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Short communication

Molecular characterization of the ITS-2 fragment of *Paramphistomum leydeni* (Trematoda: Paramphistomidae)

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ABSTRACT

Paramphistomosis has shown an increased spread over the last years in Argentina, being in some regions an emerging parasitosis, which has motivated their study and identification. Even when morphological features were reported, molecular characterization appears as a complementary and reliable tool.

Samples of *Paramphistomum leydeni* collected since 2002 from different origin, definitive hosts, natural or experimental infections and preservation method were subjected to PCR-RFLP for ITS2+ fragment, and compared to *Fasciola hepatica* and Notocotilidae cercarie. Frozen and ethanol fixed samples amplified fragments of 500 bp for *P. leydeni* and Notocotilidae samples, while *F. hepatica* eggs produced a 540 bp amplicon. Restriction fragments obtained from endonucleases *Hinf*I, *Hha*I, *BsuR*I, *Taq*I, and *Tru*I were identical for all *P. leydeni* samples, supporting the morphological classification previously performed. Four selected amplicons were sequenced and reported at GenBank, given a consensus sequence ITS-2+ of 441 bp. This first report of molecular characterization for *P. leydeni*, improves the current knowledge of the genus and establishes precedents for further specimen classifications.

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1. Introduction

Paramphistomosis, a worldwide parasitic disease affecting cattle and other ruminants, is produced by several amphistome species that can cause production losses and even mortality (Boray, 1959; Horak, 1971). Up to date, amphistome species causing paramphistomosis in Argentina were classified as *Paramphistomum leydeni* (formerly *Cotylophoron cotylophorum*) (Sanabria et al., 2009) and *Balanorchis anastrophus* (Szidat and Ostrowski de Nuñez, 1962), using standard histological methods. *P. leydeni* is found most frequently, with increased distribution over the last ten years (Sanchez et al., 2005; Sanabria and Romero, 2008).

Since amphistome species can be morphologically similar, molecular biology techniques can be helpful for classification and species identification. Some amphistome genus and species were characterized using molecular methods; Itagaki et al. (2003) characterized three different genus by ribosomal DNA (rDNA) Internal Transcribed Spacer 2 (ITS-2), whereas Rinaldi et al. (2005) applied a similar technique using the ITS-2 of *Calicophoron daubneyi* from different definitive hosts. To the best of our knowledge, molecular characterization of *Paramphistomum* genus has not been described. Therefore, the aim of this study was to perform the ITS-2 characterization of *P. leydeni* using PCR-RFLP and sequencing.

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Table 1
Characteristics and origin of samples analyzed by PCR and RFLP.

Sample	Specimen	Specimen stage	Host	Infection source	Province	Geographic location	Collection date	Preservation
1	*P. leydeni*	Adult	Cattle	N	ER	32.55 S 59.12 W	January-04	Formaline
2	*P. leydeni*	Adult	Cattle	N	ER	30.38 S 58.75 W	September-06	Formaline
3	*P. leydeni*	Adult	Cattle	N	BA	35.26 S 58.20 W	September-08	Formaline
4	*P. leydeni**	Adult	Cattle	N	BA	34.05 S 54.05 W	January-02	Ethanol
5	*P. leydeni*	Adult	Cattle	N	BA	35.34 S 58.01 W	June-08	Formaline
6	*P. leydeni*	Adult	Cattle	N	BA	35.34 S 58.01 W	August-09	Frozen
7	*P. leydeni*	Adult	Cattle	N	BA	35.34 S 58.01 W	November-08	Frozen
8	*P. leydeni**	Adult	Cattle	N	BA	35.34 S 58.01 W	November-08	Ethanol
9	*P. leydeni**	Adult	Sheep	E	BA	35.34 S 58.01 W	November-09	Frozen
10	*P. leydeni**	Adult	Sheep	N	ER	32.55 S 59.12 W	December-09	Frozen
11	Notocotylidae	Cercariae	*Drepanotrema* sp.	N	ER	32.55 S 59.12 W	December-09	Ethanol
12	*F. hepatica*	Adult	Cattle	E	BA	35.34 S 58.01 W	May-08	Formaline
13	*F. hepatica*	eggs	Cattle	E	BA	35.34 S 58.01 W	May-08	Frozen

References: N: natural; E: experimental; ER: Entre Rios; BA: Buenos Aires.
* Sequenced samples submitted to GenBank.

2. Materials and method

P. leydeni adult flukes from field findings over the years 2002–2009 were classified according to their definitive host (sheep and cattle), origin (Buenos Aires or Entre Ríos provinces), source of infection (natural or experimental), and preservation method (frozen up to −20 °C, ethanol 70 °C or Formaline 5%). Notocotylidae cercariae, as well as eggs and adult flukes of *Fasciola hepatica* were also analyzed. Half of a *P. leydeni* or *F. hepatica* adult fluke, 10 Notocotylidae cercariae and approximately 100 *F. hepatica* eggs were used for DNA extraction (Table 1). Adult flukes of *P. leydeni* were previously identified using standard procedures (Sanabria et al., 2009).

DNA extraction was performed using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. The lysis step using proteinase K was performed overnight. The ribosomal DNA (rDNA) ITS-2 plus flanking 5.8S and 28S partial sequences (ITS-2+) were amplified, using the primers ITS-2 F (5′TGTGTCGATGAAGAGCGCAG-3′) and ITS-2 R (5′TGGTTAGTTTCTTTTCCTCCGC-3′), as reported previously (Itagaki et al., 2003; Rinaldi et al., 2005), with slight differences in annealing (55 °C for 1 min). PCR products were evidenced by electrophoresis in 1% agarose gels, stained with SYBRsafe (Invitrogen), and observed in a blue transilluminator (Safe Imager, Invitrogen). A 100 bp ladder (Biodynamics) was used as standard marker.

Amplified products were individually cut with endonucleases *Bsh1285*I, *Hinf*I, *Hha*I, *Rsa*I, *BsuR*I, *Taq*I, and *Tru1*I (Fermentas Life Science, EU) using buffer solutions as indicated by the manufacturer's instructions. Briefly, reactions were performed using 5 μl of each amplicon and 0.3 μl of enzyme. Incubation was done in a dry bath, for 1 h at 65 °C for enzymes *Taq*I, and *Tru1*I, and 1 h at 37 °C for all others. Restriction products were identified using 2.5% agarose gels, and revealed as described previously, using a 100 bp ladder (Biodynamics).

Four positive samples from different geographic locations and hosts were amplified under the same conditions in 50 μl final volume. Products were purified using QIAquick® PCR purification Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol and submitted for sequencing to CIGEBA Institute (Faculty of Veterinary Sciences, National University of La Plata). Sequencing was performed in forward and reverse directions using DYEnamic sequenciator (GE Healthcare). Sequences were analyzed with CHROMAS Lite 2.01 software (Technelysium Pty Ltd.) and ClustalX 2.0.11 (http://www.clustal.org).

3. Results

Six *P. leydeni* samples and Notocotylidae cercariae produced ~500 bp fragments for the ITS-2+, while *F. hepatica* eggs produced a ~540 bp amplicon. Formalin preserved adult flukes of *F. hepatica* and *P. leydeni* were negative. Restriction products from *P. leydeni* samples were obtained by endonucleases *Hinf*I, *Hha*I, *BsuR*I, *Taq*I and *Tru*I. In comparison, *F. hepatica* restriction fragments were given only by enzymes *Hinf*I, *Rsa*I, and *Tru*I, whereas Notocotylidae was restricted by the same enzymes as *P. leydeni*, and by *Rsa*I as well (Figs. 1 and 2). All *P. leydeni* samples had an identical restriction pattern. Approximate molecular weight of fragments produced for each endonuclease is shown in Table 2. All analyzed sequences were identical and subsequently compared with others available using online BLAST (NCBI), and registered on GenBank® under accession numbers HM209064, HM209065, HM209066 and HM209067 for samples 4, 8, 9, and 10 respectively (Table 1). Thus, considering all sequenced samples in both forward and reverse directions, a consensus sequence of 441 bp was obtained. This sequence contains an ITS-2 of 282 bp plus flanking 5.8S rRNA (107 bp) and 28S rRNA (52 bp) partial sequences (Fig. 3).

4. Discussion and conclusions

As mentioned by others, characterization of ITS-2 seems to be an accurate tool for identification of amphistomes and could even be useful for other digenea (Itagaki et al., 2003; Rinaldi et al., 2005; Goswami et al., 2009). Amplified prod-

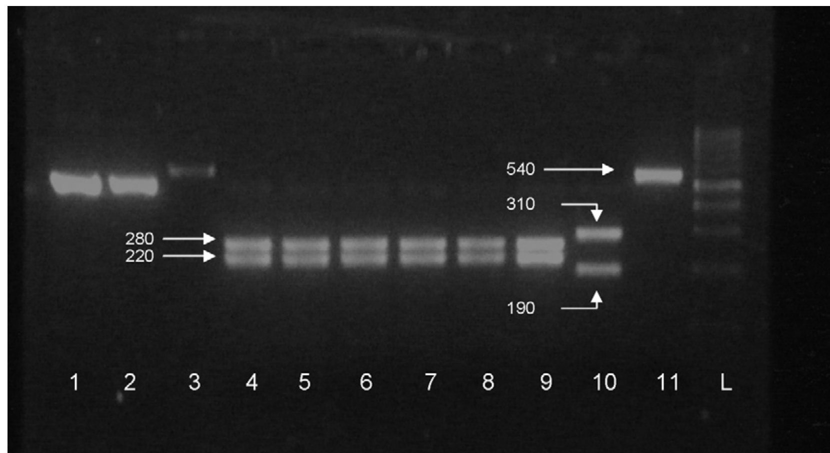

Fig. 1. Restriction pattern of ITS-2+ from *P. leydeni* using *Hha*I endonuclease. From left to right: undigested *P. leydeni*, Notocotylidae cercariae, and *F. hepatica* eggs (1, 2, 3), restriction fragments of *P. leydeni* from cattle (4, 5, 6, 7), and sheep (8, 9), Notocotylidae (10) and *F. hepatica* (11) L: 100 bp ladder. White arrows show approximate fragments' size.

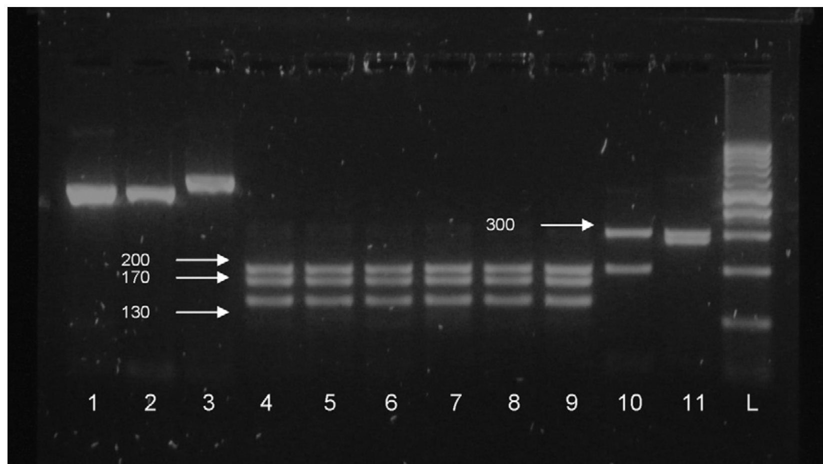

Fig. 2. Restriction pattern of ITS-2+ from *P. leydeni* using *Hinf*I endonuclease. From left to right: undigested *P. leydeni*, Notocotylidae cercariae, and *F. hepatica* eggs (1, 2, 3), restriction fragments of *P. leydeni* from cattle (4, 5, 6, 7), and sheep (8, 9), Notocotylidae (10) and *F. hepatica* (11) L: 100 bp ladder. White arrows show approximate fragments' size.

Table 2
RFLP results for each enzyme showing approximated size (bp) and number of fragments.

	*Bsh1285*I	*Hinf*I	*Hha*I	*Rsa*I	*BsuR*I	*Taq*I	*Tru1*I
P. leydeni	–	130; 170; 200	220; 280	–	100; 400	40; 460	60; 150; 290
Notocotylidae	–	200; 300	190; 310	100; 150; 250	100; 400	40; 190; 270	120–380
F. hepatica	–	270; 270	–	100; 140; 300	–	–	260–280

CAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACATCGACATCT
TGAACGCACATTGCGGCCACGGGTTTTCCTGTGGCCACGCCTGTCCGAGG
GTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGAATCTG
CCAGCTGGCGTGATCTCCTCTGTGGTTCGCCACGTGAGGTGCCAGATCTA
TGGCGTTTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCGCA
GACGAGGGTGTGGCGGTAGAGTCGTGGCTCAGTTAACTGTAATGGCAGCA
CGCTCTACTGTTGTGCCTTTGTTAGTGTAACTGGTTTGAGATGCTATTGC
TGTCCGTCCGATCATGATCACCTACTGTGGTGTTCTGCT ACCTGACCTCG
GATCAGACGTGAATACCCGCTGAACTTAAGCATATCACTAA

Fig. 3. Consensus sequence of *P. leydeni* ITS-2 (underlined characters) flanked by 5.8S rDNA (before underline) and 28S rDNA (after underline).

ucts from *P. leydeni* show no intraspecific differences when digested by RFLP, however *F. hepatica* eggs and Notocotylidae cercariae, presented different restriction patterns. The same results were obtained by Rinaldi et al. (2005) for *C. daubneyi* ITS-2+ characterization. The lack of variation among samples seems to support our previous hypothesis that *P. leydeni* affects sheep and cattle in Argentina. On the other hand, it is important to mention that all samples preserved in formaline were negative, confirming that formaline alters DNA (Magalhães et al., 2008), making it difficult to perform PCR amplification on samples preserved with traditional solutions.

The *P. leydeni* consensus sequence showed the highest similarity (98%) to *Fischoederius cobboldi*. This should not pose a problem for identification, since these two parasites are morphologically and taxonomically different (Sey, 1992). On the other hand, *P. leydeni* had 97% similarity to other closely related amphistomes, such as *C. daubneyi* or *C. calicophoron*; however, their restriction patterns are different (Rinaldi et al., 2005). These facts support the concept of differentiating amphistome species by RFLP patterns.

In conclusion, this is the first report of molecular characterization of the ITS-2 fragment of *P. leydeni* and its sequence. Although ITS-2 seems to be a valuable identification target, additional information from another segment could improve proper identification of this group and complement traditional methods. This method can be useful for analyzing samples from different locations of Argentina and border countries, in order to search for regional variations, or for identifying additional genus or species that have not been described at the moment.

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