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Research paper

Echinococcus oligarthrus in the subtropical region of Argentina: First integration of morphological and molecular analyses determines two distinct populations☆

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ABSTRACT

Echinococcosis is a parasitic zoonosis that is considered as a neglected disease by the World Health Organization. The species *Echinococcus oligarthrus* is one of the causative agents of Neotropical echinococcosis, which is a poorly understood disease that requires a complex medical examination, may threaten human life, and is frequently associated with a low socioeconomic status. Morphological and genetic diversity in *E. oligarthrus* remains unknown. The aim of this work is to identify and characterize *E. oligarthrus* infections in sylvatic animals from the Upper Paraná Atlantic Forest in the province of Misiones, Argentina, by following an integrative approach that links morphological, genetic and ecological aspects. This study demonstrates, for the first time, one of the complete life cycles of *E. oligarthrus* in an important ecoregion. The Upper Paraná Atlantic Forest constitutes the largest remnant continuous forest of the Atlantic Forest, representing 7% of the world's biodiversity. This is the first molecular determination of *E. oligarthrus* in Argentina. In addition, the agouti (*Dasyprocta azarae*), the ocelot (*Leopardus pardalis*) and the puma (*Puma concolor*) were identified as sylvatic hosts of Neotropical echinococcosis caused by *E. oligarthrus*. Mitochondrial and nuclear molecular marker analyses showed a high genetic diversity in *E. oligarthrus*. Moreover, the genetic distance found among *E. oligarthrus* isolates is higher than the one observed among *Echinococcus granulosus* genotypes, which clearly indicates that there are at least two different *E. oligarthrus* populations in Argentina. This study provides valuable information to understand the underlying conditions that favour the maintenance of *E. oligarthrus* in sylvatic cycles and to evaluate its zoonotic significance for devising preventive measures for human and animal wellbeing.

1. Introduction

Echinococcosis is a parasitic zoonosis characterized by the development of a larval tapeworm stage (metacestode) in herbivorous intermediate hosts, such as rodents and ungulates, and accidentally in humans. The adult tapeworm inhabits the small intestine of canids or felids, the definitive hosts. Infections occur in intermediate hosts when they ingest eggs that have been passed in the faeces of definitive hosts. The genus *Echinococcus* includes the species *Echinococcus granulosus* sensu lato, *Echinococcus multilocularis*, *Echinococcus shiquicus*, *Echinococcus vogeli* and *Echinococcus oligarthrus* (Nakao et al., 2013). The neotropical species *E. oligarthrus* was discovering two centuries ago in a puma (*Puma concolor*) from Brazil (Diesing, 1863). Nevertheless, its formal taxonomic classification did not come until other researcher works which contributed to include this species into *Echinococcus* genera (Cameron, 1926; Lühe, 1910) and also to clarify misclassification cases (Schantz and Colli, 1973). In Argentina, the first report in definitive hosts was in colocolo (*Leopardus colocolo*) (Schantz and Colli, 1973). Until then, seven South and Central American felids were considered to be definitive hosts of *E. oligarthrus*: jaguar (*Panthera*

☆ All sequences reported in this work are available at GenBank No. KX129801-KX129816.

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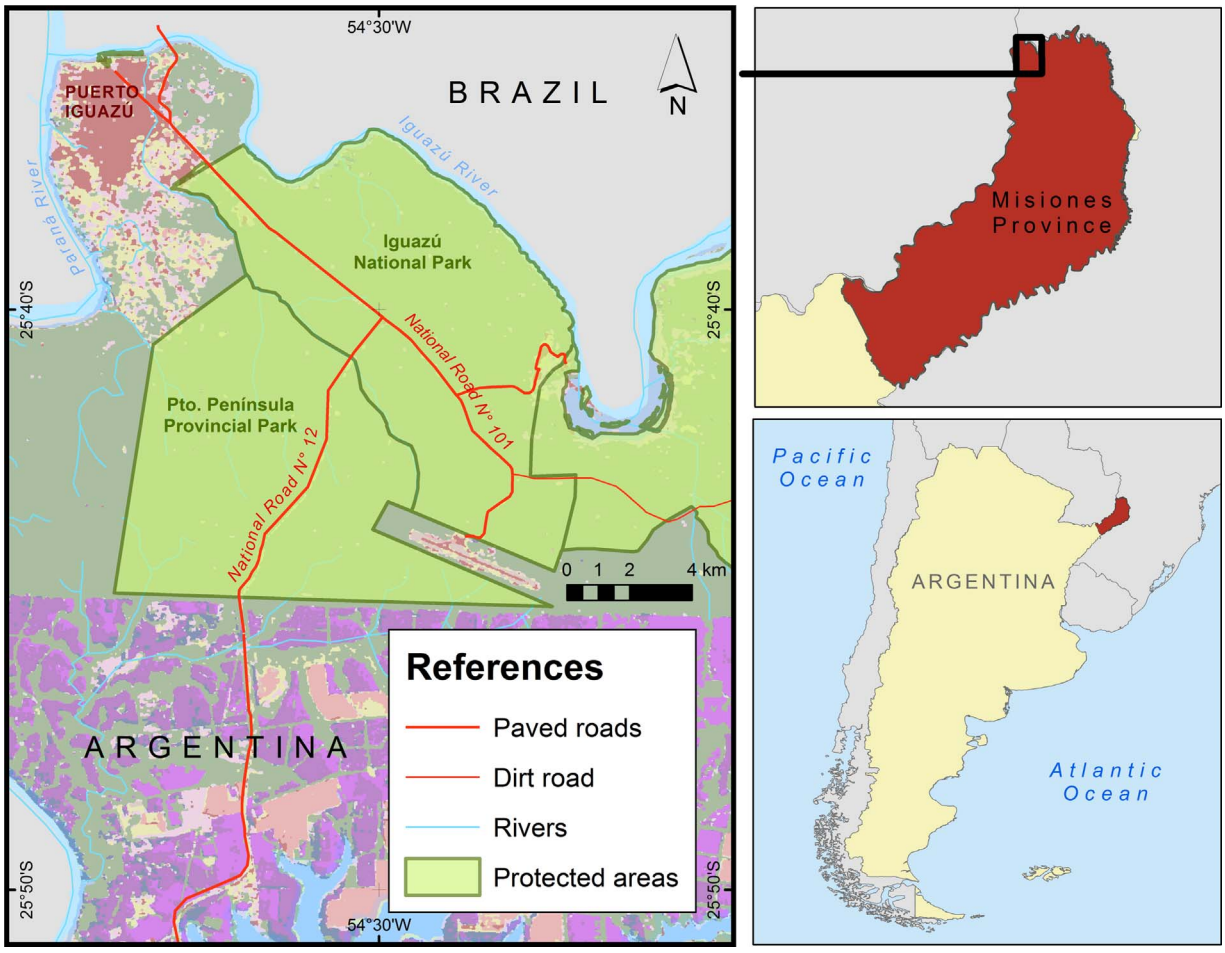

Fig. 1. Map of study area. Road-killed animals were collected from *Iguazú National Park* (INP) area which is crossed by the National routes 101 and 12 (red lines) and is placed 7 km away from the Puerto Iguazú city in the North of Misiones province, Argentina. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

onca), puma (*Puma concolor*), jaguarondi (*Puma yagouaroundi*), ocelot (*Leopardus pardalis*), geoffroy's cat (*Leopardus geoffroyi*), colocolo (*Leopardus colocolo*) and bobcat (*Lynx rufus*) (D'Alessandro and Rausch, 2008) which represent almost half of the species of felines that inhabit the Americas. Particularly in La Pampa and Rio Negro provinces from Argentina, *E. oligarthrus* was found in the small felids *Leopardus colocolo* and *Leopardus geoffroyi,* respectively (Schantz and Colli, 1973; Szidat, 1967). Regarding intermediate hosts, in Argentina it was suggested that southern mountain cavy (*Microcavia australis*) could be the intermediate host of *E. oligarthrus*, but such determination could not be confirmed. Moreover, the three species of sylvatic rodents agouti (*Dasyprocta* spp.), spiny rats (*Proechimys spp*.) and paca (*Cuniculus paca*) (D'Alessandro and Rausch, 2008) described as intermediate hosts in South America have not been described in Argentina, until now. Other intermediate host have been reported in South America; the lagomorph eastern cottontail (*Sylvilagus floridanus*) (Meléndez et al., 1984) and the marsupial opossum (*Didelphys marsupialis*) (Howells et al., 1978) in Venezuela and Colombia, respectively. Human infections seem to be described in the early 1900 (Viñas, 1903) in the Buenos Aires province of Argentina. Unfortunately, no morphological studies confirmed *Echinococcus* species (Tappe et al., 2008). Concerning human infections by *E. oligarthrus* in South America, at the present, only four cases have been reported from Suriname, Brazil, and Venezuela (Lopera et al.,1989; Basset et al., 1998; D'Alessandro and Rausch, 2008; Soares et al., 2013). In two of the cases, the disease demonstrated intra orbital tropism, causing loss of vision. Cysts were also found in heart and liver localization (D'Alessandro and Rausch, 2008). The life and the survival of affected people could be compromised by the apparent tropism of the cysts for the organs mentioned. Although the number of cases reported is low. Most of humans exposed to this disease are those living in natural areas or interface areas, as rural farmers or indigenous peoples (Noya-Alarcón et al., 2011). Underestimation of cases is usual in these groups since their limited access to the health system. Large phylogeny studies of *Echinococcus* genus based on nuclear sequences (Knapp et al., 2011; Nakao et al., 2013) and on mitochondrial genes (Nakao et al., 2007, 2013; Saarma et al., 2009) placed the neotropical *E. oligarthrus* and *E. vogeli* as basal species. Genetic diversity in *E. oligarthrus* remains unknown, since only a few isolates have been available for analyses. The few cases reported, mainly found occasionally, indicates that the life cycle is complex and not all the host species involved have been discovered. Sylvatic felids are the evident definitive hosts; however the number of different species acting as definitive and intermediate hosts could be higher than we know today. The Atlantic Forest is one of the most diverse ecosystems containing 7% of the world's species. In the province of Misiones, Argentina this diversity is maintained in the largest continuous forest remnant in the Upper Parana Atlantic Forest (UPAF), which still have 50% of the original cover of the country (Di Bitetti et al., 2003). Thus, UPAF is the region with major biodiversity in the country, which have the complete assembly of native mammals (Di Bitetti et al., 2003). An important aspect is that in the interface region adjacent to the natural area human population settlements are present a fact that contributes in maintaining the frequent interaction between domestic and sylvatic animals. Among the different genus of mammals found in UPAF region all of them have been described as hosts for *E. oligarthrus* (D'Alessandro et al., 1981; Di Bitetti et al., 2003). The aim of this work is to identify and characterize *E. oligarthrus* infections in

sylvatic animals from the UPAF, Misiones, Argentina through an integrative approach that link morphological, genetic and ecological aspects. We also attempt to understand underpinning conditions that favour *E. oligarthrus* maintenance in sylvatic cycles and to evaluate its zoonotic significance in interface areas to guide prevention measurements for human and animal wellbeing.

2. Materials and methods

2.1. Study area

The study area involved the National routes 101 and 12 from *Iguazú National Park* (INP) which is placed 7 km away from the Puerto Iguazú city in the North of Misiones province, Argentina (Fig. 1). The INP is placed at 24°41′.79″S and 54°26′55.27″W and it is included into the UPAF eco-region. 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

We actively searched for road-killed animals in the INP routes from 2015 to 2016. Animal necropsies were carried out under approved protocols by the National Parks Administration technical office. Only animals with an estimated period of discovering carcasses of 1–2 days were selected for sampling. Twenty five animals collected were analysed in this work and are summarized in Supplementary Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019. Each animal was individually packed and labelled with relevant information including place of origin, sampling date, age, 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). Each sample was individually packed and labelled with relevant information, including place of origin, sampling date, age, and sex of the animal. All samples were kept at −20 °C for at least 1 month prior to processing in order to inactivate *Echinococcus* spp. eggs. Examination of the intestinal content was performed using the sedimentation and counting technique described by Eckert, 2001 with some modifications. The small intestine was separated from the large intestine, and then each section of intestine was placed in different trays and cut lengthwise. Coarse material and large parasites of the small intestine were removed. Then, each intestine section was immersed in 9% saline solution at 37 °C for 30 min. Intestinal walls were scraped with a microscope slide, and all the intestinal 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. Intermediate hosts were thoroughly examined for cysts in all organs, tissues and cavities and with special attention to the locations described in the bibliography including ocular orbit (which was only described in human cases). Detected cyst were removed from infected organs, counted and the tissues were fixed and preserved in fresh, 10% formalin or ethanol 70% for further analysis.

2.4. Morphology studies

Adult worms and protoscoleces were analysed under optical Primo Star (Carl Zeiss Gmbh, Göttingen, Germany) microscope using Axion Cam ERc 5s camera (Carl Zeiss Gmbh, Göttingen, Germany). Each sample was registered with 4×, 10× and 40× using Carl Zeiss Vision software for image analysis. For adults, number of proglotids and total length were registered. The hooks from protoscoleces were visualized under optical microscope, took a picture at 100X and hooks morphometric technique was implemented as in Rausch et al. (1978). Statistical analysis was made for all the parameters, blade and handle lengths for both, large and small hooks. Student *t*-test was performed for independent samples in order to determine whether the means of the measurements of the hooks from the two agoutis' samples obtained differ. Cyst 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), Periodic acid–Schiff (PAS), Giemsa (G) and Ziehl-Neelsen (Z–N). The slides were analysed under optical microscope and picture was taken at 4×, 10× and 40×.

2.5. Genetic analysis

Total parasite genomic DNA was prepared from fresh or 70% ethanol preserved isolates using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). Five molecular markers were used to determine *Echinococcus* species. Two were from nuclear genome, exon region and intron region from ezrin-radixin-moesin-like protein (*elp*) gene (Knapp et al., 2011) and three were from mitochondrial genome, cytochrome oxidase subunit I (*cox1*) gene (Cucher et al., 2013), NADH dehydrogenase subunit 1 (*nad1*) gene (Bowles et al., 1995) and ATP synthase subunit 6 (*atp6*) gene (Yang et al., 2005). The selection criteria were the existence of already described sequence in *Echinococcus oligarthrus* and/or have more than 10 single nucleotide polymorphisms (SNPs) within *Echinococcus* species. The genomic region, primer sequence and product length are shown in Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019. The amplification reaction was done as described previously (Kamenetzky et al., 2000). Briefly, The PCR was performed in a final 50 µl volume containing sample DNA (10–50 ng), 200 µM each dNTP (Pharmacia LKB, Uppsala, Sweden), 2.5 mM $MgCl_2$, 50 pmol of each primer (sequences are shown in Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019) and 1.5 U of GoTaq DNA polymerase in reaction buffer (Promega, Madison, WI). The PCR conditions were as follows: an initial denaturing step (95 °C for 180 s) followed by 30 cycles of 95 °C for 60 s (denaturation), 55 °C for 60 s (annealing), 72 °C for 90 s (extension), and a final extension step (72 °C for 180 s). For each isolate and molecular marker employed the PCR-product obtained was sequenced and aligned with ClustalX (v2.0.12) with all *Echinococcus* sequences available on GenBank. Multiple alignments were edited with BioEdit (v7.1.3). Phylogenetic analyses of individual (cox1) and concatenated markers were performed using Bayesian method implemented in MrBayes 3.1.2 (Ronquist et al., 2011). The evolutionary model was set on generalised time reversible substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + G + I model) with at least 200 samples from the posterior probability distribution, and diagnostics calculated every 1000 generations. We use an exponential prior on the branch length with mean = 0.1 substitutions/site. All available sequence data from *Echinococcus* spp. were included in the analysis. The cox1 data resulted in 345 bp length for 19 taxa. The concatenated data resulted in 1965 bp length for 16 taxa.

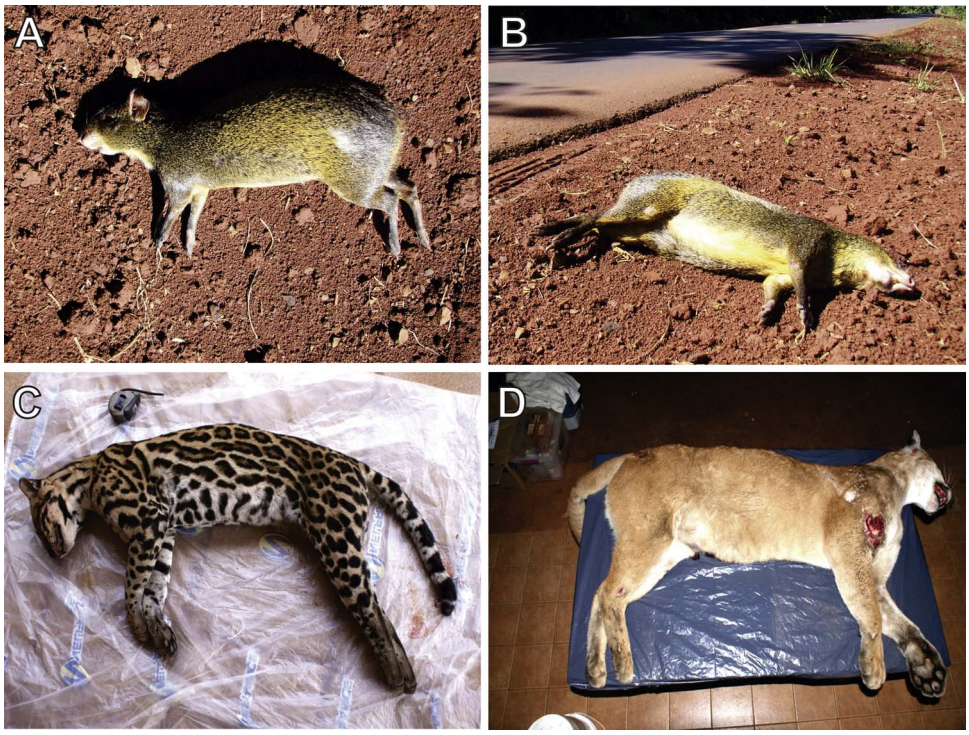

Fig. 2. Road-killed animals with *Echinococcus oligarthrus* infections. (A) and (B) Adults females of Agouti (*Dasyprocta asarae*), intermediate host of E. oligarthrus.; (C) Adult male of Ocelot (*Leopardus pardalis*); (D) Adult male of puma (*Puma concolor*), both felids are definitive hosts of E. oligarthrus.

3. Results

3.1. Morphological evidence of Echinococcus oligarthrus

Morphological studies allowed the detection of the *E. oligarthrus* in four animals: two adult female agoutis (*Dasyprocta azarae*), one adult male ocelot (*Leopardus pardalis*) and one adult male puma (*Puma concolor*) (Fig. 2, Supplementary Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019). Multiple metazoan parasitic unilocular polycystic hydatid cysts were identified during the necropsy performed on the agoutis. Most of the cysts were intact but some of them were collapsed. Abundant viable protoscoleces were found when hydatid fluid was aspired. Twenty-five cysts were observed in the first agouti (DAMi1), the affected organs being liver, lung, kidney, spleen and heart. The second agouti (DAMi2) had seven cysts: six in the liver and one in the heart. Cysts in liver tissue were generally ovoid and measured 5–10 mm in diameter. Cysts in the spleen were similar however they were up to 20 mm in diameter, thereby causing organ dystrophy. Heart and kidney cysts were 10 mm in diameter. Multiple small cysts of up to 1 mm were found in the lung (Fig. 3). Protoscoleces and cyst tissue from DAMi2 were further analysed. Protoscoleces were ovoid, the small diameter ranging from 73.1 to 111.9 µm ($\bar{X}$ = 91.2 ± 8.8) and the large diameter ranging from 91.7 to 150.3 µm ($\bar{X}$ = 125.7 ± 12.5). The histological analyses are presented in Fig. 4 The pictures show structures that are compatible with the metacestode of cystic echinococcosis (Fig. 4A–H). The sections that were stained with Giemsa and H & E clearly present a more outer adventitial layer followed by a more internal laminar layer (Fig. 4C and G). The laminar layer is also clearly identified by PAS staining as a complex thick layer composed of wavy thin layers (Fig. 4E). Towards the centre of the cyst follows the germinal layer which is connected to the brood capsules (Fig. 4C–G). The nuclei of this layer were also clearly seen with Giemsa and H & E staining techniques. Almost all of the protoscoleces were invaginated and connected to the germinal layer of the brood capsule by a stalk. Protoscoleces were identified based on several structures, such as suckers, hooks and calcareous corpuscles. Two types of hooks were observed, small and large, which were positive for Giemsa and Z–N staining. The general morphology of the hooks was typical of *E. oligarthrus* being large with linear handle and the blade length representing a bit more than 50% of the total hook length (Fig. 5H). To classify the agoutis' cysts in an accurate manner, the size and dimension of the hooks were carefully analysed (Table 1).The total length of the hooks in both agoutis showed no significant differences ($p > 0.1$). Blade/total length (BL) and handle/total length (HL) proportions were also similar: in agouti 1, 56.88% (BL) and 43.11% (HL) for large hooks, and 53.6% (BL) and 46.39% (HL) for small hooks; in agouti 2, 57.37% (BL) and 42.60% (HL) for large hooks, and 51.16% (BL) and 48.8% (HL) for small hooks. These values are consistent with the values obtained by other authors for *E. oligarthrus* identification (D'Alessandro et al., 1995; Rausch et al., 1978; Rodríguez et al., 2000).

Both felids, the ocelot (LPMi) and the puma (PCMi), were found to contain hundreds of worms, including some gravid specimens. Worms consisted of a scolex, one or two anterior immature segments and a posterior gravid segment (Fig. 5). A total of fifty-five and forty adult worms from the ocelot and the puma showed similar measures of median total length (2.2–2.5 mm) and range (min. 1.55, max. 3.95–3.98 mm), respectively (Table 2). These parasite populations were identified as *E. oligarthrus* based on their presence in felid hosts and on the morphological criteria described previously (Salinas-López et al., 1996).

3.2. Genetic molecular marker analysis

Larval and adult parasites in each host were analysed by five molecular markers. The samples were selected as follows: one liver cyst from agouti DAMi1, one liver cyst from agouti DAMi2, five adult worms from ocelot LPMi, and three adult worms from puma PCMi. The PCR product sequencing confirmed the presence of *E. oligarthrus* in the UPAF, Misiones, Argentina. Interestingly, cox1 sequences from the two agouti cysts analysed have 100% identity with the sequence reported by Soares et al. (2013) in a human patient from Brazil (GenBank access No. JN367278). Additionally, all the ocelot worms analysed have 100%

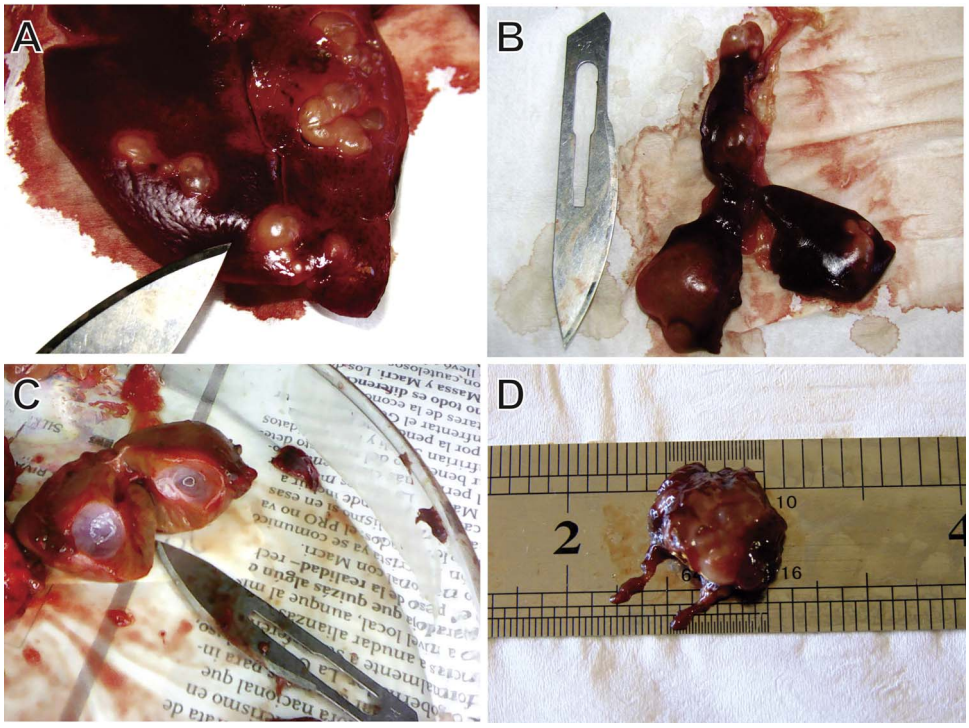

Fig. 3. Hydatid cysts from agoutis *Dasyprocta asarae*. (A) Liver with multiple cysts distributed in the liver parenchyma; (B) Atrophied spleen by the number and size of cysts; (C) Dissected kidney, can be seen that a single cyst occupies two thirds of the organ; (D) Isolated hepatic cyst with 10 mm diameter.

identity with parasite samples from agoutis in the four molecular markers examined (Table 3, GenBank access No. KX129801-KX129816). Therefore, our results show for the first time, the existence of one of the complete life cycles of *E. oligarthrus* in the region. Furthermore, cox1 sequences from puma isolates are 93.9% (328/349) identical to the sequences obtained from ocelot-agoutis isolates, and 96.8% (338/349) identical to cox1 sequences from the complete *E. oligarthrus* mitochondrial genome reported in a *Mus musculus* laboratory host from Panama (GenBank no. AB208545, Nakao et al., 2007), which demonstrates that *E. oligarthrus* is not a homogeneous population in Argentina. All of the adult worms that were isolated from the puma have 100% identity with the three molecular markers employed (Supplementary Figs. S1 and S2 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019). Phylogenetic analyses of the five molecular markers used confirmed that there are two *E. oligarthrus* populations in Argentina, one in agouti-ocelot hosts and the other one in puma host. The phylogenetic topology obtained with cox1 sequences is shown in Fig. 6 which is consistent with previously phylogenetic analyses (Nakao et al., 2013) placing *E. oligarthrus* and *E. vogeli* as basal species from *Echinococcus* genus. The same phylogenetic topology was observed when all of the sequences from the molecular markers employed in this work where concatenated (Supplementary Fig. S3 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019). The concatenated data matrix of the five gene fragments was ~80% complete. There were no missing data in the cox1 and atp6 matrices. Missing data resulted from molecular markers that

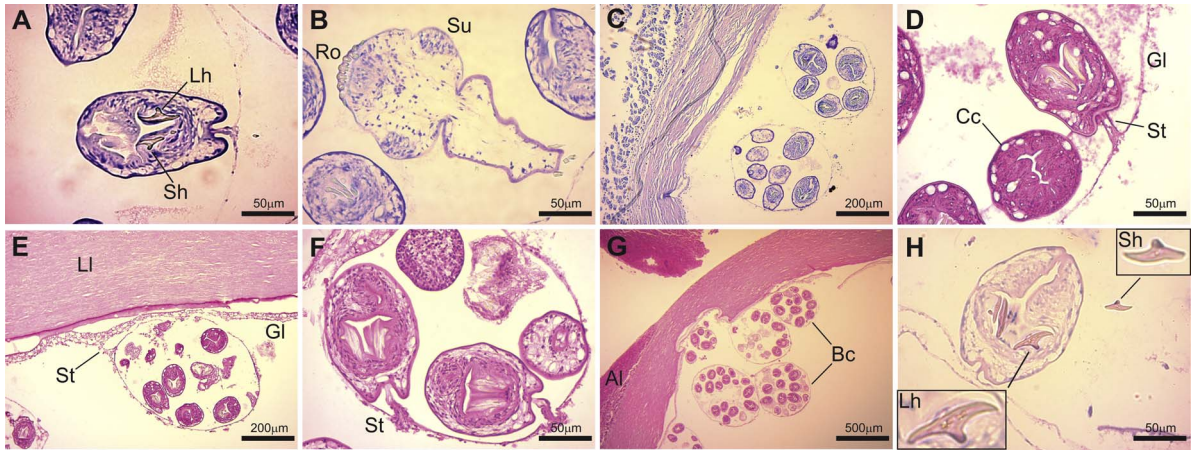

Fig. 4. Histological sections of different parasitic structures stained with different histological techniques viewed under light microscopy. The images show structures compatible with the metacestode stage of *Echinococcus oligarthrus*. (A) Invaginated protoscolex and associated germinal layer, Giemsa stained. Size bar: 50 µm; (B) Evaginated protoscoleces, Giemsa stained. Size bar: 50 µm; (C) Two brood capsules with invaginated protoscoleces and associated membranes, Giemsa stained. Size bar: 200 µm; (D) Two invaginated protoscoleces and associated germinal layer, Periodic Acid-Schiff (PAS) stained. Size bar: 50 µm; (E) Brood capsule and associated membranes, PAS stained. Size bar: 200 µm; (F) Brood capsule, Haematoxylin and Eosin (H & E) stained. Size bar: 50 µm; (G) Brood capsules and associated membranes, H & E stained. Size bar: 500 µm; (H) Invaginated protoscolex and associated germinal layer, Ziehl Neelsen stained. Size bar: 50 µm. Inset: large hook (Lh) and small hook (Sh) viewed under higher magnification. Abbreviations: Al, Adventicial layer; Bc, brood capsule; Cc, calcareous corpuscles; Gl, germinal layer; Lh, large hook; Ll, laminar layer; Ro, rostellum; Sh, small hook; St, stalk; Su, sucker.

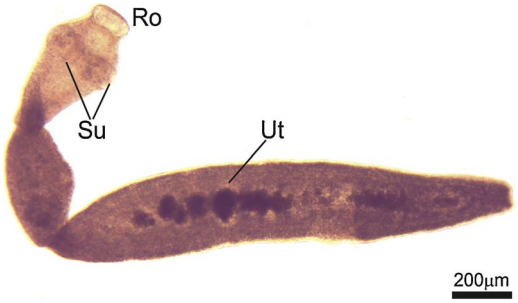

Fig. 5. Micrograph of *Echinococcus oligarthrus* adult worm obtained from the small intestine of the ocelot (*Leopardus pardalis*). Ro, rostellum; Su, sucker; Ut, uterus.Size bar: 200 µm.

could not be amplified from PCMi or LPMi isolates (Table 3), probably due to the low quantity of DNA. However, polymorphism between the target sequences and the primer annealing regions cannot be ruled out. Interestingly, the genetic distance obtained when analysing cox1 sequences in *E. oligarthrus* was higher (up to one order) than the genetic distance in *E. granulosus sensu stricto* genotypes (Supplementary Table S3 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.03.019). Nevertheless, more samples have to be analysed to confirm these results.

4. Discussion

This study represents the first molecular evidence of *E. oligarthrus* in Argentina. In addition, the integration of molecular, morphological and ecological analyses allowed us to describe, for the first time, one of the complete life cycles of *E. oligarthrus* in the rainforest region of the province of Misiones. *E. oligarthrus* was frequently found in key species such as ocelot, puma, jaguar and jaguarondi as definitive hosts, and in agouti, spiny rat and paca as intermediate hosts in Central and South America (D'Alessandro and Rausch, 2008). Furthermore, in the Americas six species of carnivores seem to be refractory to *E. oligarthrus* infections, namely *Leopardus tigrinus, Leopardus guttulus* (southern tiger cat), *Leopardus wiedii* (margay), *Leopardus jacobitus* (Andean mountain cat), *Leopardus guigna* (kodkod) and *Lynx canadensis* (Canada lynx). In the south region of Argentina, morphological studies evidenced *E. oligarthrus* in small felids such as *Leopardus geoffroyi* and *Leopardus colocolo* (Schantz and Colli, 1973). In this work, we enlarge *E. oligarthrus* distribution and add two felids to the country list of definitive hosts: *Leopardus pardalis* and *Puma concolor*. Regarding intermediate hosts, the occurrence of metacestodes of *E. oligarthrus* has been well established in the *Dasyproctae* gender described in the neotropics. Species of this gender are considered to be the usual intermediate host of the parasite (D'Alessandro, 1997). Therefore, this work evidenced, for the first time, the infection caused by *E. oligarthrus* in the *Dasyprocta azarae* species, adding a new species of sylvatic rodent as intermediate host to the country. In Argentina, beyond the geographic range of agoutis and pacas, other species of rodents must serve as intermediate hosts of *E. oligarthrus*, which may be involved in the infections of small felids (*L. geoffroyi* and *L. colocolo*) (Schantz and Colli, 1973). Thus, more intermediate host species need to be analysed in the UPAF region to appropriately identify the range of intermediate hosts involved in the transmission of *E. oligarthrus*. Hydatid cysts cause dysfunction of the affected organs in intermediate hosts, leading to a general weakening that hinders their ability to escape from predators. Therefore, this parasite/host interaction process could modulate population growth by affecting the lifespan of the affected animals or by exposing them to be preyed on since this situation ensures the perpetuation of the parasite in nature. Therefore, increasing our knowledge about intermediate host spectra will shed light on crucial aspects of *E. oligarthrus* life cycle through the trophic interactions that are occurring in this natural area. In our analysis of agoutis' parasites, we found morphological characteristics similar to those previously described for *E. oligarthrus* (D'Alessandro and Rausch, 2008; Rausch et al., 1978; Rodríguez et al., 2000; Zimmerman et al., 2009). In this work we found cysts in liver and internal organs from agoutis whereas previous findings of *E. oligarthrus* from Brazilian agoutis indicated that most of the cysts were located subcutaneously (Zimmerman et al., 2009). Regarding histological analyses, all the parameters evaluated were consistent with previous findings (Rodríguez et al., 2000; Zimmerman et al., 2009).Considering these features and the nature of the intermediate host, the data provides evidence that the hydatid cysts arose from the *E. oligarthrus* species. Furthermore, an ecological and morphological comparison with previous reports indicates that the adult worms that were found in the ocelot and in the puma also belonged to the *E. oligarthrus* species (Madrigal and Herrera, 1973; Mendes and Vasconcellos, 1987; Salinas-López et al., 1996; Schantz and Colli, 1973).

In regard to genetic information, nothing is known about the genetic diversity of *E. oligarthrus*. Until this study, only two isolates had been analysed by sequencing: one from Panama (Nakao et al., 2013) and one from Brazil (Soares et al., 2013). The genetic sequences obtained from the new isolates analysed in this work form a node that clusters all the *E. oligarthrus* samples. Furthermore, by using molecular techniques we

Table 2
Total lengths (in millimetres) of adult worms from *E. oligarthrus* obtained from sylvatic felids.

Host	Origin	n	$\bar{X}$	SD	Min	Max
Ocelot	Misiones, Argentina	55	2.2	0.53	1.55	3.98
Puma	Misiones, Argentina	40	2.5	0.65	1.55	3.95

Table 3
Molecular marker alleles obtained for *Echinococcus oligarthrus* isolates.

Host	Sample ID	number of isolates	Molecular marker[a]				
			COX1	ATP6	NAD1	ELP	E10
Agouti	DAMi	2	cox1_1	atp6_1	nad1_1	elp_1	E10_1
Ocelot	LPMi	5	cox1_1	atp6_1	nad1_1	–	E10_1
Puma	PCMi	3	cox1_2	atp6_2	–	elp_2	–

[a] numbers before molecular marker ID means different allele sequences.

Table 1
Lengths (in micrometers) of rostellar hooks from protoscoleces of *Echinococcus oligarthrus* from agouties.

	Sample	Total length				Blade					Handle				
		Min	Max	$\bar{X}$	SD	Min	Max	$\bar{X}$	SD	BL(%)	Min	Max	$\bar{X}$	SD	HL(%)
Agouti (DAMi 1)	Large hooks (N = 31)	35.09	45.01	37.02	2.58	20.39	27.37	21.06	2.54	56.88	14.7	17.64	15.96	1.61	43.11
	Small hooks (N = 22)	30.04	34.96	33	1.52	12.34	22.08	17.69	2.42	53.6	12.88	17.7	15.31	1.89	46.39
Agouti (DAMi 2)	Large hooks (N = 46)	38.21	43.05	41.36	1.03	22	26.9	23.7	1.01	57.37	15.2	19.67	17.6	1.02	42.6
	Small hooks (N = 38)	27.83	34.96	32.7	1.21	15	21.8	16.7	1.17	51.16	11.7	17.37	15.9	1.12	48.8

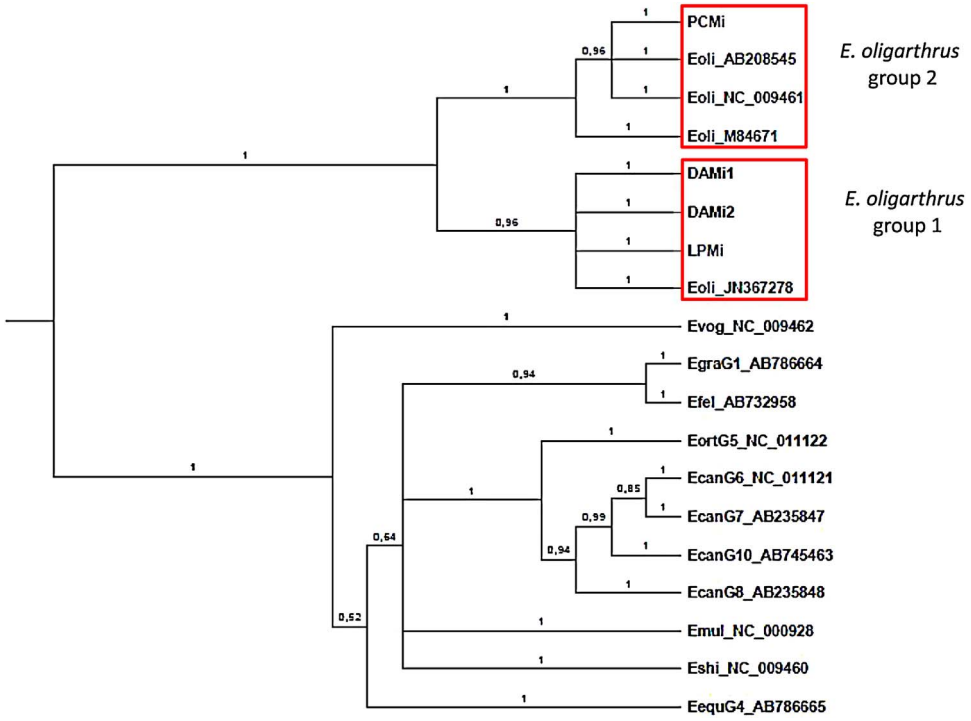

Fig. 6. Phylogenetic tree based on cox1 sequences from *Echinococcus oligarthrus*. The tree topology was inferred by Bayesian Phylogenetics implemented in MrBayes software (Ronquist et al., 2011). Posterior probabilities are shown at nodes. Each *E. oligarthrus* genetic groups are marked with red squares. All *Echinococcus* spp. cox1 sequences used as references are labelled with the Genbank number. Abbreviations: DAMi, agouti isolate; LPMi, ocelot isolate; PCMi, cougar isolate; Eoli_JN367278, Brazilian human isolate (Soares et al., 2013); AB208545, Panama mouse isolate (Nakao et al., 2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were able to distinguish that the isolates belong to different populations. In addition, the genetic distance observed in *E. oligarthrus* sequences from Argentina has higher levels than the one detected between the *E. granulosus sensu stricto* genotypes (Kamenetzky et al., 2002).The results obtained in this work show the advantage of employing molecular techniques to identify different populations of parasites that could not be detected at the morphological level. The fact that we found identical *E. oligarthrus* sequences in the ocelot and the agouti is consistent with the diet habits of the ocelot, which include agouti as its main rodent pray (Moreno et al., 2006). Furthermore a work conducted in the north of Misiones showed that agoutis make up most of the biomass (16.48%) in an ocelot's diet compared with other main preys, such as the white-and-black lizard (10.57%) and the akodon (9.96%) (Palacio et al., unpublished data). Nevertheless, the differences found in *E. oligarthrus* sequence from the puma isolates lead us to consider the wider range of prey preferences of this carnivore. Thus, as we stated above, more species included in a puma's diet (i.e., brocket deer and pacas) need to be analysed in order to assign them a specific role in the transmission cycle of neotropical echinococcosis. Based on our findings, we were able to describe one of the *E. oligarthrus* sylvatic cycle which is maintained in the Argentinian UPAF. The genetic studies conducted in this work showed that the *E. oligarthrus* life cycle is more complex than we knew and that it could be circumscribed to definitive and intermediate specific hosts, giving rise to genetically different populations of *E. oligarthrus*. Therefore, more isolates from different hosts need to be analysed with more molecular markers to confirm this results. Although there is plenty of information concerning gene sequences for other species of *Echinococcus* spp. that are present in Argentina (Cucher et al., 2016; Kamenetzky et al., 2002), nothing is known for *E. oligarthrus*. Understanding the genetic variation of parasitic organisms has great medical significance. Implementation of molecular marker analyses could help to determine these genetic variations (Thompson, 2008). Therefore, knowing specific genetic information could be relevant for the prevention, diagnosis and treatment of the disease.

Neotropical echinococcosis is a poorly understood disease and requires a complex medical examination to determine the appropriate type of intervention. Only four cases of echinococcosis that were unequivocally identified as being caused by *E. oligarthrus* have been reported. Notably, most humans exposed to Neotropical echinococcosis are those living in natural or interface areas, such as rural farmers or indigenous peoples (Noya-Alarcón et al., 2011). In these places, the total number of cases of this illness may be underestimated both because of limited access to the health system and the chronic course of the disease. This socio economic situation is a common scenario in several regions of Argentina. Particularly, in Misiones province, people carry out several activities within the natural habitat of wild felids, such as hunting and wood extraction (Di Bitetti et al., 2003). Consequently, they are exposed to the infected faeces of wild felines or to the consumption of undrinkable water. In addition, domestic cats living in interface regions may be considered as a source of Neotropical human echinococcosis. According to our observations, it is very usual for residents of interface regions to own domestic cats. That could promote a sylvatic-domestic cycle in which cats would be involved through their hunting of wild animals. Despite living with humans, domestic cats keep their hunting habits, thus being more vulnerable to acquire parasitic diseases. Interestingly, domestic cats have similar hunting habits and prey preferences to *Leopardus geoffroyi*, which is a natural host of *E. oligarthrus* (Schantz and Colli, 1973) and is also genetically related to domestic cats (Sunquist and Sunquist, 2002). It would be interesting to evaluate if domestic cats may be susceptible to natural infection by *E. oligarthrus*.

Finally, further analyses in neighbouring regions where the sylvatic cycle could be present may help understand the actual epidemiological situation of this neglected disease. The information provided in this work represents a valuable resource to gain a greater knowledge of Neotropical echinococcosis from an integrative approach that links morphological, molecular and ecological data. The analyses presented constitute pivotal tools to understand the eco-epidemiological aspects of this neglected disease, as well as to improve preventive measures in favour of human and animal health.

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