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Occurrence of *Diplostomum pseudospathaceum* Niewiadomska, 1984 and *D. mergi* Dubois, 1932 (Digenea: Diplostomidae) in Danish freshwater snails: ecological and molecular data

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Abstract: Freshwater pulmonate snails from three locations in Lake Furesø north of Copenhagen were screened for infection with furcocercariae (by shedding in the laboratory) and recovered parasite larvae were diagnosed by molecular methods (by performing PCR of rDNA and sequencing the internal transcribed spacer [ITS] region). Overall prevalence of infection in snails was 2%. Recovered cercariae from *Lymnaea stagnalis* (Linnaeus) were diagnosed as *Diplostomum pseudospathaceum* Niewiadomska, 1984 (prevalence 4%) and cercariae from *Radix balthica* (Linnaeus) as *D. mergi* (Dubois, 1932) (prevalence 2%). Pathogen-free rainbow trout were then exposed to isolated cercariae and infection success and site location of metacercariae in these fish were determined. Infection experiments confirmed that both species could infect rainbow trout with the eye lens as infection site for the metacercarial stage although infection success differed. Combination of molecular and biological assays may contribute to improvement of our knowledge on diagnosis, distribution and biology of diplostomids in fish.

Keywords: Digenea, fish host, site specificity, parasites, molecular study

Eye flukes of the genera *Diplostomum* von Nordmann, 1832 and *Tylodelphys* Diesing, 1850 parasitise mainly freshwater fish (Betterton 1974, Sweeting 1974, Shariff et al. 1980, Kennedy 1981, Höglund and Thulin 1992, Owen et al. 1993, Buchmann and Uldal 1994, Field and Irwin 1995, Larsen et al. 2005). The life cycle involves pulmonate snails as first intermediate hosts (infected by sporocysts releasing cercariae), fish as second intermediate hosts (metacercariae in the eyes) and fish-eating birds as final hosts (carrying the adult fluke in the intestine).

Wild and cultured fish species in Danish aquatic regions with fresh- and brackish water have previously been reported infected with eye flukes. Thus, cultured rainbow trout (Buchmann and Bresciani 1997), freshwater eels (Køie 1988) and even marine fishes caught in Baltic brackish waters, such as eel pout (Guildal 1982) and Baltic cod (Buchmann 1986), have been diagnosed with eye flukes. Recently, also flounders in the North East Atlantic were found infected by diplostomids (Cavaleiro et al. 2012). Early Danish reports (Guildal 1982, Buchmann 1986, Køie 1988, Buchmann and Bresciani 1997) noted mainly the occurrence of *Diplostomum spathaceum* (Rudolphi, 1819) and *Tylodelphys clavata* (von Nordmann, 1832), but a later SEM and morphometric study of cercariae released from snails showed the occurrence in Denmark of *D. pseudospathaceum* Niewiadomska, 1984 and *D. baeri* Dubois, 1937 (see Larsen et al. 2005), suggesting that biodiversity is higher than previously reported. No molecular confirmation of any of these parasite diagnoses has yet been performed but the advent of molecular techniques has provided parasitologists with tools to clarify the occurrence and distribution of *Diplostomum* species.

We have therefore conducted a study on the occurrence of cercariae in Danish pulmonate snails and performed a molecular diagnosis of the recovered cercariae combined with infection studies to confirm the identity, infectivity and site location in the fish host.

Snails. Pulmonate snails, *Radix balthica* (Linnaeus) and *Lymnaea stagnalis* (Linnaeus), were collected in the eutrophic freshwater lake Furesø (maximum water depth of 37 m), located immediately north of Copenhagen, Denmark. Snails were collected (September/October 2011) along the shoreline at three locations (1 – 55°47'06"N, 12°24'27"E [southern shore]; 2 – 55°48'42"N, 12°26'24"E [eastern shore]; 3 – 55°48'16"N, 12°22'46"E [western shore]).

Individual snails were identified based on Glöer (2002), brought to the laboratory and incubated in 400 ml beakers containing 30 ml tap-water (Frederiksberg County) for 12 h at 20 °C under artificial illumination. Cercarial shedding was evaluated under a Leica MZ95 stereo-microscope at 6–63 × magnification. A total of 702 snails were collected and 15 of these shed furcocercariae (overall prevalence of infection 2%). Two out of 50 *Lymnaea stagnalis* (prevalence 4%) and 13 out of 652 *Radix balthica* (prevalence 2%) were positive. Samples of cercariae were preserved in 96% ethanol (Kemetyl, Denmark) for molecular analysis.

PCR and sequencing. DNA was extracted from cercariae from all 15 snails but successful amplification was only achieved with cercariae from one *L. stagnalis* and two *R. balthica*. These three cercarial isolates were then used for experimental infection of fish. Crude lysates of three individual furcocercariae were used for PCR and sequencing of the internal transcribed spacer (ITS) region (rDNA). The extract of three individual cercariae (recovered from ethanol preservation) from the three different snails were prepared by lysis in a buffer containing Proteinase K (0.45%) with Tween 20, 10 mM Trizbase and 1 mM EDTA at 55 °C and 800 RPM until complete digestion as judged visually by microscopy. The reaction was stopped by heating to 95 °C

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Table 1. Infection success recorded following exposure of fish to cercariae of two species of *Diplostomum*. Rainbow trout (one fish examined per cercarial type per sampling time point) were exposed to approximately 200 cercariae and their eyes examined at various time intervals post-exposure. Numbers represent the number of metacercariae recovered from both lenses of exposed fish.

Parasite species	GenBank accession number	Snail host	3 h post-exposure	6 h post-exposure	9 h post-exposure	12 h post-exposure
D. pseudospathaceum	JX494232	*Lymnaea stagnalis*	8	ND	17	60
D. mergi	JX494231	*Radix balthica*	ND	2	ND	6
D. mergi	JX494233	*Radix balthica*	ND	0	ND	8

ND: not determined.

Table 2. Comparison of ITS1 sequences from Danish *Diplostomum* species with *Diplostomum* species from other locations. Gaps (insertions and deletions) were included in calculation of similarities.

This Study*	GenBank*	Country	Species	Similarity (%)	Coverage[a] (bp)	Substitutions/ Gaps
JX494231	JF775690	Finland	*D.* cf. *mergi*	100	605	0/0
	AF419279	Poland	*D. mergi*	99.3	580	3/1
	AY386135	Scotland	*D. mergi*	99.0	593	3/3
JX494233	AF419279	Poland	*D. mergi*	99.5	580	2/1
	JF775690	Finland	*D.* cf. *mergi*	99.3	605	4/0
	AY386135	Scotland	*D. mergi*	99.0	593	3/3
JX494232	JF775729	Finland	*D.* cf. *pseudospathaceum*	100	604	0/0
	JQ665456	Germany	*D. pseudospathaceum*	100	604	0/0
	AF419273	Poland	*D. pseudospathaceum*	100	604	0/0

*GenBank accession number; [a] some ITS1 sequences retrieved from GenBank were incomplete.

for 10 min. Sequences comprising the ribosomal ITS region were amplified by PCR in 60 µl reactions using 6 µl of crude DNA extract, 1 mM of dNTPs, 1.5 mM MgCl$_2$ and 1 unit of Biotaq™ DNA Polymerase (Bioline, DNA Technology A/S, Aarhus, Denmark) in the NH$_4$ reaction buffer, and 0.5 mM of forward and reverse primers targeting the 3' end of 18S rRNA and ITS2, respectively. Forward and reverse primers were D1 (Galazzo et al. 2002) and BD2 (Luton et al. 1992), respectively. PCR conditions were as follows: a pre-denaturation step at 95 °C for 5 min and 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec and finally a post-elongation step at 72 °C for 7 min. Aliquots of 5 µl of the products were analyzed by electrophoresis on 2% agarose and visualized by ethidium bromide staining. PCR products were purified by the means of Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare, Brondby, Denmark) according to the manufacturer's instructions. Sequencing of PCR products was performed by Macrogen, Seoul, South Korea. The primers BD2 (Luton et al. 1992) and Diplost_R1 (5'-GAA TGA GAA CAT ATA GGT TTG C-3') (own construct) were used as sequence primers.

Phylogenetic analysis. A molecular phylogenetic analysis was performed based on three new ITS1 sequences from the present study together with 19 ITS1 sequences of *Diplostomum* spp. and two outgroup sequences of *Tylodelphys scheuringi* (Hughes, 1929) (GenBank accession No. FJ469596) and *Alaria taxideae* Swanson et Erickson, 1946 (GenBank accession No. JF820609) retrieved from GenBank. The rationale behind choosing the above 19 *Diplostomum* spp. sequences was that these have all been published in peer-reviewed literature (Galazzo et al. 2002, Niewiadomska and Laskowski 2002, Locke et al. 2010, Rellstab et al. 2011), and the origin of parasites and the methodology used for species identification can thereby be corroborated. These sequences were chosen as representatives for certain species/isolates and it should be noted that identical

GenBank sequences, which did not add further to the present analysis, were not included. Sequences were aligned with ClustalX v2.1 (Larkin et al. 2007) and the phylogenetic analysis was conducted in MEGA5 (Tamura et al. 2011) using the Maximum Likelihood method based on the K2+G model (suggested as the best-fit model in MEGA5). A bootstrap consensus tree was inferred from 1000 replicates.

Infection procedure. Naïve specimens of rainbow trout fry *Oncorhynchus mykiss* (Walbaum) (body weight ranging from 0.45–1.16 g) were used for infection studies. The fish were hatched from eggs, originating from Fousing trout farm, Denmark, at the Bornholm Salmon Hatchery (Nexø, Denmark), which is a recirculated system supplied with municipal freshwater. Three groups of fish (each comprising at least four rainbow trout) were used, one for each isolate. Each fish was incubated individually at 20 °C in a beaker (1000 ml volume) containing 200 ml aerated freshwater and newly released cercariae. Sampling of one fish (for each parasite isolate exposure) was done at two to three times between 3 and 12 h post exposure according to Table 1 and subjected to parasitological examination for metacercariae. At 6–12 h post-exposure fish from each infection group were fixed for histology (see below).

Parasite recovery from fish. Eyes from exposed hosts were opened by a scalpel incision and *corpus vitreum* and lens placed in a glass Petri dish for examination under a Leica MZ95 dissection microscope (6–63× magnification) with sub-illumination. The number and location of metacercariae were noted.

Histology. Trout exposed to cercariae were fixed in neutral formalin for 24 h and then preserved in 70% ethanol. Following dehydration in graded ethanol series and xylene fish were embedded in paraffin and sectioned (4 µm). Slides with sections were de-paraffinized, stained with haematoxylin/eosin and mounted in Depex (Gurr, UK). Sections were studied and photographed at 200× magnification in a Leica DMLB microscope.
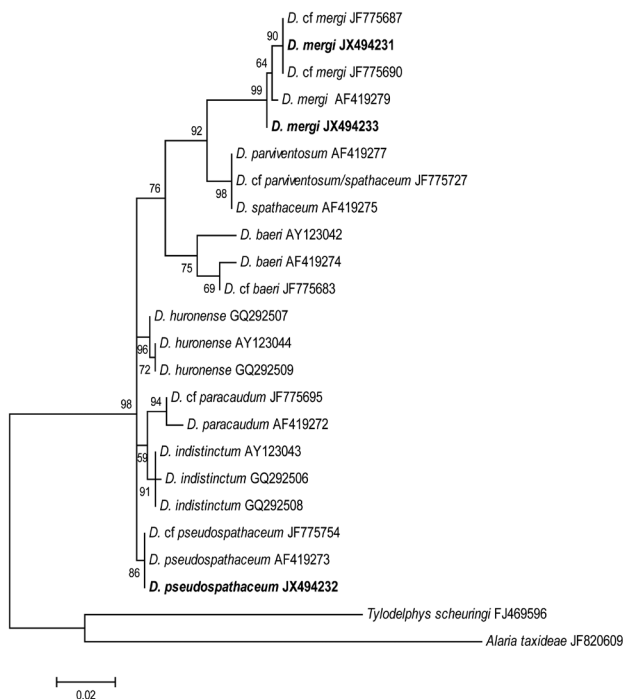
Fig. 1. Phylogenetic analysis inferred by using the Maximum Likelihood method based on an alignment comprising 22 ITS1 sequences of *Diplostomum* spp. and two outgroup sequences. Danish isolates of *D. pseudospathaceum* and *D. mergi* are shown in bold. Numbers above nodes are bootstrap values; only values above 50% are shown.

Complete ITS1 sequences of three *Diplostomum* specimens were obtained from three out of 15 cercarial isolates recovered from infected snails. This suggests that the local biodiversity in the habitat is higher due to the fact that the new records presented here may only represent a minor part of the species present. One of the three isolates (JX494232) was 100% identical to sequences of *D. pseudospathaceum* from Finland, Germany and Poland (Table 2). The second sequence (JX494231) was 100% identical to *D.* cf. *mergi* from Finland and had high similarity to *D. mergi* from Poland and Scotland, differing at only four and six positions, respectively (corresponding to 99.3% and 99.0% identity). The third sequence (JX494233) was highly similar to *D. mergi* from Poland, Finland and Scotland with similarities of 99.5%, 99.3% and 99.0%, respectively. The similarity between the two Danish isolates of *D. mergi* was 99.3% due to four substitutions. The Polish isolates were identified on the basis of morphological characters (Niewiadomska and Laskowski 2002). The phylogenetic analysis showed that the Danish isolates branched within clades comprising their respective species with high support (Fig. 1).

The present investigation thus confirms that *D. pseudospathaceum* occurs in Denmark (GenBank accession number JX494232). The snail intermediate host was *L. stagnalis* (shell length 18 mm). The presence of the parasite *D. pseudospathaceum* recovered from the same snail species in Denmark has previously been documented based on morphometric and scanning electron microscopy analyses (Larsen et al. 2005). In addition, a new geographic record of *D. mergi* is presented here. Two isolates of this parasite species (GenBank accession
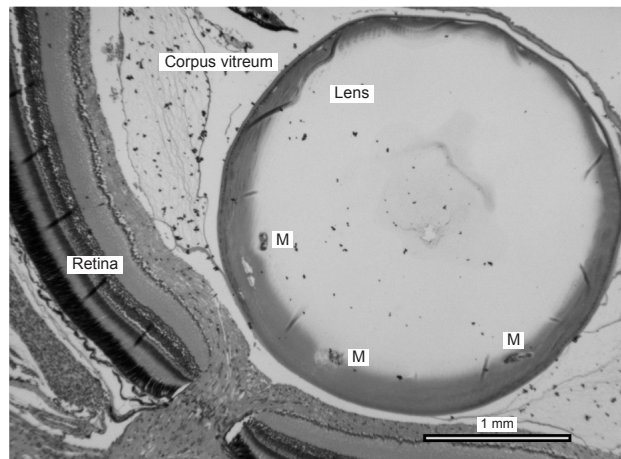
numbers JX494231 and JX494233) were recovered from two *R. balthica* (shell length 17 and 19 mm, respectively; Fig. 1). Both *D. pseudospathaceum* and the two isolates of *D. mergi* cercariae were demonstrated to infect rainbow trout and migrate to the lens to develop into the metacercarial stage (Table 1, Fig. 2).

This infection site in fish has previously been noted (Niewiadomska 1986, Larsen et al. 2005, Rellstab et al. 2011, Cavaleiro et al. 2012). However, the infection success differed markedly as a higher proportion of *D. pseudospathaceum* cercariae reached the metacercarial stage in the lens when compared to *D. mergi.* This is in line with previous studies indicating that different species of *Diplostomum* may reach the lens microhabitat in a certain fish host species with different rates (Larsen et al. 2005).

Identification of metacercariae within the genus *Diplostomum* has previously been based on morphological and morphometric studies (Niewiadomska 1986, Höglund and Thulin 1992, Niewiadomska and Niewiadomska-Bugaj 1995, 1998) but molecular methods providing data for sequences of DNA sequences encoding ribosomal RNA have provided in recent years higher resolution when diagnosing these metacercariae (Galazzo et al. 2002, Niewiadomska and Laskowski 2002). This approach was used in the present investigation. Although previous studies have demonstrated the lens as an important site for these two *Diplostomum* species, it may be worth combining molecular diagnostics and infection success studies.

Thus, it cannot be excluded that innate immune and/or physiological factors in a certain fish host can affect the infection success of different *Diplostomum* species differentially, which will lead to different rates of metacercarial establishment in the lens. Therefore, the present approach could serve as a precise tool in faunistic and parasito-ecological studies, especially when describing new species and/or isolates. Of particular future interest is elucidation of the origin and identities of *Diplostomum* species in marine hosts such as eel pout (Guildal 1982), cod (Buchmann 1986) and performing comparative analyses with isolates from flounder (Cavaleiro et al. 2012). This basic approach should be supplemented by use of additional target sequences comprising mitochondrial sequences for differentiation of isolates.

Fig. 2. Longitudinal histological section of rainbow trout fry experimentally infected with *Diplostomum pseudospathaceum* showing metacercariae in lens. M – metacercaria. Haematoxylin staining. Scale bar: 1 mm.

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