Brazil (Justo *et al.* 2017), and “*M. lineatus*” from Argentina (Fugassa, 2020) and Chile (Oyarzún-Ruiz *et al.* 2020). Also, larvae and adult worms of *Mesococestoides* sp. have been recorded from Argentina (González *et al.* 2013; Hamann *et al.* 2014; Hamann & González 2015), Chile (Oyarzún-Ruiz *et al.*

2020), and Peru (Angulo-Tisoc *et al.* 2020). In addition, tetrathyridia have been found parasitizing lizards from Argentina (García *et al.* 2015), Brazil (Justo *et al.* 2017), Costa Rica, and Nicaragua (Burseley *et al.* 2012). However, none of these studies supported the morphological diagnoses with genetic evidence. Therefore, there is an evident need to elucidate the life cycles of these tapeworms by applying an integrative taxonomic approach, i.e., combining morphological and molecular analyses to delimit species (Kubečka *et al.* 2018; McAllister *et al.* 2018; Jesudoss Chelladurai & Brewer 2021).

In Chile there are 131 species of reptiles, including genus *Liolaemus* (Liolaemidae) with 72 species, of which 57 are catalogued as endemic (Mella 2017). The helminth parasites for this genus of lizard have been recorded in seven species. However, the identification to the species level of these parasites is not given (San-Martín-Órdenes *et al.* 2019). The present work aimed to include new data to the helminth fauna of native reptiles and to discuss, through a phylogenetic framework, the systematics of this intricate genus of tapeworms in the Neotropics.

Material and Methods

During March 2019, a total of 18 braided tree iguanas (*Liolaemus platei*), 8 females and 10 males, were collected in 2 sites from the Coquimbo region, northern Chile; Farellón Sánchez (31°26.8'S 71° 1.1'O) and Cañas de Michío (31°38.2'S 71°3.8'O) (Supplementary Figure S1). The capture permit was provided by the state-owned organism Servicio Agrícola y Ganadero, Chile (334/2019). Lizards were captured with a sliding noose (Mella 2017), kept inside individual cloth bags, and transported to the Laboratory of Ecological Interactions, Facultad de Ciencias, Universidad de Chile. Lizards were euthanized with an intracoelomic inoculation of 0.5% sodium thiopental (100 mg/kg). Parenchymatous organs and the coelomic cavity were dissected under the stereomicroscope for the presence of larval stages of tapeworms. Tapeworm larvae were washed in distilled water and fixed and preserved in ethanol 96%. Larvae were counted under a stereomicroscope, and different morphotypes were separated for morphological and molecular analyses. Larvae were stained with Carmine Alum, dehydrated, diaphanized, and mounted in Canada balsam following Lutz *et al.* (2017). Tetrathyridia were deposited in the collection of Museo de Zoología, Universidad de Concepción, under access number: MZUC-UCCC 47352.

Total genomic DNA was extracted from single worms for every morphotype by employing DNeasy blood and animal tissue kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. DNA quantity and quality for each sample was tested with an EpochTM Microplate Spectrophotometer (Santodomingo *et al.* 2022). The extracted DNA was preserved at -24°C until analysis.

Conventional PCR (cPCR) was performed to amplify partial sequences of 18S rRNA and 12S rRNA loci, following Oyarzún-Ruiz *et al.* (2022). Primers and cPCR thermal conditions are provided in Supplementary Table S1. Amplicons of expected size were purified and sequenced in both directions at Macrogen (Seoul, South Korea). Sequences were verified for quality and edited with Geneious Prime[®] v.2021.2.2. (<https://www.geneious.com>). Basic local alignment searches were performed using the BLASTn tool (<https://blast.ncbi.nlm.nih.gov>), and similar sequences were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov>). Alignments were constructed with MAFFT algorithm in Geneious Prime to then extract the informative regions, as suggested by Santodomingo *et al.* (2022). The phylogenetic analyses for both loci were performed using maximum likelihood method (ML) and

Bayesian inference (BI) with IQ-TREE v1.6.12 and MrBayes v3.2.6, respectively (see Oyarzún-Ruiz *et al.* 2022). Consensus tree for ML and BI was generated for every locus following Santodomingo *et al.* (2022).

Genetic pairwise distances between the sequences of the present study and sequences of valid *Mesocestoides* taxa (Padgett *et al.* 2005; Berrilli & Simbul 2020; Jesudoss Chelladurai & Brewé 2021) were computed using MEGA7 (Kumar *et al.* 2016). The sequences obtained in the present study were deposited in the NCBI GenBank database under the following access numbers: OQ701082-OQ701086 for 12S rDNA locus and OQ701087-OQ701089 for 18S rDNA locus.

Results and Discussion

Only one male lizard (prevalence = 5.5%) from the Cañas de Michío site was found parasitized with a total of 138 tapeworm larvae, morphologically compatible with tetrathyridia of *Mesocestoides* (Skirnisson *et al.* 2016b; Kubečka *et al.* 2018; McAllister *et al.* 2018). All tetrathyridia were free throughout the coelomic cavity; none was found encysted in the parenchyma or adhered to the serosa of any organ. Three different morphotypes were detected (I, II, and III), all with a well-developed excretory pore and deep invagination canal. Morphotype I had an anterior end wider and more prominent in comparison to its hindbody, with few calcareous corpuscles, mostly on the anterior end. Morphotype II was slender with a smaller anterior end in comparison to its hindbody. Calcareous corpuscles were numerous and concentrated mostly in the anterior end and anterior half of the hindbody. Morphotype III was wider and spherical, with few or no calcareous corpuscles observed (Supplementary Figure 2). Measurements are detailed in Table 1.

Our 18S sequences showed a 98.96% identity (1145/1157, 100% query cover, 0 gaps, 0 E-value) with *Mesocestoides melesi* (MN512707) isolated in a bank vole (*Myodes glareolus*) from Poland, while for 12S locus, our taxon showed 93.22% identity (330/354, 100% query cover, 5 gaps, 1E-141) with *Mesocestoides* sp. (MG214761) isolated in a Northern bobwhite (*Colinus virginianus*) from the USA. According to the topology of 18S tree, our sequences were included into the *Mesocestoides* clade with high nodal support (98% for ML and 0.99 for BI), supporting the morphological diagnosis (Supplementary Figure S3). Conversely, despite the morphological differences abovementioned, the phylogeny of both loci suggests that all three morphotypes are conspecifics (Figures 2, S2), which was also confirmed through the genetic pairwise distances of 12S with 0% of intra-specific differences (Supplementary Table S2). Our sequences formed a monophyletic clade for 12S, with robust nodal support (98% for ML and 1 for BI); meanwhile, for 18S there was mild support for ML (81%) and robust for BI (0.72). The genetic pairwise comparison for 12S showed that our sequences differed by 2.4 to 15.9% of nucleotide positions from other *Mesocestoides* taxa (Supplementary Table S2).

According to the topology of 12S locus (Figure 1), our sequences were closely related to taxa of *Mesocestoides* clade C (Padgett *et al.* 2005) (100% for ML, 1 for BI), which includes taxa mostly from the USA, followed by Kazakhstan and Russia. Although there are no available sequences of clade C for 18S locus (see Padgett *et al.* 2005), our sequences showed as a sister taxon (97% for ML, 0.88 for BI) to a clade composed by unidentified *Mesocestoides* taxa parasitizing domestic dogs, rodents, and shrews from the USA and Russia.

Table 1. Measurements of the three morphotypes of tetrathyridia isolated in the braided tree iguana (*Liolaemus platei*). Mean and standard deviation, with range between parentheses.

	Morphotype I (n = 9)	Morphotype II (n = 3)	Morphotype III (n = 8)	All** (n = 20)
Length	2.16 ± 0.23 (1.80–2.55)	2.48 ± 0.39 (2.20–2.93)	1.64 ± 0.25 (1.15–1.99)	2.00 ± 0.41 (1.15–2.93)
Width anterior end	1.13 ± 0.12 (0.85–1.24)	0.92 ± 0.09 (0.82–0.98)	1.26 ± 0.09 (1.15–1.40)*	1.15 ± 0.16 (0.82–1.4)
Width hindbody	0.97 ± 0.16 (0.71–1.25)	1.04 ± 0.08 (0.95–1.09)	–	1.05 ± 0.18 (0.71–1.4)

*Diameter.

**Measurements of the three morphotypes.

The discrepancy between the BLASTn results and the phylogenetic inferences for 18S rRNA could be explained because BLASTn only conducts pairwise sequence comparisons based on local alignment, whereas a phylogenetic approach tries to establish evolutionary relationships using optimal global alignment of all sequences represented, specialized algorithms, and robust statistical methods. Therefore, while BLASTn can be a useful exploratory tool, it should not be considered a substitute for phylogenetic analyses when the goal is to understand the evolutionary relationships among different taxa (Mata *et al.* 2017; Hall 2018). On the other hand, the high value of similarity for 18S in the BLASTn result is because this locus is highly conserved in comparison to 12S, which is the reason the former is only informative at the highest level (Crosbie *et al.* 2000; Kubečka *et al.* 2018).

The lack of reliable morphological traits for the specific identification of *Mesocestoides* tetrathyridia has been previously suggested (Skirnisson *et al.* 2016a, b; McAllister *et al.* 2018). As a consequence, the morphological differences of tetrathyridia here reported could be related to different stages of development (Skirnisson *et al.* 2016b), although no larva was determined as a pre-larval stage (see McAllister *et al.* 2018). García *et al.* (2015) also recorded different morphotypes from a parasitized leopard iguana (*Diplolaemus leopardinus*) from Argentina; however, the diagnosis was not complemented with a molecular analysis to determine if these were conspecific. Furthermore, the authors described an acephalic morph, which probably corresponded to tetrathyridia with an invaginated scolex (e.g., Skirnisson *et al.* 2016b; Kubečka *et al.* 2018; present study), because acephalic morph has been found only in domestic dogs (Padgett & Boyce 2004; Padgett *et al.* 2005).

The prevalence here recorded agrees with the global prevalence of tetrathyridia in lizards, which is near 4% (Jesudoss Chelladurai & Brewer 2021). The parasitic load recorded is concordant with previous reports, from a few worms to several hundred (Padgett & Boyce 2004; Skirnisson *et al.* 2016b). A high number of tetrathyridia could occur during asexual proliferation, as seen in experimental trials with reptiles and micromammals (Padgett & Boyce 2004). However, none of the three morphotypes isolated here showed any sign of this, i.e., duplicated scolices or buds (Skirnisson *et al.* 2016b; Kubečka *et al.* 2018), agreeing with previous records in wild lizards from Europe and North America (McAllister *et al.* 2018; Berrilli & Simbula 2020). Although asexual proliferation has been recorded for certain populations of *Mesocestoides*, it seems to be rare in nature (Padgett & Boyce 2004;

Skirnisson *et al.* 2016b; Kubečka *et al.* 2018; McAllister *et al.* 2018; Berrilli & Simbula 2020).

All tetrathyridia were found free in the coelomic cavity, mostly distributed over the digestive tract and liver. No tetrathyridium was found encysted or attached to any organ or to the coelomic wall, corroborating that tissular invasion is not a common strategy in *Mesocestoides* spp. (Padgett & Boyce 2004; present study). Nonetheless, questions arise as to whether this attribute is specific for this taxon or related to the immune response of the host.

Of relevance to the source of infection, the braided tree iguana is catalogued as an insectivorous reptile (Mella 2017). Thus, if the life cycle requires an arthropod as the first intermediate host (i.e., a three-host life cycle) (Padgett & Boyce 2004), this would explain the infection in lizards. On the other hand, if there is no need for an arthropod (i.e., a two-host life cycle), the lizard could get infected through the ingestion of proglottids, wherein the eggs are protected and concentrated in high numbers inside the paruterine organ (McAllister *et al.* 2018).

The definitive hosts recorded for clade C are canids, including the domestic dog, Island foxes (*Urocyon littoralis*), and wolves (*Canis lupus*) (Padgett *et al.* 2005). Considering that our sequences were closely related to this clade, a similar definitive host would be expected. However, this suggestion must be taken with caution because wild felids have also been recorded with adult tapeworms in South America (Fugassa 2020).

Parasitological surveys on braided tree iguanas count only ectoparasites (San-Martín-Órdenes *et al.* 2019). In consequence, this study represents the first report related to its helminth fauna. Moreover, although there are records of tetrathyridia in other Neotropical reptiles (Burseley *et al.* 2012; García *et al.* 2015; Justo *et al.* 2017), this is the first mention of *Liolaemus* species acting as the intermediate host of *Mesocestoides*, expanding the spectrum of intermediate hosts in this region. Furthermore, this is the first attempt to molecularly characterize a tapeworm of genus *Mesocestoides* from the Neotropics and the third study reporting sequences from a lizard (McAllister *et al.* 2018; Berrilli & Simbula 2020).

Virtually, all vertebrate preys have the potential to harbour tetrathyridia (Jesudoss Chelladurai & Brewer 2021). This was supported by the topology for both loci, with the absence of a pattern related to the use of intermediate hosts (Padgett & Boyce 2004; Burseley *et al.* 2012; Skirnisson *et al.* 2016a, b; Kubečka *et al.* 2018). Some families of lizards that host tetrathyridia elsewhere are also present in Chile, e.g., Gekkonidae, Teiidae, and Scincidae (McAllister *et al.* 2018; Burseley *et al.* 2012; Mella 2017). Thus, studies

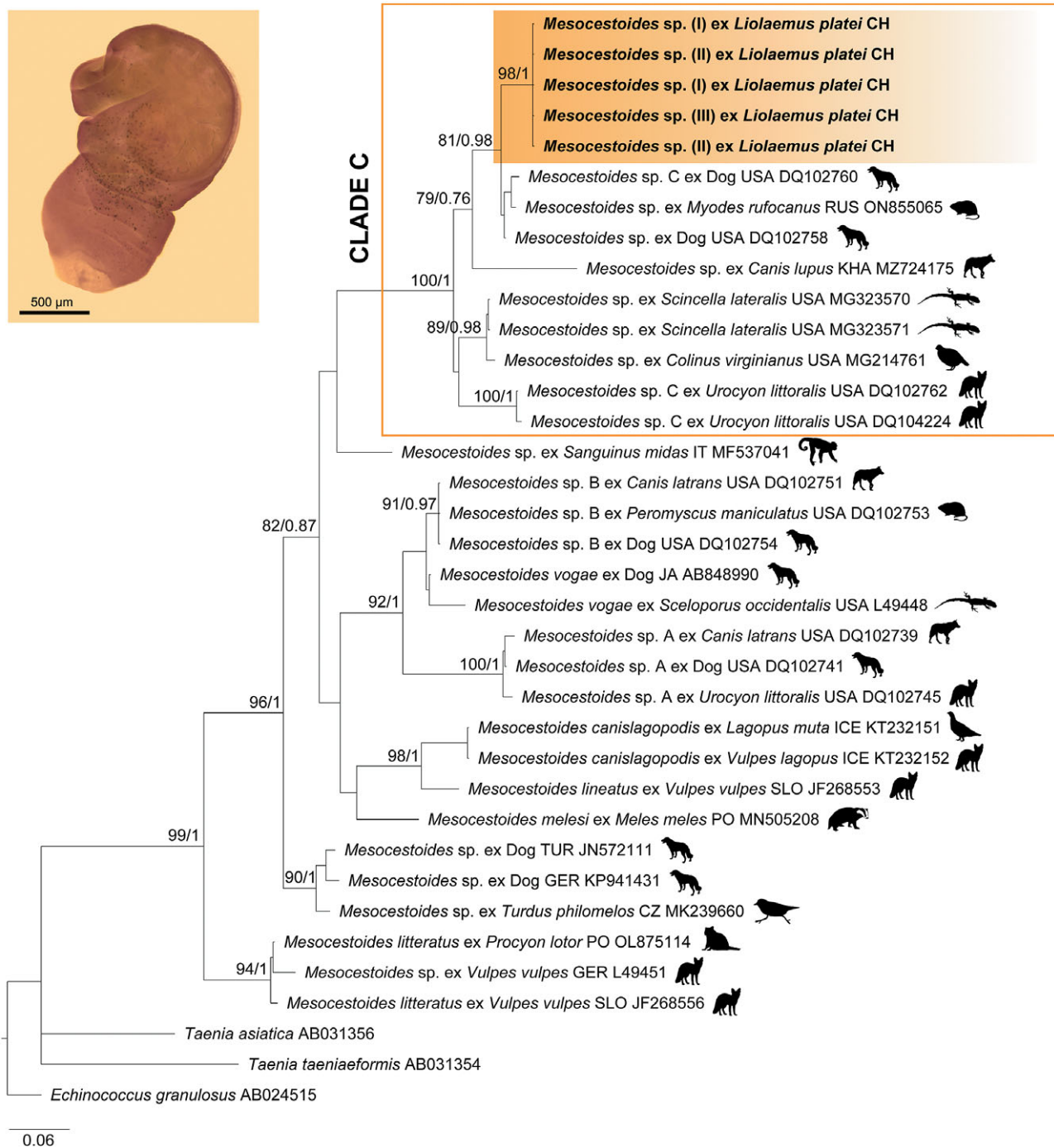


Figure 1. Consensus tree of maximum likelihood (ML) and Bayesian inference (BI) phylogenies inferred for 12S rRNA gene, using an alignment of 315 bp. Calculated substitution models for ML and BI were TPM3u+F+G4, and M_{85} , M_{15} , M_{90} , M_{50} , and M_{177} , respectively. The best models were chosen using the Bayesian Information Criterion (BIC). Bootstrap values and Bayesian posterior probabilities are indicated above each branch. The sequences from the present study are highlighted within an orange box. Abbreviations: CZ, Czech Republic; GER, Germany; ICE, Iceland; IT, Italy; PO, Poland; JA, Japan; KA, Kazakhstan; RUS, Russia; SLO, Slovakia; TUR, Turkey.

focused on the helminthological fauna of these and other families should consider determining the presence of tetrathyridia.

In Chile and Peru there are previous mentions of “*M. lineatus*” in a domestic dog and Andean fox, respectively (see Fugassa 2020; Oyarzún-Ruiz et al. 2020). Considering that this species is restricted to Central Europe (Jesudoss Chelladurai & Brewer 2021), it is possible that it represents a different taxon. Another conflictive species in the Neotropics is *M. variabilis* which, besides its natural distribution in the USA, was recorded in an Andean fox from Peru

(Angulo-Tisoc et al. 2020). According to Padgett et al. (2005), this taxon would be synonymous with *M. vogae*; however, there is no molecular data from the Neotropics to support this statement.

There is the possibility that our specimens represent an undescribed taxon, considering the restricted distribution of the parasitized lizard and the putative wild definitive hosts (Mella 2017; Oyarzún-Ruiz et al. 2020). In addition, future studies in other potential intermediate hosts should prevent overlooking tetrathyridia during necropsy, considering the challenge imposed by its small size and

variable morphology, especially with low parasitic loads (e.g., García *et al.* 2015; Skirnisson *et al.* 2016b; McAllister *et al.* 2018).

This is the first contribution of molecular data for this genus of tapeworm in the Neotropics. Keeping in mind its intricate taxonomic history, additional sequences from this zoogeographical realm could answer questions related to the evolutionary relationships of the genus, particularly those from the Americas (Padgett & Boyce 2004; Padgett *et al.* 2005; McAllister *et al.* 2018).

Supplementary material. The supplementary material for this article can be found at <http://doi.org/10.1017/S0022149X23000329>.

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Competing interest. None.

Ethical standard. Procedures performed in this study were verified and approved by the Bioethics Committee of Universidad de Chile (Form CICUA N°18202-SCS-UCH). Capture of lizards was authorized by the Servicio Agrícola y Ganadero (SAG; Resolution No. 334/2019).

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