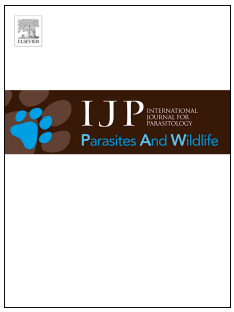

First identification and molecular phylogeny of *Sparganum proliferum* from endangered felid (*Panthera onca*) and other wild definitive hosts in one of the regions with highest worldwide biodiversity

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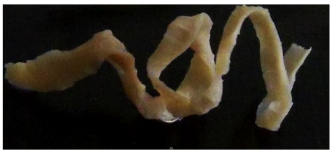
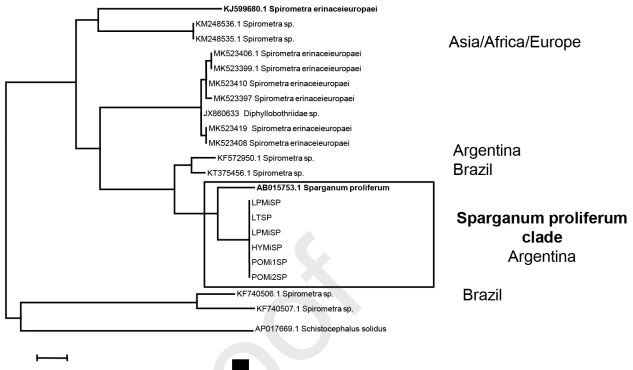
Endagered wild felid host : *Panthera onca*



Larvae and adult morphological features extraction

Molecular phylogeny
Mitochondrial genes



Species determination
Sparganum proliferum

Title

First identification and molecular phylogeny of *Sparganum proliferum* from endangered felid (*Panthera onca)* and other wild definitive hosts in one of the regions with highest worldwide biodiversity

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*All sequences reported in this work are available at GenBank nºAccession: MK976918-MK976920.

Abstract

After decades of being neglected, broad tapeworms now attract growing attention thanks to the increasing number of reports from humans but also thanks to many advancements achieved by application of molecular methods in diagnosis and epidemiological studies. Regarding sparganosis, unfortunately general uniformity of most species, their high intraspecific variability and lack of agreement among researchers has led to confusion about the classification of *Spirometra/Sparganum* species. For the first time we determined adult, eggs and plerocercoid life cycle stages and the molecular phylogeny of *Sparganum proliferum* obtained from endangered wild felids (*Panthera onca*, *Leopardus pardalis*, *Leopardus guttulus* and *Herpailurus yagoaurondi*) in one of the largest continuous remnants of worldwide biodiversity, the Atlantic Forest from South America. Our results showed that at least 57% of total species of wild felids in this natural area could act as definitive hosts of *Sparganum proliferum*. We conclude that the availability of more morphological characteristics are needed in order to secure reliable characterization and diagnosis of sparganosis. The integration of these data with molecular analysis of mitochondrial DNA sequences will be useful for species discrimination.

keywords: endangered fauna; *Sparganum proliferum*; wild carnivores; mitochondrial genes

1. **Introduction**

Sparganosis is an emerging parasitic zoonotic disease mainly caused by the second larva stage (plerocercoid) of diphyllobothriid cestodes such as, *Spirometra* ssp. and *Sparganum proliferum* **(**Noya et al. 1992, Kokaze et al. 1997; Miyadera et al., 2001, Brabec, Kuchta and Scholz, 2006; Kuchta et al., 2008;Schauer et al. 2014;, Oda et al. 2016; Kikuchi, T., & Maruyama, H. 2020; Kikuchi et al. 2020; Hong *et al.*, 2020). *Sparganum proliferum* is a cryptic parasite which phylogeny and life cycle are poorly understood. The adult stage of *S. proliferum* has not been observed and the precise taxonomic relationships of *S. proliferum* with other tapeworms remain unclear because few genes have been sequenced (Noya *et al.*, 1992; Miyadera *et al.*, 2001; Okamoto *et al.*, 2007). Recently, the genome and transcriptomic analysis of plerocercoid of *S. proliferum* was reported and confirmed that *S. proliferum* and *Spirometra erinaceieuropaei* are closely releted but different species (Kikuchi et al., 2020). In addition to taxonomic considerations, the pathogenicity of *S. proliferum* (proliferative sparganosis) and plerocercoids of Diphyllobothriidae tapeworms, including those of *S. erinaceieuropaei* (non-proliferative sparganosis) are different (Kikuchi & Maruyana 2020). Sparganosis cases are reported worldwide, but it has been predominantly diagnosed in Southeast Asia, mainly in China (Dorny *et al.*, 2009; Qiu and Qiu, 2009; Liu *et al.*, 2015). Human sparganosis frequently occurs by consuming raw or undercooked meat of infected reptiles or amphibians, drinking water contaminated with copepods as well as direct contact with the skin of infected frogs or snakes (Li *et al.*, 2011; Liu *et al.*, 2015; Oda *et al.*, 2016, Okino *et al.*, 2017; Zhang et al., 2020). Also, a case of human infection by adult of *S. erinaceieuropaei* has been reported in Vietnam (Le *et al.*, 2017). In Argentina, three cases of sparganosis have been reported in individuals from border countries. Two with cerebral location (Boero, Garaguso and

Navarr, 1991; Jones *et al.*, 2012) and one with cutaneous location (De Roodt *et al.*, 1993). Moreover, in Argentina there are few reports of *Spirometra* spp. in animals. *Spirometra mansonoides* has been found in cats (Santa Cruz and Lombardero, 1987), *S. erinaceieuropaei* in cats (Venturini, 1980, 1989) and dogs (Denegri, 1993). In wildlife, Martínez et al. (2010) has identified eggs of *S. mansonoides* in the felines *F. pardalis*, *F. yagouaroundi*, *Panthera onca* and *Puma concolor*. *Spirometra* has been reported in Pampas fox (*Lycalopex gymnocercus*) (Reigada, Bisceglia and Miño, 2012; Petrigh *et al.*, 2015). Despite numerous attempts to clarify its taxonomy, host specificity and geographic distribution (Faust et al, 1929; Wardle et al, 1974), the genus remains one of the most complicated groups of tapeworms, and several lines of evidence concluded that it is very difficult and almost impossible to distinguish some of the 50 nominal species of *Spirometra* based solely on morphological characteristics (Iwata, 1934, 1972; Mueller, 1974; Daly, 1981; Odening, 1985; Kuchta and Scholz, 2017). In Asia, there are several studies of *S. erinaceieuropaei* lineages (Zhang et al., 2016), particularly in wild frogs in China the prevalence is above 10% in some regions. (Hong et al., 2020; Zhang et al., 2017; Zhang et al., 2020). Regarding Africa, there are molecular reports of findings of *Spirometra sp.* in human infections in South Sudan and Ethiopia (Eberhard *et al.*, 2015). In Europe, there are also molecular records of *S. erinaceieuropaei* plerocercoid larvae in wild fauna from Poland (Kołodziej-Sobocińska *et al*., 2019). In Brazil, there have been reports of *Spirometra* spp. larval stages in cold-blooded animals (Rego and Schäffer, 1992) humans (Liu *et al.*, 2015) and adult stages in wild felids (Vieira et al, 2008). The occurrence of a particular *Spirometra* lineage in South America has been reported (Almeida *et al.*, 2016) and the molecular sequences obtained were phylogenetically cluster in a separate node and distant to the Asian *S. erinaceieuropaei* lineage.

The objective of this work is to identify and characterize diphyllobothriid infections in wild animals of the Atlantic Forest of Misiones, Argentina through an integrative approach that links morphological, genetic and ecological aspects. Here we report, for the first time, the presence of adult and eggs of *S. proliferum* in wild animals in South America, confirmed by molecular analysis. Our results could be useful to understand some of the underlying aspects of the life cycle of *S. proliferum* and evaluate the zoonotic importance in the interface areas to guide prevention measures for human and animal welfare.

2. Methods

2.1. Study area

The study area contains one of the largest continuous remnants of Atlantic Forest (AF) in the World. It is located in northern Misiones province, Argentina, 54°15′30.60″W, 25°55′52.32″S). The area is 220 m in altitude and presents subtropical climate with annual rain precipitations between 1700 and 2100 mm (Ligier, 2000).

2.2. Animal samples

Road-killed animals were actively searched on national routes 101 and 12 that cross the Iguazú National Park between the years 2015 to 2016. Animal necropsies were carried out under approved protocols by the National Parks Administration technical office (NEA 423 Rnv ex DCM 483 Dispo 23/2015). Only 1 –2 days old animal carcasses were selected for sampling. Five animals were collected and analysed in this work and are summarized in Table 1. Each animal was individually packed and labelled with relevant information including place of origin, sampling date, age category, and sex of the animal.

2.3. Parasite samples

The intestinal tracts of the analysed carnivores were carefully removed from each carcass and subsequently isolated by ligatures (pylorus and rectum). All samples were kept at −20 °C for at least 1 month prior to processing in order to inactivate possible parasite eggs from other species (Scioscia *et al.*, 2013). Examination of the intestinal content was performed as previously (Arrabal *et al*., 2017) using the modification of the technique described originally by Eckert (2001). Briefly, the small intestine was separated from the large intestine, and then each section was placed in different trays and cut lengthwise. Coarse material and large parasites of the small intestine were removed. Then, each section was immersed in 9% saline solution at 37 °C for 30 min. Intestinal walls were scraped with a microscope slide, and all the content of each section were poured into individual glass bottles and left to stand for 20 min. The supernatant was discarded and physiological saline solution was added to dilute the sediments. This procedure was repeated several times until the supernatant was almost translucent. Obtained sediments were examined in small portions of 5 –10 ml round petri dishes with magnifier lens at ×65 to identify small helminths. The helminths found were cleaned with saline solution and deposited in recipients with either 4% formalin or 70% ethanol for further taxonomic and molecular examination, respectively.

2.4. Morphology studies

Strobilas of adult tapeworms, larvae and eggs were analyzed under optical Primo Star (Carl Zeiss Gmbh, Göttingen, Germany) microscope using Axion Cam ERc 5s camera (Carl Zeiss Gmbh, Göttingen, Germany). Each sample was whole mounted and registered with 4×, 10× and 40× using Carl Zeiss Vision software for image analysis. Moreover, strobilas and larvae tissue sections were prepared in paraffin and were sectioned in serial sections of 4 –5 μm, mounted on glass slides, and stained with hematoxylin-eosin (HE). The slides were analysed under optical microscope and picture

was taken at 4×, 10× and 40×. The main features analyzed in the larvae were pleomorphism, color, symmetry and presence or not of scolex (Noya et al., 1992). The main features analyzed in eggs were size, shape and presence or not of cap and pointed ends (Mueller, 1936).

2.5. Molecular identification and phylogenetic analysis

Total parasite genomic DNA was obtained using the DNeasy Blood &Tissue Kit (Qiagen GmbH, Hilden, Germany). Three molecular markers from mitochondrial genome were used to determine species. Cytochrome c oxidase subunit I (cox1) gene, NADH dehydrogenase subunit 1 (nad1) gene and ATP synthase subunit 6 (atp6) gene were selected since we know and used them in previously reports from cestodes (Kamenetzky et al., 2002; Arrabal et al., 2017) and were demonstrated to be useful to classify isolates of *Spirometra* in previously reports (Almeida et al., 2016; Zhang et al.; 2017, 2020). The genes were amplified by polymerase chain reaction according to Arrabal *et al.* (2017) (Supplementary Table 1). The PCR-product obtained was sequenced and firstly aligned with ClustalX (v2.0.12) with *Spirometra* and *Sparganum* sequences extracted from complete mitochondrial genomes available on GenBank and considered as reference genomes (International Helminth Genomes Consortium; 2019). To get insight into an accurate phylogenetic analysis of our parasite linage we downloaded cox1 sequences form Asia, Africa, Europe and South America totalling 275 cox1 sequences (Lavikainen et al., 2013; Zhang et al., 2017, 2020; Jeon & Eom 2019; Kolodziej-Soboeinska et al., 2019; Hong et al., 2020). After several sequence redundancy removal 42 cox1 sequences were retained. These data set includes *Spirometra* cox1 sequences from wild frogs that were described to have a high pairwise genetic distance with the reference mitochondrial genome (Zhang et al., 2017). Multiple alignments were edited with BioEdit (v7.1.3). Maximum likelihood phylogeny was

performed using MEGA7. A discrete gamma distribution was used to model evolutionary rate differences among sites. Branch lengths were measured as the number of substitutions per site. All positions with less than 80% site coverage were eliminated. There was a total of 296 positions in the final dataset. Additionally, Bayesian phylogeny was implemented by using BEAST. Substitution model HKY+G+X with gamma distribution was selected with PartitionFinder. Changes in the evolutionary rates among branches were performed by using random local clock model (Drummond and Suchard, 2010). For earlier tree a basic coalescent model was selected. MCMC run was performed with tree parameter values sampled every 1000 steps over a total of 100,000.000 steps (Zhang et al., 2017).

3. Results

3.1 Morphological identification of *Sparganum proliferum* in wild carnivores

The analysis for intestinal tracts of wild carnivores allowed as isolating tapeworms morphologically compatible to *Spirometra* in wild carnivores, this being the first report of this parasites in the eco-region of Atlantic Forest. One *Leopardus pardalis* (ocelot), one *Panthera onca* (jaguar), one *Leopardus guttulus* (tirica) and one *Herpailurus yagoaurondi* (yaguarundi) (Figure 1). Parasites were identified according to morphological features, the individual selected for further analysis has a resemblance with *Spirometra* by their general appearance and size (Figure 2). The larva has the following major macroscopic features: pleomorphism, white color; length <5 mm, lack of bilateral symmetry and without scolex (Figure 2D) accordingly to previously *Sparganum proliferum* larvae features described so far (Noya et al., 1992). Although numerous worms were found it was not feasible to identify all specimens based on morphological features because most of them were fragmented and were not suitable for morphological examination. Regarding strobilas corresponding to adult tapeworms the

193 major differentiating features of the eggs found were land shape and the evident cap
194 and pointed ends attributable to the genus (Mueller, 1936) (Figure 3). The average eggs
195 measures were 67.02 μm by 34.95 μm (n = 50). Histological sections of strobilas were
196 performed. The main characteristics (based in mature and gravid proglottids) were i)
197 presence of anterior and posterior uterine coils in the longitudinal median line of the
198 proglottids ii) ventral middle uterine pore in the third of the gravid proglottid iii) uterus
199 opened by a pore well separated from and posterior to the vagina, and presence a
200 varying number of loops in the terminal heavy walled portion in an "S" shape (iv)
201 uterus consisted of 5-7 loops and the dumbbell-shaped ovary connected to the uterus
202 and situated near the posterior margin v) vagina passed traversing from its vestibule in
203 an approximately straight path in the median line thrown into lateral undulations of
204 different amplitude viii) cirrus surrounded by the seminal receptacle and opens out
205 separately from vagina and near to the uterine pore (Figure 4). In this section the ratio of
206 width and length of gravid proglottids and uterine morphology were consistent to
207 *Spirometra* spp. (Iwata et al., 1972; Mueller, 1974).

Table 1. Percentage divergence between mitochondrial sequences from samples of wildlife *Sparganum proliferum* and reference genes of *Spirometra erinaceieuropaei* (KJ599680) and *Sparganum proliferum* (AB015753).

Sample ID	Host[&]	Parasitic stage in host	Percentage Divergence (SE)[+]					
			Cox1		Nad1		Atp6	
			Spirometra erinaceieuropaei	*Sparganum proliferum*	*Spirometra erinaceieuropaei*	*Sparganum proliferum*	*Spirometra erinaceieuropaei*	*Sparganum proliferum*
LPMiSP	Ocelot	Adult	14.4 (2.4)	4.2 (1.3)	12.5 (2.3)	--*	26.9 (2.6)	--*
LTMiSP	Tirica	Plerocercoid	14.4 (2.4)	4.2 (1.3)	12.5 (2.3)	--*	26.9 (2.6)	--*
HYMiSP	Yaguarundi	Adult (fragment)	14.4 (2.4)	4.2 (1.3)	Nd	--*	nd	--*
POMiSP1	Jaguar	Adult	14.4 (2.4)	4.2 (1.3)	Nd	--*	nd	--*
POMiSP2	Jaguar	Adult (fragment)	14.4 (2.4)	4.2 (1.3)	Nd	--*	nd	--*

*There is no sequence information from *Sparganum proliferum* (AB015753) nad1 and atp6 genes.

[+] Pairwise genetic distance was calculated with MEGA7 using Tamura-Nei (1993) model

[&]POMiSP1 and POMiSP2 samples belongs to the same individual host

[+] Genbank accession numbers: MK976918 (cox1), MK976919 (nad1) and MK976920 (atp6).

3.2 Molecular characterization of *Sparganum proliferum* in wild felids

First, we analysed by PCR and sequencing the adult obtained from the ocelot (sample LPMiSP) by three molecular markers. The sequences obtained from cox1 (295 nt), nad1 (343 nt) and atp6 (594 nt) mitochondrial genes were concatenated resulting in a dataset of 1322 nucleotides to analyze the complete information in an integrated phylogeny. Multiple sequence alignment comparisons with all mitochondrial reference genomes were performed in order to identify the ocelot mitochondrial sequence. Redundant reference sequences were removed and a total of 10 orthologous sequences from mitochondrial complete genomes were finally included (Supplementary Table 2). The phylogenetic tree constructed based on the multiple alignment showed that LPMiSP belongs to *Spirometra* lineage near to *S. erinaceieuropaei* (KJ599680) isolated from a human in Korea (Supplementary Figure 1). The genetic divergence between LPMiSP and *S. erinaceieuropaei* was 14.4% for cox1, 12.5% for nad1 and 26.9% for atp6 (Table 1). Since *S. erinaceieuropaei* cox1 non redundant sequences available in GenBank have an average genetic divergence of 8.8% and the genetic distance obtained between LPMiSP and *Spirometra* spp. was relatively higher (14.4%) we couldn't classify it as belonging to the same species. To get insight the presence of *Spirometra* in wild felids we assessed to amplify and sequence the same three molecular makers from more samples. The cox1 sequences from jaguar (samples POMiSP1 and POMiSP2), tirica (sample LTMiSP) and yaguarundi (sample HYMiSP) and additional nad1 sequence from sample LTMiSP were obtained. The nad1 sequence obtained from tirica host was 100% identical to the previously sequenced obtained from LPMiSP-nad1. Additionally, all cox1 sequences were 100% identical to each other. Since atp6 was not possible to be amplified, we hypothesize that several SNPs are present between mitochondrial genomes from Argentinean wild felids and *Spirometra* spp. mitochondrial genomes reported, and may be

different species. To test this hypothesis, we retrieve a broader set of cox1 sequences available for *Spirometra/Sparganum* in GenBank and performed multiple alignments. The number of SNPs between cox1 sequences from parasites from Argentinean wild hosts is shown in Supplementary Figure 2. One interesting finding was that that cox1 sequences from wild felids from Argentina have 4.2% of genetic divergence of to *Sparganum proliferum* cox1 sequence (AB015753) (Table 1). This finding was consistent with the phylogeny obtained from the multiple sequences alignment. Even the tree topology indicates that a taxonomic revision of some sample is needed (some *Spirometra decipiens* clustered with *S. erinaceieuropaei* sequences). Parasite samples obtained in this work shared common ancestor with *Sparganum proliferum* (Figure 5). Besides this, the sequences that were characterized as *Sparganum*, including those obtained in this study, are included within the same clade as a *Spirometra* lineage registered in South America (KF572950 and KT375456). These sequences have 6.4% and 6.7% of genetic divergence with LPMiSP, respectively. The *Spirometra* sequences from the next near node (e. g. KF988137) have 12.0% genetic divergence with LPMiSP. Taking into account the tree topology and the genetic distance between *Sparganum* and *Spirometra* cox1 sequences we suggest that KF572950 and KT375456 accession numbers also belongs to *S. proliferum* species. We confirmed our results by Bayesian phylogenetic analysis (Supplementary Figure 3). In this phylogeny numbers along branches indicate posterior probabilities that support the groups mentioned before. Moreover, the effective sample size (ESS) values for all parameters were above 200 giving confidence to the analysis.

4. Discussion

After decades of being neglected, broad tapeworms now attract growing attention thanks to the increasing number of human cases but also thanks to considerable advance achieved by

application of molecular methods in diagnosis and epidemiological studies (Scholz *et al*, 2019). Regarding sparganosis, general uniformity of most species, their high intraspecific variability and lack of agreement among investigators has led to confusion about the classification of *Spirometra/Sparganum* (Mueller, 1974; Daly, 1981; Kuchta and Scholz, 2017). Moreover, most of the available material was obtained from host examined long time post mortem or even from decomposed carcasses, which may have caused significant morphological changes (Hernández-Orts *et al.*, 2015). As a result, morphological and biometrical data in some species descriptions may be misleading. Similarly, most clinical samples of larval stages were not characterised molecularly and were described under different names. This work showed that *S. proliferum* and *S. erinaceieuropaei* species have dissimilar cox1, nad1 and atp6 sequences. The molecular results of this work are in concordance with previous analysis where both species were clearly distinguished by cox1, nuclear sdhB and 18S rDNA V2 region gene sequencing (Miyadera *et al.*, 2001, Kikuchi et al., 2020). Also, discrepancies in *Spirometra* phylogeny and a possible new species were also reported by Zhang et al. (2017; 2020) studying parasites from wild fauna. Moreover, the low identity and high genetic distance between atp6 sequences obtained from ocelot (LPMiSP) and the reference atp6 sequence from *S. erinaceieuropaei* support these findings. Mitochondrial atp6 sequences from *S. proliferum* are not available in public databases to make the necessary comparisons with the results obtained in the present work.. The isolates analyzed in this work are not closely related to *S. erinaceieuropaei* or other Asian *Spirometra* lineage, but instead, might display close affinities to one of the lineages described as *Spirometra* from South America and with *S. proliferum* (AB015753) mitochondrial reference genome. Our source of parasites are dead animals on the road, it should be noted that the high temperature of the region under study favours the decomposition rate of carcasses. For this reason, helminth

specimens not suitable for ideal morphological identification are the most common outcome. We overcome these problems by implementing an integration of morphological and molecular data analysis. Morphologically similar species presenting a spiralled uterus (*S. decipiens, S. gracilis, S. longicollis, and S. mansoni*) were reported in wild felids from Brazil, as well as in proglottids of *Spirometra* spp. (Almeida et al., 2016); however, the vagina of *S. erinaceieuropei* is considered to lie next to the midline and descends in waves of different amplitude (Palmer *et al.*, 2008). Also, the shape of the uterus lacks uniformity in the number of turns (between three and seven loops) having irregular arrangement and size (Iwata, 1932; Mueller, 1974; Okino *et al.*, 2017). Our results showed that the proglottids of *S. proliferum*, and also the eggs found, presented the same morphological characteristics that *S. erinaceieuropei* and it is because of that, these species need to be evaluated using molecular markers. Recently, Kuchta et al. (in press) suggested *Sparganum proliferum* belongs to a lineage of *S. decipiens* described in South America. However, since their results are based only in genetic data and we analyzed not only this data but also novel adult morphological features that classification may be revised. More isolates analyzed with other molecular markers such as nuclear genes or complete genome sequences are needed to confirm the presence of *S. proliferum* in wild hosts. meanwhile the three mitochondrial genes employed in this work could be used as molecular markers for epidemiology studies. The sequence comparison among *Spirometra* from Brazil and *Sparganum* from Argentina indicates that they are different lineages. Species of *Sparganum* occur in warmer latitudes similar to the region analyzed here (Mueller, 1974; Daly, 1981). Fatal proliferative sparganosis was reported from domestic cats in North America (Buergelt, Greiner and Senior, 1984; Woldemeskel, 2014) and dogs in Europe (Stief and Enge, 2011). However, the impact of diphyllobothriid cestodes in wild animals is not clear yet. Our findings showed for the first time

S. proliferum adults and larvae in the intestine track of the wild felids. In *Spirometra* species it was already described that once in the secondary vertebrate host, the procercoid can develop into a plerocercoid larva in different tissues, which can survive predation and reach a wide variety of vertebrates (Mueller, 1974; Opuni and Muller, 1974; Liu *et al.*, 2015). We found *S. proliferum* plerocercoides larvae in tirica intestine, indicating that the preys it feeds on are harbouring pleroceroides that survive the gastric digestion. Regarding the unknown complete life cycle of *S. proliferum*, the human activities in the region under study like the conversion of natural landscapes to urban areas may increase predation by domestic dogs and cats on wild amphibian and reptile populations, thus potentially enhancing the incidence of proliferative sparganosis (Borteiro *et al.*, 2015). The possible role of amphibians and reptiles may have in the occurrence of human cases in South America is an issue not being well investigated yet. In Argentina, there are few reports of *Spirometra* spp., mostly in domestic definitive hosts Venturini, (1980, 1989); Santa Cruz and Lombardero, (1987); Denegri (1993). Regarding wildlife, identified *S. mansonoides* eggs were reported in felids (Martínez *et al*, 2010), *Spirometra* spp. in the Pampas fox (*Lycalopex gymnocercus*) (Reigada, Bisceglia and Miño, 2012; Scioscia *et al.*, 2014; Petrigh *et al.*, 2015).The present work showed that at least four different species of wild felids, out of the six existing in the natural area under study are involved in the sylvatic life cycle of *S. proliferum* and could act as definitive hosts in the Atlantic Forest. This region is shared with other groups of carnivores (canids, mustelids and procyonids) that could also be participating in the cycle. *Sparganum proliferum* is a good model for ecological interaction studies, which allows us to understand and define the trophic levels of the intermediate and definitive hosts, and then to establish the distribution of parasites within a host population (Denegri, 2008). For these reasons, the knowledge of prevalence of *Sparganum* in wild animals of Argentina is necessary, due to

ongoing changes on the environment that affect parasite ecology and its transmission dynamics. In Argentina few cases of human sparganosis have been reported but the real prevalence is unknown in the country. In the meantime, a reliable taxonomic criterion based on morphological characteristics integrated with molecular analysis of mitochondrial DNA sequences will be useful for species discrimination.

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Legends to the figures

Figure 1.Animals road-killed in Iguazú National Park, Misiones, Argentina. The intestinal tracts of each carnivore felid were necropsied and parasites removed from intestine. A- *Panthera onca* (jaguar); B-*Herpailurus yagouaroundi* (yaguarundí); C-*Leopardus guttulus* (tirica); D-*Leopardus pardalis* (ocelot).

Figure 2. *Sparganum proliferum* worms from intestinal tracts of wild cats. A- Larvae from tirica; B- Adult from ocelot; C- Adult from jaguar; D- Larva or plerocercoid from tirica. (A-C. Macrocopical images, D. Stereoscopic magnifying glass image).

Figure 3. Whole mounted samples from *Sparganum proliferum* adult found in wild felids host A- Proglottids showing uterus with eggs from ocelot. B, C and E - Light brown eggs with evident cap and pointed ends attributable to the genus from jaguar. D- Gravid uterus from ocelot *VA: vagina; UP: uterine pore; U: uterus

Figure 4. Histological cut from mature proglottid showing the uterus (U) and vagina (VA) and cirrus sac (CS). Showing the cirrus (C), uterus (U), genital pore (GP), vaginal pore (VP), uterine pore (UP), and ovary (OV) . Aceto carmine stain.

Figure 5. *Sparganum proliferum* COX1 phylogeny. A total of 48 sequences from different host species and geographic origin were analyzed by Maximum Likelihood method including 296 positions in the final dataset. Genbankaccession number are shown. The codes of the samples obtained in this work are the same as Table 1. References species are marked with a black dot.

Supplemenetary Figure 1: Phylogenetic analyzes of *Sparganum* samples. Maximum Likelihood method of three molecular markers concatenated from parasite ocelot isolate (LPMiSP) and 10 orthologous sequences from reference genomes. There were a total of 1245 positions in the final dataset.

376 Supplementary Figure 2: Multiple sequence alignment of cox1 *Sparganum* and *Spirometra*
377 species perfomerd with ClustalX (v2.0.12). Dots indicates identical nucleotides.

378 Supplementary Figure 3: Bayesian phylogeny of *Spirometra* and *Sparganum* based on the data
379 set of cox1. The numbers along branches indicate posterior probabilities. The ESS value for all
380 parameters were above 200. Sequences ID are the same as Figure 5.

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