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First report of *Spirometra* (Eucestoda; Diphyllobothriidae) naturally occurring in a fish host

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Abstract: *Spirometra* Faust, Campbell et Kellogg, 1929 is a genus of cestodes belonging to the family Diphyllobothriidae. To date, amphibians, reptiles, and mammals are known second intermediate hosts of these parasites; humans can also be infected (the zoonotic disease is known as sparganosis or spirometrosis). Although the number of phylogenetic studies on *Spirometra* spp. has increased worldwide in recent years, there are few in South America. Specifically in Uruguay, molecular studies have shown that tapeworms of *S. decipiens* (Diesing, 1850) complexes 1 and 2 are present in this country. In this study, we characterised the larvae of *Spirometra* present in the annual fish *Austrolebias charrua* Costa et Cheffe. Phylogenetic analysis of the cytochrome c oxidase subunit I (COI) sequences of these larvae showed that they belong to *S. decipiens* complex 1. This is the first report of teleost fishes serving as a second intermediate host for tapeworms of the genus *Spirometra* in nature.

Keywords: Parasites, South America, molecular characterisation, *Austrolebias*, *Spirometra decipiens*, Uruguay

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Spirometra Faust, Campbell et Kellogg, 1929 is a genus of cestodes belonging to the family Diphyllobothriidae. These organisms have a complex allogenic life cycle that includes small crustaceans as first intermediate hosts, vertebrates (amphibians, ophidians, mammals) as second intermediate hosts, and carnivorous foxes or felids as definitive hosts (Waeschenbach et al. 2017, Scholz et al. 2019, Arrabal et al. 2020). Humans have been reported to become infected with this parasite as incidental hosts and contract the zoonotic disease known as sparganosis (Holtz and Gilman 2013, Liu et al. 2015, Waeschenbach et al. 2017, Scholz et al. 2019). This zoonotic disease is not very common in South America, possibly due to underdiagnosis; there are only a few cases in Brazil, Argentina, Venezuela, Paraguay, and Uruguay (Sakamoto et al. 2003, Oda et al. 2016, Kikuchi and Morayama 2019), while it is ubiquitous in the wild as reported by several authors (Scioscia et al. 2014, Petrigh et al. 2015, Almeida et al. 2016, Arrabal et al. 2020, Armúa-Fernández et al. 2021, Brabec et al. 2022, Fredes et al. 2022).

Recent work on the taxonomy of *Spirometra* spp. has yielded a plausible species-level classification with at least six molecularly distinct taxa (Kuchta et al. 2021). This classification was originally consistent with the geographical distribution of taxa identified by molecular characterisation, with *Spirometra decipiens* (Diesing, 1850) complex 1 and 2 being the species occurring in the Americas. However, recent reports have indicated that *Spirometra mansoni* (Cobbold, 1883) may also occur in South America (Brabec et al. 2022), suggesting that more extensive sampling may eventually reveal further complexity in the systematics and distribution of the taxon.

In Uruguay, larval forms of *Spirometra* sp. (also called sparganum, pl. spargana) have been found infecting the coelom, musculature, or subcutaneous tissues of mammals, reptiles, and amphibians, and have always been identified by morphology (Oda et al. 2016). More recently, Armúa-Fernández et al. (2021) identified *Spirometra decipiens* complex 1 in reptiles and mammals, and *S. decipiens* complex 2 in reptiles from Uruguay by molecular analyses.

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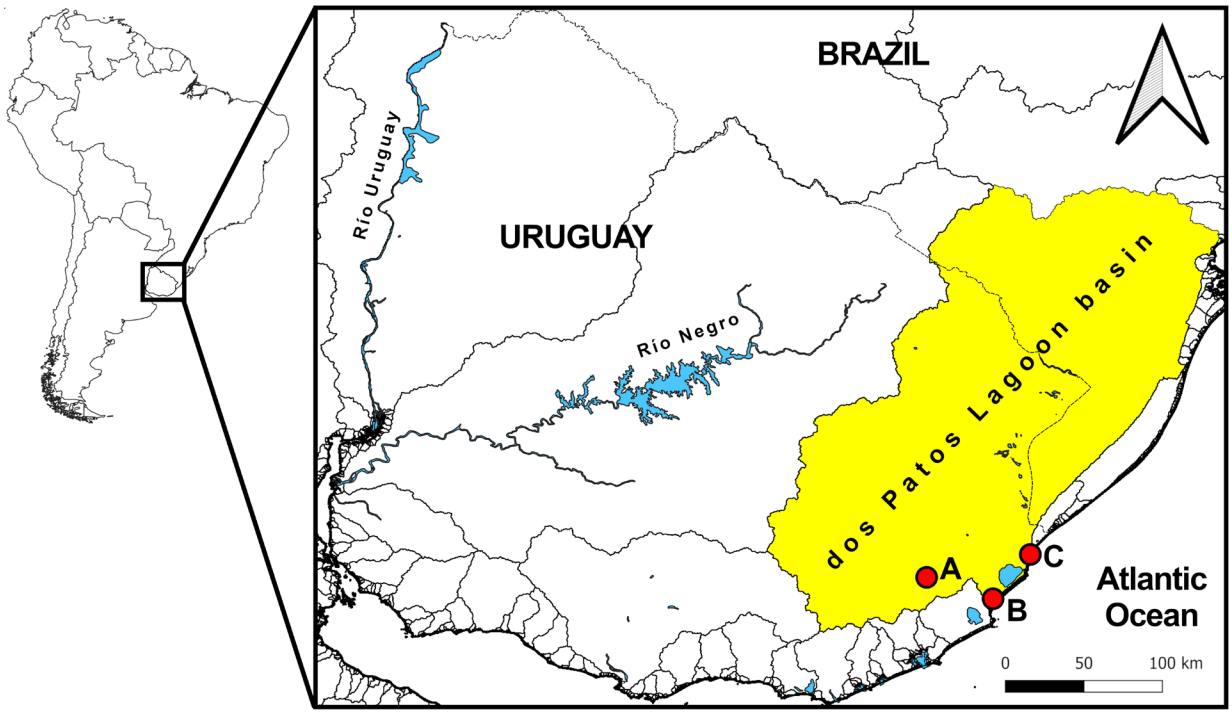

Fig. 1. Hydrological basin map of Uruguay. Painted yellow is Dos Patos Lagoon basin, located in eastern Uruguay and southern Brazil. Red dots show the three localities from where *Austrolebias charrua* Costa et Cheffe were collected in this study. A – India Muerta; B – Ruta 9 km 272; C – La Coronilla.

The anuran second intermediate hosts reported for *Spirometra* in Uruguay are known to inhabit wetlands and temporary ponds (Berois et al. 2015, Oda et al. 2016). These ecosystems are abundant in Uruguay, and an important component of their fauna is the large diversity of freshwater annual fishes (Cyprinodontiformes, Aplocheiloidei), particularly of the genus *Austrolebias* Costa, 1998. These organisms are an attractive model for various topics in biology due to their particular life cycle and the ability of their drought-resistant eggs to survive dry periods through diapauses in their embryonic development (Costa 2002, 2003, 2006, Loureiro et al. 2004, 2011, Arezo et al. 2007, Berois et al. 2015). However, a neglected aspect of the biology of this genus is its parasite fauna, with only seven published parasitological reports (Pereira and Vaz 1933, Taberner et al. 2003, Luque et al. 2011, Delgado and García 2015, Montes et al. 2017, Marcotegui et al. 2018, Vettorazzi et al. 2020).

To increase knowledge of the biology and distribution of the genus *Spirometra* and to supplement reports of parasites infecting *Austrolebias*, we conducted a molecular identification study to characterise spargana found in the annual fish *Austrolebias charrua* Costa et Cheffe.

MATERIALS AND METHODS

Twenty-six annual fish of the species *Austrolebias charrua* were collected in October 2019 from temporary ponds at three sites of the Dos Patos Lagoon basin (*sensu* Albert and Reis 2011) in Uruguay (Fig. 1). Fish were kept alive in the laboratory until euthanasia, according to guidelines approved by the corresponding Honorary Commission on Animal Experimentation (CHEA; Facultad de Ciencias, Universidad de la República, Uruguay), based on the International Guidelines for Fish Euthanasia (AMVA; Leary et al. 2013).

After euthanasia and necropsy of *A. charrua* specimens, cestodes were manually removed from the body cavity, fixed, and preserved for molecular analysis in 95% ethanol and stored at 4 °C until use. Parasites were observed morphologically to identify them *a priori*, using the taxonomic descriptions in Noya et al. (1992) and Waeschenbach et al. (2017) as reference. Ecological indices were calculated following Bush et al. (1997), using the open-license software QPweb (Reiczigel et al. 2019) to obtain confidence intervals for prevalence using the Blaker method (for deviation from normality) and bias-corrected and accelerated bootstrap confidence intervals for mean intensity of infection. Voucher specimens were deposited at the Museo Nacional de Historia Natural (MNHN) in Montevideo, Uruguay, under lot number MNHN 4262.

Molecular identification

Total genomic DNA was extracted from whole specimens by proteinase K digestion, followed by extraction with sodium chloride and ethanol precipitation (modified from Medrano et al. 1990). Amplification by polymerase chain reaction (PCR) began with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 49 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Primers used for PCR were based on Bowles et al. (1992): 2575 (5'TTT TTT GGG CAT CCT GAG GTT TAT3') and 3021 (5'TAA AGA AAG AAC ATA ATG AAA ATG Y3'), which were designed to amplify a cytochrome oxidase subunit 1 (COI) region of approximately 446 bp (Bowles et al. 1992). Genomic sequencing of the amplicons was performed

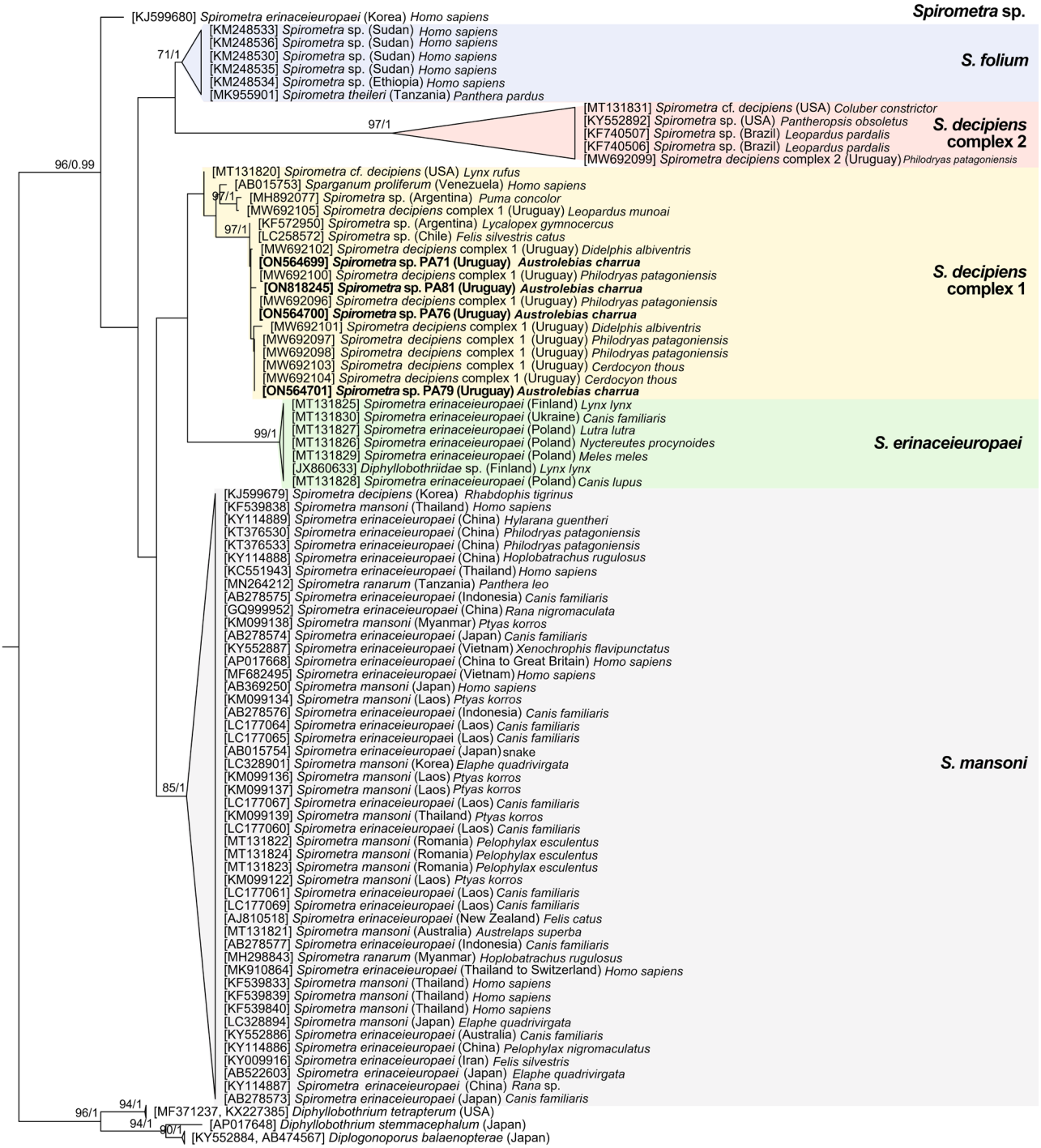

Fig. 2. Phylogenetic tree resulting from the Maximum Likelihood (ML) and Bayesian Inference (BI) methods used to analyse partial sequences of cytochrome c oxidase subunit I (COI) corresponding to the six accepted species of *Spirometra* and the spargana found in annual fish from Uruguay. Both phylogenetic approaches yielded the same topology. The node supports shown on the branches correspond to the bootstrap values resulting from 1,000 pseudo-replicates of the ML method (in percentage), and posterior probability values of the BI method. Only bootstrap support values above 70 and the corresponding posterior Bayesian probability are shown. Taxa are shown in the form "species name (locality) host". Specimens studied in this work are in bold.

using the standard sequencing service of Macrogen Inc (Korea) standard sequencing service (ABI 3730xl system).

A preliminary nBLAST search of the GenBank database was performed to determine if phylogenetic analysis could be restricted to only one genus, resulting in high percentages of identity with *Spirometra* species. In accordance with the methodology of Armúa-Fernández et al. (2021), multiple sequences of species of *Spirometra* available in GenBank were downloaded to create a final data alignment, and the resulting database was analysed using the open-license software DnaSP v.6 (Rozas et al. 2017) to identify haplotypic redundant sequences that could be removed. Three *Diphyllobothrium* species *D. tetrapterum* (von Siebold, 1848), *D. stemmacephalum* Cobbold, 1858, and *D. balaenopterae* (Lönnberg, 1892) were selected as outgroups based on the

Table 1. Sampling information of *Austrolebias charrua* Costa et Cheffe captured in October 2019 at three temporary ponds in different localites in southern Dos Patos Lagoon basin (southeastern Uruguay). Confidence intervals were calculated by Blaker's method for prevalence and bias-corrected and accelerated bootstrap for mean intensity. Confidence intervals for locality A could not be estimated due to the low number of samples.

	India Muerta	Ruta 9 km 272	La Coronilla
	-33.655368, -54.062627	-34.203320, -53.788991	-33.900624, -53.526706
Number of hosts (NH)	2	12	12
Total number of parasites per temporary pond (NP)	4	11	60
Prevalence (P%)	50	42 (18–71)	58 (29–83)
Mean intensity (MI)	4	2.2 (1.4–4.4)	8.6 (3.6–13.9)

currently accepted phylogenies of the family Diphyllobothriidae (Waeschenbach et al. 2017; Fraija-Fernández et al. 2021). Alignment of the sequences was performed with MUSCLE algorithm in the program MEGA v.10 (Kumar et al. 2018). The nucleotide substitution model for the Akaike Information Criterion (Akaike 1974), the corrected Akaike Information Criterion (Sugiura 1978), and the Bayesian Information Criterion (Schwarz 1978) was TrN + G, which was selected as the best model using JModeltest2 (Darriba et al. 2012).

A heuristic Maximum Likelihood (ML) search was performed using Subtree Pruning and Regrafting (SPR) and the robustness of the nodes was determined using Bootstrap analysis (1,000 pseudoreplicates) with PhyML 3.0 software (Guindon et al. 2010). In addition, Bayesian Inference (BI) with four Markov chains Monte Carlo (MCMC) was performed for 12 runs of 20 million generations using MrBayes software (Huelsenbeck and Ronquist 2001) in the CIPRES portal (Miller et al. 2010). The Tracer v1.7 package (Rambaut et al. 2018) was used to check stationarity of MCMC. The genetic distance between lineages included in the final alignment was calculated by computing the mean uncorrected p-distance (1,000 bootstrap pseudo-replicates) between eight predefined groups of taxa: the six accepted species of *Spirometra*, the new sequences obtained in this study, and the outgroup. This was performed in MEGA v.10 (Kumar et al. 2018).

RESULTS

A total of 75 spargana were found free in the body cavity (coelom) of *Austrolebias charrua* collected from three sites in southeastern Uruguay, with locality C (La Coronilla) being the one with the highest parasitological indices (Table 1). The larval parasites were tentatively identified as *Spirometra* sp. This was based on general morphological features such as the simple, enlarged scolex with distinct transverse folds (Scholz et al. 2019), the absence of aberrant proliferation, and the presence of an apical slit as opposed to two longitudinal bothria across the entire scolex (Waeschenbach et al. 2017) (see Supplementary Material 1). In this case, no further identification could be made because the specimens were not sexually developed.

Molecular identification

Six new sequences for the mitochondrial marker COI were obtained (accession numbers ON564699, ON564701, ON818245, ON818247), but only the sequences longer than 300 bp were used for the analyses, resulting in an average length of 357 (311–375) bp (ON564699, ON564700, ON564701, ON818245). The preliminary nBLAST search in the GenBank database revealed 99% identity with Uruguayan sequences of *Spirometra decipiens* complex 1 obtained from amphibians and reptiles (Armúa-Fernández et al. 2021), confirming the morphological classification *a priori* and restricting the study to the genus *Spirometra*.

The final alignment consisted of 90 partial sequences of the mitochondrial COI gene: 81 sequences of *Spirometra* spp. downloaded from GenBank, four new sequences obtained in this study, and five sequences corresponding to the outgroup (see Supplementary Material 2). On average, the sequences had a length of 381 (311–391) bp, 139 variable sites, and 125 parsimony informative sites. Both phylogenetic approaches used (ML and BI) showed a topology of six defined lineages, consistent with the results of previous authors (Armúa-Fernández et al. 2021, Kuchta et al. 2021). The specimens studied in this work belonged to the monophyletic clade of *S. decipiens* complex 1, with a ML bootstrap support of 97 and a BI posterior probability of 1 (Fig. 2). They were also more closely associated with sequences from southern South America (Argentina, Chile, and Uruguay), which indicates some geographic consistency in the phylogeny. Genetic distance analysis supported this result by yielding the lowest uncorrected p-distance value between the spargana found in *A. charrua* and those of *S. decipiens* complex 1 (mean p-distance = 0.0228 ± 0.0041).

DISCUSSION

This work is the first report in the world of a *Spirometra* species (*S. decipiens* complex 1 *sensu* Kuchta et al. 2021) occurring naturally in a fish host, namely *Austrolebias charrua*. The term 'natural' is used here to emphasise that this parasitic association between *S. decipiens* and *A. charrua* was found in natural systems and not experimentally enforced. This clarification is necessary because artificial infections have been performed in fish in the past to investigate the ability of *Spirometra* sp. to exist in these hosts (Joyeux et al. 1934, Yutuc 1951, Bearup 1957, Mueller 1960, Odening and Bockhardt 1982). While some authors were able to obtain a few spargana from artificially infected fish (Joyeux et al. 1934, Odening and Bockhardt 1982), most failed to obtain successful infections. For example, all attempts by Yutuc (1951) were unsuccessful because the spargana were either regurgitated, stuck in the posterior chamber of the buccal cavity, or digested.

Bearup (1957) used procercoid-bearing copepods to infect fish and also reported failure in all experiments. Finally, Mueller (1960) cultured spargana in fish sera and injected them intramusculary into fish. After having no success, he concluded that fish could not serve as intermediate hosts for *Spirometra* spp. under any circumstances. While it is true that the partially successful infections were early indicators of the potential ability of *Spirometra* sp. to develop in fish hosts, such reports are not comparable to what we found in *A. charrua*. In these annual fish, spargana thrived in the host body cavity, suggesting successful development

of procercoids into plerocercoids as well as active migration through the intestinal wall.

Although annual fishes are a poorly studied system in terms of their parasites, they are known to harbour predominantly larval stages of endoparasites, as shown by parasitological studies of the African counterpart of the rivulids, the nothobranchiids. This may be due to their annual life cycles, which result in annual local extinction events that most adult endoparasites cannot survive (Nezhybová et al. 2016). This is consistent with *A. charrua* acting as a second intermediate or accidental host of *S. decipiens* complex 1, with larvae most likely ingested as procercoids in small crustaceans and developing into plerocercoids after migrating through the gut wall. Although there are no official reports of mammals or reptiles consuming annual fish as part of their diet in Uruguay, the finding of *Spirometra* in *A. charrua* may indicate a possible trophic relationship between these hosts.

The disparity in the number of spargana found at the various sites draws attention. A likely explanation could be that the samples were taken in October, the end of the rainy season in Uruguay, and the beginning of the drought season (Berois et al. 2015). This meant that the water of the temporary ponds was in an advanced state of evaporation, resulting in heterogeneous scenarios in the different temporary ponds due to their irregular shapes and morphologies. This led to an inevitable sampling bias in the number of hosts collected (only two hosts could be found in locality A), which was obviously reflected in the infection indices. Some other variables that may have affected the number of spargana found in annual fish would be host competition (presence of amphibian larvae that could also act as second intermediate hosts for spargana), proximity to other water sources, availability of definitive hosts in the area, and anthropological influence.

At the observational level, anthropological effects appeared to vary among the temporary ponds sampled in this study. Locality C (La Coronilla) is closest to human settlements, so domestic animals and opportunistic mammals such as some foxes, e.g., *Cerdocyon thous* (Linnaeus) may be more abundant in this area, likely resulting in high numbers of spargana in fish. Locality B (Ruta 9 km 272) is located near a highway, possibly affecting the abundance of definitive hosts in this area that reach the temporary pond, and hence the lower numbers of spargana. Locality A (India Muerta) is the least anthropologically impacted, but was nearly dry at the time of sampling, making it difficult to draw further conclusions.

Knowledge of the life cycles of *Spirometra* spp. infecting wildlife in South America, especially in Uruguay, is still scarce (Armúa-Fernández et al. 2021). With *A. charrua* as a new link in the trophic interactions that the parasite uses to reach adulthood, the scenario becomes even more complex. This highlights the need for further studies that should encompass a broader range of hosts and environments. Exploring the link between *Spirometra decipiens* complex 1 and *A. charrua* from an ecological perspective could also deepen the understanding of this novel niche and thus contribute to the understanding of the life history of these parasites.

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Author contributions. R.V. conceived the idea, carried out the experiments, corresponding analyses, and wrote the manuscript. W.N. and S.M. worked as overseers of the research process and contributed to the correction of the draft. W.N. co-administered the project, supervised and helped in the visualization and writing of the manuscript. G.G. reviewed the manuscript. S.M. aided R.V. in the initial taxonomic identification by morphology. N.R. reviewed the manuscript, oversaw, and helped plan the experiments, aided R.V. in carrying out the phylogenetic analyses and in writing the manuscript.

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