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Molecular diversity of *Trichobilharzia franki* in two intermediate hosts (*Radix auricularia* and *Radix peregra*): A complex of species

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ABSTRACT

Recently, the systematic use of the molecular approach as a complement to the other approaches (morphology, biology, life cycle) has brought help for the identification of species considered as different in the past to be regrouped and synonymised, and distinctions to be drawn between species similar at the morphological level. Among these species, we tried to clarify the situation of *Trichobilharzia franki* Müller and Kimmig, 1994, species that today include more than 50 haplotypes notably coming from larval stages isolated from intermediate hosts belonging to gastropods of the *Radix* genus. Cercariae were isolated in France and Iceland from various molluscs, before being analyzed, with their hosts, by molecular analysis of various fields such as the D2 and ITS of the ribosomal DNA and the COX1 of mitochondrial DNA. We thus show the presence of two clades depending on the specificity of their intermediate host in which they were isolated (*Radix auricularia* or *Radix peregra*), thus allowing separation of the species *T. franki* that had been described in the past as a probable new species.
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1. Introduction

Among schistosomatids, representatives of the genus *Trichobilharzia*, with more than 40 described species, are probably the most frequent in the field. The parasite life cycle comprises various birds (mainly waterfowl) and water snails as definitive and intermediate hosts, respectively. Due to a complicated life cycle and the difficulty of isolation and morphological identification of the adult worms, the taxonomy within this genus is difficult. In the past, characterization of the species was based mainly on morphology of the larval (cercariae) and adult stages. Unfortunately, in many cases cercariae and adults were described separately with regard to their further development in definitive or intermediate hosts, respectively, meaning that the morphology of all developmental stadia of such species remained unknown. Valuable data were obtained by studies on schistosomatids developing in experimentally infected hosts, but numerous problems such as unavailability of compatible hosts or isolation of adult flukes from the birds (Kolářová et al., 2010) limited the wide use of these methods. The use of recent molecular tools such as the 28S and ITS of the ribosomal DNA and the COX1 of the mitochondrial DNA (Littlewood and Johnston, 1995; Mollaret et al., 1997; Snyder and Loker, 2000; Picard and Jousson, 2001; Lockyer et al., 2003; Olson et al., 2003; Snyder, 2004; Brant et al., 2006; Littlewood et al., 2006; Webster et al., 2007) finally allowed definitive and precise taxonomical identification of the parasites.

On the basis of morphological and molecular characteristics, including intermediate-host specificity, three *Trichobilharzia* species maturing predominantly in waterfowl (for a review see Horák et al., 2002) are currently recognized in Europe: *Trichobilharzia szidati* Neuhaus, 1952 developing in *Lymnaea stagnalis*, *Trichobilharzia franki* Müller and Kimmig, 1994 developing in *Radix auricularia* and *Trichobilharzia regenti* Horák Kolářová and Dvořák, 1998 developing in *Radix peregra*. Four species that had been discovered some time ago were recently characterized in North America – *Trichobilharzia physellae* (Talbot, 1936) McMullen and Beaver, 1945 developing in *Physa parkeri*, *Trichobilharzia querquedulae* McLeod, 1937 developing in *Physa acuta* (experimentally), *Trichobilharzia stagnicolae* (Talbot, 1936) McMullen and Beaver, 1945 developing in *Stagnicola emarginata* and *Trichobilharzia brantae* Farr and Blankemeyer, 1956 developing in *Gyraulus parvus*. For the latter, phylogenetic analysis of DNA suggests that this species probably does not belong in the genus *Trichobilharzia* (Brant and Loker, 2009).

T. franki has been reported in many European countries: Czech Republic (Kolářová et al., 1997; Rudolfová et al., 2005), Germany

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(Brant and Loker, 2009), Switzerland (Picard and Jousson, 2001), France (Ferté et al., 2005; Jouet et al., 2008, 2009), Iceland (Skírnisson and Kolářová, 2008; Skírnisson et al., 2009; Aldhoun et al., 2009a), Russia (Semyenova et al., 2005), Belarus (Chrisanfova et al., 2009), Poland (Rudolfová et al., 2005) and Finland (Aldhoun et al., 2009b). At present, many haplotypes of cercariae and/or adults originating from naturally infected snails and aquatic birds, respectively, are available in GenBank. Except for one record of *T. franki* isolated from *L. stagnalis* by Rudolfová et al. (2005), the larval stages were found only in snails of genus *Radix*: mainly *R. auricularia* but also *R. peregra* were then identified as the intermediate hosts.

Since *T. franki* has been found in a variety of geographical areas in a large number of hosts and considering the increasing number of sequences available in the databases (in particular the recent description of new American species), we studied the possible variations of the parasite within the species and revised the complex of parasites with regard to species-type described by Müller and Kimmig (1994). We attempted to clarify the position of the haplotypes of parasites found in various *Radix* snails, using molecular analysis.

2. Materials and methods

This study was carried out for an international project (EGIDE) in the aim of studying the common parasites between France and Iceland. Snails of the genus *Radix* were collected between 2000 and 2009 in France and during summer and autumn 2009 in Iceland. Cercarial emergence was stimulated according to Kolářová et al. (2010). Ocellate furcocercariae were preserved in 95% ethanol and frozen (−20 °C) until the DNA analysis. Positive snail hosts were frozen directly at −20 °C in individual sterile bags for storage. Only the samples from which cercariae and their corresponding snails were sequenced were used for molecular analyses. The sequenced samples are listed in Table 1. Photographs and measurements of cercariae were made on fresh material or preserved in formalin, with a digital camera (Leica DC 300) attached to a microscope (Leica DMLB or Olympus BX50) equipped for differential interference contrast or Nomarski system.

After removing all ethanol from samples, DNA was extracted using the Qiamp DNA Mini Kit (Qiagen, Germany) following manufacturer's instructions. During the first step (tissue lysis), cercariae or a small part of the foot of each positive snail were crushed using a piston pellet (Treff, Switzerland). The DNA was eluted in 50 μl of the buffer provided. Polymerase Chain Reaction was performed in a 50 μl volume using 5 μl of DNA, and 50 pmol of each of the primers. The PCR mix contained (final concentrations) 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 200 μM dNTP each base, and 1.25 units of *Taq* polymerase (Eppendorf, Germany).

Sequencing of the D2 domain of the 28S subunit, internal transcribed spacers (ITS-2 and ITS-1) of ribosomal DNA and COX1 domain of the mitochondrial DNA were used for the identification of avian schistosomes obtained from naturally infected snails. PCR was performed under conditions that had been published previously (Jouet et al., 2009).

Ribosomal DNA: ITS-2 and ITS-1 of furcocercariae were amplified using primers ITS3Trem (5′-GCG TCG ATG AAG AGT GCA GC-3′), ITS4Trem (5′-TCC TCC GCT TAT TGA TAT GC-3′), ITS2Trem (5′-GCT GCA CTC TTC ATC GAC GC-3′) and ITS5Trem (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) (Dvořák et al., 2002). The D2 domain was amplified using the primers C2′B (5′-GAA AAG TAC TTT GRA RAG AGA-3′) and D2 (5′-TCC GTG TTT CAA GAC GGG-3′) according to Mollaret et al. (1997).

Mitochondrial DNA: The domain COX1 was amplified using the primers SchistoCox1-5′ (5′-TCT TTR GAT CAT AAG CG-3′) and SchistoCox1-3′ (5′-TAA TGC ATM GGA AAA AAA CA-3′) (Webster et al., 2007).

ITS-2 was also used for snail identification: Amplification of the positive snails was made by using the specific primers of *Radix* spp. DIX1 (5′-CGC GCT CTG GWC CKT CGC GGC-3′) and DIX2 (5′-ATY TYG TYC GAT TTG AGG TTG-3′) (Jouet et al., 2008).

PCR products were directly sequenced in both directions with the primers used for DNA amplification (QIAGEN, Germany). The sequences are deposited in GenBank under the accession numbers HM131131–HM131205 and HQ003220–HQ003235.

Sequences were aligned using the ClustalW routine included in the MEGA version 3.1 software (Kumar et al., 2004) and checked by eye. The D2 domain (554 bp) and ITS region (1637 bp) of the ribosomal DNA and the COX1 domain (822 bp) of the mitochondrial DNA for the parasite and ITS-2 (440 bp) of the rDNA for the snails were used for tree construction and rooted with the outgroup taxon (*Bilharziella polonica* (D2), *T. szidati* (ITS and COX1) and *L. stagnalis* (ITS-2)).

Phylogenetic analyses were performed using haplotypes obtained in this study (Table 1) and sequences available in GenBank: *T. regenti*, *T. szidati*, *T. physellae*, *T. querquedulae*, *Trichobilharzia* sp., *T. stagnicolae*, *T. brantae*, *Allobilharzia visceralis*, *Dendritobilharzia pulverulenta*, *Gigantobilharzia huronensis*, *B. polonica* for ocellate furcocercariae; *R. auricularia*, *Radix balthica* (=*peregra*, *ovata*), *Radix ampla*, *Radix lagotis*, *Radix labiata* and *Radix* sp. for snails (Tables 2 and 3). Phylogenetic reconstruction using Neighbour-Joining (NJ) with the Kimura-2 parameter and uniform rates among sites was performed using the MEGA version 3.1 software (Kumar et al., 2004). Maximum Likelihood (ML) analysis was performed in Phyml online (Guindon et al., 2005). The model and the parameters were chosen using the hierarchical likelihood ratio test implemented in Modeltest 3.7 (Posada and Crandall, 2001). Maximum Parsimony (MP) analysis was performed in MEGA version 3.1 software (Kumar et al., 2004) using the Maxi-mini Branch and Bound method. For all NJ, ML and MP analyses, gaps were treated as missing data and internal node support was assessed by bootstrapping over 500 replicates.

3. Results

3.1. Morphological comparison of cercariae

Measurements of the cercariae isolated from *R. auricularia* and *R. peregra* show variations between them and relative to the cercariae of *T. franki*, *T. querquedulae* and *T. physellae*, particularly in the overall length of the cercariae (Table 4). However, cercariae of avian schistosomes are contractile, which may explain such variability. It is thus impossible to determine to which species the parasites belong on the basis of morphological criteria alone.

3.2. Molecular analyses of cercariae

The molecular analysis of the D2, ITS and COX1 domains are congruent (Figs. 1–3): they show that the cercariae isolated from *Radix* belong to *Trichobilharzia*. Our analyses clearly show that the haplotypes *T. franki* "*auricularia*" and *T. franki* "*peregra*" belong to two distinct clades, separated by the haplotypes corresponding to the recently described American species: *T. querquedulae* and *T. physellae*.

For the D2 domain, each clade ("*auricularia*" and "*peregra*") is composed of 100% homologous haplotypes. In spite of the conserved character of this domain (specific level of the D2), three variations are present between these two haplotypes. In comparison, *T. franki* "*auricularia*" and *T. physellae* are also separated by three variations, two variations between *T. franki* "*peregra*" and *T. physellae*, and also between *T. franki* "*peregra*" and

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