Parasitology International xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Prevalence and sequence comparison of *Phyllodistomum folium* from zebra mussel and from freshwater fish in the Ebro River

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ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 6 October 2010
Accepted 8 October 2010
Available online xxxx

Keywords:
Phyllodistomum folium
Dreissena polymorpha
Trematoda
DNA
ITS1-5.8S-ITS2 region
Cyprinid

ABSTRACT

We utilised DNA analysis to detect the presence of the digenean *Phyllodistomum folium* in three cyprinid species, *Scardinius erythrophthalmus*, *Cyprinus carpio* and *Rutilus rutilus*. DNA sequencing of the region containing the genes ITS1-5.8S-ITS2 revealed 100% sequence identity between DNA from the sporocysts found in zebra mussels and DNA from adults located in the urinary system of 29 cyprinid fish. A second genetically different (variation = 1.6%) sequence was observed in two samples from *R. rutilus*. In our opinion, the existence of a complex of species reported as *P. folium* is supported by recent genetic studies, including our own results. The overall prevalence of *P. folium* in mussels from the Ebro River was 4.67% in 2006, although during the summer months the rates frequently exceeded 10%.

1. Introduction

Following the discovery of zebra mussels *Dreissena polymorpha* in the lower Ebro River in Spain, we carried out further studies to determine if this exotic bivalve had introduced nonindigenous species of parasites in this basin. After macroscopic and histological observation, only one helminth species was identified in the bivalves examined, *Phyllodistomum folium*, whose sporocysts were detected in 0.14% of the mussels [1].

The development of the digenean trematode *P. folium* requires two hosts, one of which is the freshwater mussel *D. polymorpha*. Adult stages are fish parasites exhibiting a close morphological similarity and a great intraspecific variability in most morphological characters used for species differentiation [2–4]. A wide range of host fishes have been recorded for *P. folium*, and obvious disagreement exists regarding the degree of specificity of this helminth species.

Because the morphological characterisation of *Phyllodistomum* spp. provides a limited number of criteria for species determination, better comparative markers are necessary to provide additional information for the taxonomic characterisation of the species [4]. As an alternative to the classical approaches, molecular tools (usually DNA sequencing) are thought to allow quick and accurate identification of genetically distinct but morphologically similar species [5].

This study aimed to identify the fish species that harbour the adult stages of *P. folium* in the Ebro River and to establish the genetic relationship between the adult stages and sporocysts in zebra mussel gills by sequencing the internal transcribed spacer 1 region, the 5.8S ribosomal gene and the internal transcribed spacer 2 (ITS1-5.8S-ITS2) region.

2. Materials and methods

2.1. Mussels

Zebra mussels were collected from February to October in 2006 from the following four sites on the Ebro River: Ribaroja dam, Flix dam, Flix meander and Ascó meander (Fig. 1). After collection, the bivalves were transported to the laboratory, where they were measured and opened. The gills were dissected to observe the presence of helminth sporocysts and histological sections from this tissue were used for taxonomical identification [1].

Sporocyst infected gills from four specimens of *D. polymorpha* were stored at −20 °C for DNA analysis. Two of these mussels were collected from the Flix water reservoir in March 2006, and the other two mussels were collected from the meander of Flix in June 2006.

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doi:10.1016/j.parint.2010.10.004

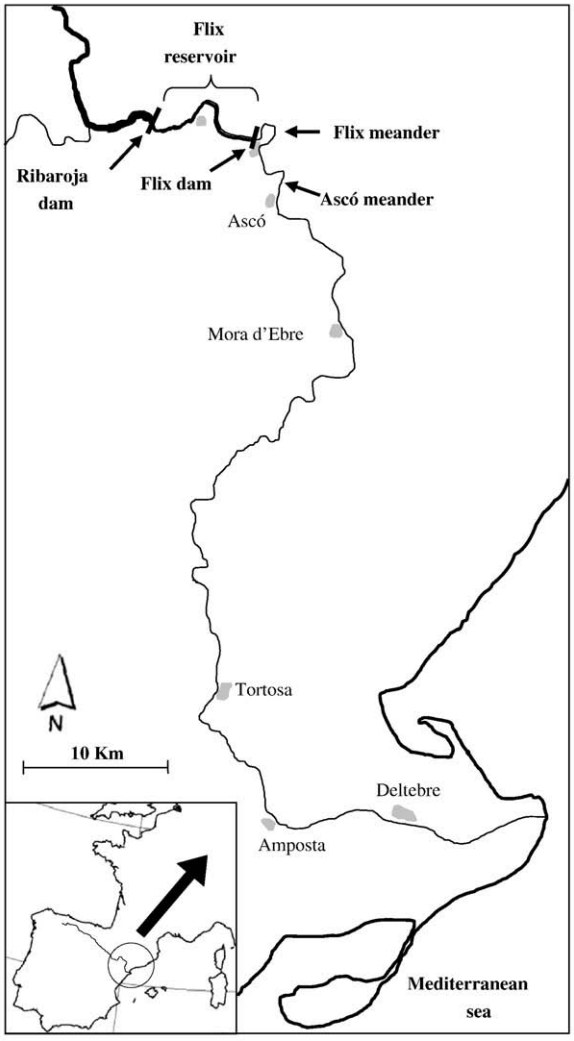

Fig. 1. Map of the low course of the Ebro River indicating the sampling sites. The positions of dams are indicated by black bars and the main towns (grey areas) are also shown.

2.2. Fish

Fish were sampled in July and October of 2006 and in February of 2007 by daylight boat electrofishing in the littoral zone of the Flix Reservoir. The fish were divided in two groups. In group A, 67 fish specimens were transported alive to the laboratory, where they were dissected for parasite examination. The urinary systems of the fish were examined microscopically for helminth detection, and when the presence of adult phyllodistomes was detected, the ureters and urinary bladder were preserved at −20 °C for DNA analysis. For group B, 75 additional fish specimens were collected. The ureters and urinary bladders were not microscopically examined but were preserved at −20 °C to investigate the presence of phyllodistomes using only DNA analysis.

2.3. DNA extraction

Frozen infected mussel gills and frozen fish urinary tracts were homogenised with a rotor–stator homogeniser in 500 µl of RSB buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, and 25 mM EDTA). Then, 20 µl of 25% SDS and 10 µl of 2 mg/ml proteinase K were added, and the homogenates were incubated at 55 °C for 2 h. DNA was purified with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol.

2.4. PCR amplification and sequencing

The analysis of the ITS1-5.8S-ITS2 region was performed by PCR amplification and direct sequencing. Using Primer Express software (Applied Biosystems) and the previously reported sequence of *Phyllodistomum* sp. Lithuania-VS-2003 from *Pisidium amnicum* (GenBank accession no. AY277703) [4], a set of primers was designed to amplify a 569-bp fragment from the ITS1 to the ITS2 region (forward: TACCTGATGGTGGTAACGC; reverse: ATCACGTCAGCTGGCATTAC).

PCR amplifications were carried out in 25 µl in an ABI 2700 thermocycler (Applied Biosystems) using 50 ng of genomic DNA, standard PCR buffer, 1.5 mM $MgCl_2$, 100 µM each dNTP, 150 nM each primer and 1.25 U Taq DNA polymerase (Invitrogen). The PCR profile included an initial denaturation step of 95 °C for 5 min and a final extension step of 72 °C for 5 min. Cycling conditions were 40 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. PCR products were enzymatically purified with ExoSAP-IT (Amersham) according to the manufacturers' instructions, followed by bi-directional sequencing.

Sequence reactions were performed in a total volume of 5 µl using 1 µl of the BigDye Terminator V3.1 Cycle Sequencing Kit reagent (Applied Biosystems), 250 nM each primer and 3–5 ng of purified PCR product. Samples were analysed in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). ClustalW multiple sequence alignment software (http://www.ebi.ac.uk/clustalw/) was used to obtain consensus sequences by multiple sequence alignment. To compare the consensus sequences obtained, BLAST searches were carried out (http:// www.ncbi. nlm.nih.gov/blast/Blast.cgi).

3. Results and discussion

3.1. Prevalence of P. folium in zebra mussels

The overall prevalence of *P. folium* sporocysts in mussels from the Ebro River was 4.7% in 2006 (Table 1), although during the summer months rates frequently exceeded 10%. These results are consistent with those in previous reports, which have noted that the prevalence of *P. folium* infection in zebra mussel populations tends to be low: less than 5% [6–9] or less than 12.3% [10,11], with higher prevalences being infrequent [12]. In a recent investigation of several thousand mussels from all over Europe, only 11 specimens were confirmed to be infected by *P. folium* [13].

The increasing prevalence of *P. folium*, which was 0.14% in 2003–2004 (early phase of colonisation after the introduction of zebra mussels in late 1990s) and 4.7% in 2006, may represent the successful colonisation of the Ebro River by this helminth species when intermediate and final hosts are present.

The larger mussels (>30 mm) from the Flix meander reached the maximum infection prevalence (32%) during September sampling, which can be related to two factors: the water temperature in the Mediterranean Ebro River and the mussel size. One factor influencing the prevalence of infections in zebra mussel populations is water temperature. In one study of mussels from two sites of Lukomskoe Lake (Belarus), a rate 13% higher was found where the water was heated by discharge from a hydroelectric plant, with differences between the two sites around 5–6 °C [11]. The prevalence of infection also tends to be higher in larger mussels. In Lukomskoe Lake, the youngest mollusc to be found infected had a shell height of more than or equal to 8 mm, and the prevalence increased steadily in mussels up to 28 mm in length [11]. Additionally, in the Volga Basin (Russia), the infection was present only in 23- to 29-mm mussels [8].

3.2. DNA Phyllodistomum analysis in zebra mussel and fish

The ureters and urinary bladders of 67 fish (group A) were microscopically observed, and adults of *Phyllodistomum* sp. were identified in five of these fish (Table 2). The urinary systems of these

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Table 1
Prevalence of *Phyllodistomum folium* infection in zebra mussel gills.

Location month	Positive mussels/dissected mussel (classified by length)							
	11–15 mm	16–20 mm	21–25 mm	26–30 mm	31–35 mm	36–40 mm	Total	%
Flix dam								
February			1/20	1/32	0/1		2/53	3.8
March	1/49	6/313	5/108	2/141	0/1		14/612	2.3
June		1/11	10/112	13/124	2/41 26/288		9.0	
July	0/8	1/38	7/123	18/112	2/270/1	28/309	9.1	
September	0/4	0/9	8/63	15/98	7/37		30/211	14.2
October	0/13	0/44	10/160	6/186	3/24 0/1	19/428	4.4	
Flix meander								
February			0/18	0/9			0/27	0
June		0/34	4/281	3/256	1/85 0/2		8/658	1.2
July	0/1	0/41	5/160	11/149	1/38	0/1	17/390	4.4
September	1/21	1/21	8/78	8/120	6/19		24/259	9.3
October	0/3	0/35	1/88	8/162	0/46	0/2	9/336	2.7
Ribaroja dam								
June			0/96	0/32			0/128	0
July	0/6	0/54	0/30				0/90	0
Ascó meander								
September		1/7	0/16	0/1			1/24	4.2
Total	2/105	10/607	59/1353	85/1422	22/319	0/7	178/3813	4.7
%	1.9	1.6	4.4	6.0	6.9		4.7	

five fish were processed to isolate DNA for PCR analysis. DNA extraction from the *Rutilus rutilus* specimen was unsuccessful, but DNA and PCR amplification was successful in the two carps, *Cyprinus carpio*, and the two rudds, *Scardinius erythrophthalmus*.

DNA from the frozen urinary systems of 75 fish (group B) not previously observed by microscopic examination was also PCR amplified to investigate the presence of *Phyllodistomum* sp. DNA. Twenty-seven of the 75 fish were found to be positive using this technique (Table 2): five *S. erythrophtalmus* specimens, three *C. carpio* specimens and 19 *R. rutilus* specimens. The *Silurus glanis* specimens were negative for parasite presence by microscopic observation and by PCR amplification.

The ITS1-5.8S-ITS2 region was sequenced from six pieces of gills obtained from four specimens of zebra mussels infected with larval stages of *P. folium* and from 31 samples of urinary system tissue from fish (Table 2). The sequences obtained from the gills were 100% identical among all samples. The nucleotide sequences obtained from 29 of the 31 fish samples were 100% identical, but two samples from *R. rutilus* (from group B) differed by nine nucleotides (1.6% of variation) (Fig. 2). Multiple sequence alignment allowed us to obtain a consensus sequence for each DNA fragment obtained from the gills and the 29 fish urinary tissues (Fig. 2).

Table 2
Phyllodistomum folium infection in fish.

Fish species	Group A			Group B			Total sequenced
	n	MP	PP	*n*	MP	PP	*n*
Scardinius erythrophthalmus	9	2	2	23	NE	5	7
Cyprinus carpio	33	2	2	5	NE	3	5
Rutilus rutilus	16	1	—	42	NE	19	19
Silurus glanis	9	0	—	5	NE	0	0
Total	67	5	4	75	0	27	31

Two different groups of fish samples were used in this study to evaluate *Phyllodistomum folium* infection. Group A: samples were microscopically analysed for the presence of adult phyllodistomes in the ureters and urinary bladder. Microscopically positive (MP) samples were subjected to DNA extraction and PCR amplification. Group B: the ureters and urinary bladders of these fish were not microscopically examined (NE), but all samples were directly subjected to DNA extraction and PCR amplification. PCR positive (PP) samples, from with the ITS1-5.8S-ITS2 region was amplified, are indicated in each group. Samples in which DNA analysis was not possible or was unnecessary are indicated (—). In the last column, the sequenced samples are indicated.

Our DNA consensus sequences for the ITS1-5.8S-ITS2 region resulted in 100% sequence identity between samples from the zebra mussel gills and the samples from the urinary systems of 29 of the 31 fishes. Comparison of our consensus sequence with nucleotide databases using BLAST showed that this sequence was 100% identical to the ITS1-5.8-ITS2 region of the species *P. folium* in zebra mussels originating in Lepelskoe Lake and Lukomskoe Lake (Belarus) (GenBank accession nos. AY288831 and AY288832) [14]. They also showed a 98% identity to two different sequences reported to be a genetically different variant of *P. folium* from *D. polymorpha* (GenBank accession nos. AY278565 and AF533015) [14]. The two samples of *R. rutilus* showed a sequence 100% identical to a different variant of *P. folium* from *D. polymorpha* (GenBank accession nos. AY278565 and AF533015) [14].

When compared with sequences of larval phyllodistomes from *P. amnicum* (GenBank accession no. AY277703) or *Sphaerium corneum* (GenBank accession no. AY277704), the sequence identity decreased to 94% and 85%, respectively. Nucleotide sequence identity from adult *P. folium* from the three-spined stickleback, *Gasterosteus aculeatus*, (GenBank accession no. AY277705) was determined to be only 85% [4]. A phylogenetic tree was constructed using all of the sequences mentioned previously (Fig. 3).

The nucleotide sequences of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (rDNA) have been proven to be a useful tool for differentiating closely related digenean species [15–17]. According to these studies, this fragment is stable within species, but, at the same time, is characterised by a relatively high interspecific variability, which makes it a good target for the differentiation of congeneric digenean species. A review of 63 studies that reported partial or complete ITS sequences for approximately 155 named digenean species from 19 families found levels of interspecific variation greater than 1% except for the genus *Schistosoma*, were they observed an interspecific variation of 0.8% [5]. In this way, the 1.6% variation between our two sequences is interpreted to be an interspecific variation in the phyllodistome species complex that parasitises cyprinid fish.

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                10        20        30        40        50        60        70        80        90       100
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D. polymorpha   TACCTGATGGTGGTAACGCGTTGCCTACGTACAGTCAAGCCAATGGTGGGGTGCCTATCTGTCTGATGCCTTTCGACATGCTCGTGGCCTTTGGTCACCA
Fish            ....................................................................................................
R. rutilus      ..................................A..G.............................................T...A.......

               110       120       130       140       150       160       170       180       190       200
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D. polymorpha   GTCCATGATTGTGGGCGACAGAGTGTACTACCTTTGACGGTAGTGCTAGGCTCAATGAGTGGTTGCACATCAAGGGCTACGGCCCGGACACCGCCCCATT
Fish            ....................................................................................................
R. rutilus      .......C............................................................................................

               210       220       230       240       250       260       270       280       290       300
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D. polymorpha   CCAGTTTTATACTTTACCATACACTGTTGAGCGAACCGTGTCTTCGGACTCGATTCAATACTCATGTGTGCCCGGCTTCGCTGGGTTGCACTCAGTCACC
Fish            ....................................................................................................
R. rutilus      ..........................................T.............A.....................................

               310       320       330       340       350       360       370       380       390       400
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D. polymorpha   TGGCGTGGCCTTCCCCCAGGCTTGACTGAAATACTCGGATTTTCCGAGTATTGTACAACTCTGTGCGGTGGATCACTCGGCTCGTGTGTCGATGAAGAGC
Fish            ....................................................................................................
R. rutilus      ..........................................T.........................................................

               410       420       430       440       450       460       470       480       490       500
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D. polymorpha   GCAGCCAACTGTGTGAATTAATGTGAACTGCATGCTGCTTTGAACATCGACATCTTGAACGCATATTGCGGTCACAGGTTAGCCTGTGGCCACGTCTGTC
Fish            ....................................................................................................
R. rutilus      ....................................................................................................

               510       520       530       540       550       560
                |....|....|....|....|....|....|....|....|....|....|....
D. polymorpha   CGAGAGTCGGCTTACATATTATCACGACGCCCAAAAAGTCGTGGCTTGGGTAATGCCAGCTGACGTGAT
Fish            ...........................................................
R. rutilus      ..................................T........................
```

Fig. 2. *Phyllodistomum folium*. Multiple sequence alignment of the ITS1-5.8S-ITS2 region of *P. folium* consensus sequences from mussel zebra gills and fish urinary system of different species. The sequences from the two different *Rutilus rutilus* specimens, from group B, that differed in nine nucleotides from all the other analysed sequences are depicted. Dots represent identities with respect to the first sequence.

The taxonomic status of phyllodistomes remains unclear due to synonymy and homonymy. Adult specimens of *P. folium*, the type species, collected from *Esox lucius* were described by Olfers in 1817 (cit. from [18]). Many of the phyllodistomes found by subsequent researchers in the bladders and urinary ducts of various fishes have been referred to as this species, probably because the mature worms have few distinctive qualitative differences and because the anatomical features that have been used taxonomically do not remain constant during the maturation of members of the same species [3,19]. This great morphological variability occurring within *P. folium* indicates, that either the species is extremely variable [20,21] or the older taxa consist of assemblages of numerous species [4]. As a consequence of this confusing situation, the degree of specificity of *P. folium* in vertebrate hosts and the relationships between cercariae or metacercariae and sexually mature phyllodistomes are not well known. Pigulewsky [22] stated that pike *E. lucius* is the only vertebrate host of *P. folium*, whereas the trematodes reported under this name from cyprinids represent an independent species, *P. dogieli*. However, a wide range of host fishes for *P. folium* including cyprinids, esoxids, percids, salmonids and silurids have been recorded [23]. Finally, *P. folium* is also considered a parasite of the three-spined stickleback (*G. aculeatus* L.) [4,24–26].

In our opinion, the existence of a complex of species reported as *P. folium* is supported by recent genetic studies, including our own results. One of the species could correspond to the species reported by Petkevičiūtė et al. [4]. Comparing sequences and karyotypes, they

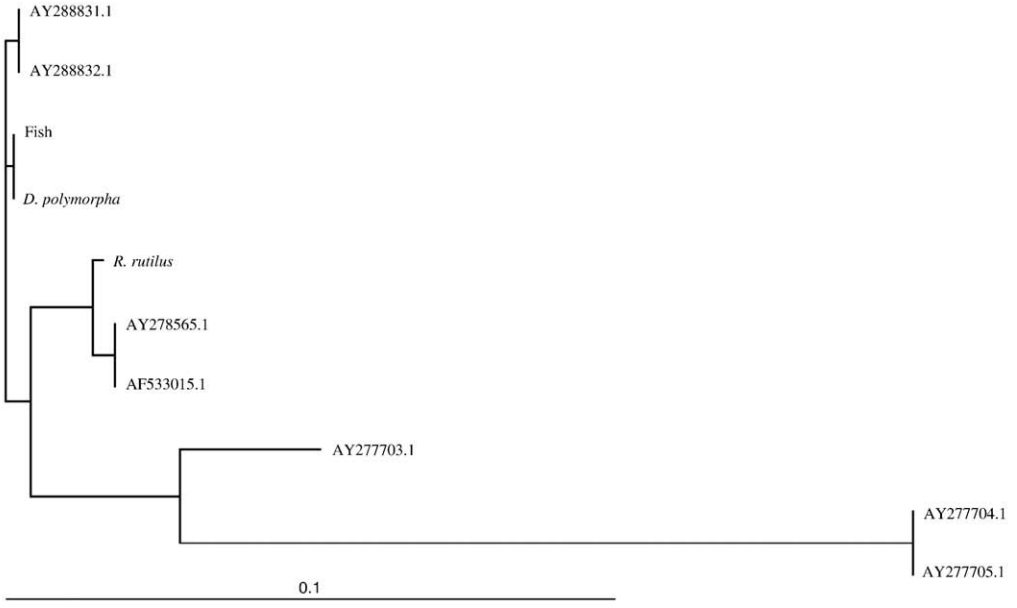

Fig. 3. Phylogenetic tree constructed using NeighbourcJoining methodology and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2). "Fish" represents the sequence that we obtained in 29 fish. This sequence is identical with our sequence from *D. polymorpha* but is different from the sequence from *R. rutilus*. The rest of the sequences are from the cited references.

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showed that the final host of *P. folium* is *G. aculeatus* and that the intermediate host is *Sphaerium corneum* (GenBank accession nos. AY277704 and AY277705). However, this species did not seem to correspond with larval phyllodistomes from zebra mussel gills.

Our sequencing comparison strongly supported the hypothesis that, in the Ebro River, zebra mussels and three cyprinid species are utilised by the same species of the *P. folium* complex. Sequences of *P. folium* from *D. polymorpha* gills have also been reported (GenBank accession nos. AY28831, AY288832, AY278565 and AF533015) [14]. Unfortunately, these sequences were not associated with any fish host species. The identity between those sequences and the sequences from our study was 100%.

Recent DNA studies provide a new approach to sort out the taxonomic status of phyllodistomes. However, we consider it necessary to make a great effort to collect samples from different families of fish and bivalves, to sequence small pieces of *Phyllodistomum* specimens, to mount these specimens and to compare the morphological and genetic data to puzzle out the confusing classification and life cycles of this genus.

Acknowledgements

The authors would like to thank the Spanish Electric Company "Endesa" for the financial help, to the members of "Grupo Especial de Actividades Subacuáticas de la Guardia Civil" for diving into the Ebro River and collecting the mussels for this study and to C. Cons for the technical assistance.

This study was partially financed by the Spanish Ministry of the Environment and the Catalan Water Agency (MOBITROF project to J.O. Grimalt). LB held a doctoral fellowship from the University of Girona.

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Please cite this article as: Peribáñez MA, et al, Prevalence and sequence comparison of *Phyllodistomum folium* from zebra mussel and from freshwater fish in the Ebro River, Parasitol Int (2010), doi:10.1016/j.parint.2010.10.004