International Journal for Parasitology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

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

Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity ☆

Simona Georgieva [a,b], Miroslava Soldánová [a,c], Ana Pérez-del-Olmo [c,d], Daniel R. Dangel [c], Jiljí Sitko [e], Bernd Sures [c], Aneta Kostadinova [a,f,*]

[a] *Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic*
[b] *Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic*
[c] *Department of Aquatic Ecology, University of Duisburg-Essen, Universitätsstrasse 5, D-45141 Essen, Germany*
[d] *Departament de Biologia Animal, de Biologia Vegetal i d'Ecologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain*
[e] *Komenský Museum, Horní nám. 7, 750 11 Přerov 2, Czech Republic*
[f] *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Street, 1113 Sofia, Bulgaria*

ARTICLE INFO

Article history:
Received 11 August 2012
Received in revised form 29 October 2012
Accepted 29 October 2012
Available online xxxx

Keywords:
Cryptic species
Digenea
Diplostomum
Barcoding
cox1
ITS
Europe

ABSTRACT

We believe this study is the first attempt to address molecular prospecting for species diversity of *Diplostomum* (Digenea: Diplostomidae) in Europe. A database linking sequences from the barcode region of the cytochrome *c* oxidase subunit 1 (*cox*1) mitochondrial gene and from the internal transcribed spacer cluster (ITS1-5.8S-ITS2) of the rRNA gene was generated for larval and adult parasites of snails, fish and gulls from central Europe. Analyses of the novel *cox*1 dataset revealed the presence of six genetically distinct *Diplostomum* lineages in the snail and fish populations studied in the River Ruhr drainage (Germany). ITS1-5.8S-ITS2 sequences from a representative subset of isolates supported the delineation detected by *cox*1. Molecular elucidation of the life-cycles of *Diplostomum spathaceum* and *Diplostomum pseudospathaceum* in central Europe was achieved by matching multiple sequences for isolates from natural infections in snails, fish and birds identified on the basis of the morphology of all life-cycle stages. Comparative analyses restricted to the ITS1 rDNA region and incorporating sequences for six European and seven North American *Diplostomum* spp. retrieved from GenBank, corroborated the results of the molecular prospecting based on the *cox*1 dataset. Taken together, these analyses depicted 20 molecularly characterised species and lineages of *Diplostomum* including three complexes of genetically distinct lineages i.e. '*Diplostomum mergi*', '*Diplostomum baeri*' and '*Diplostomum huronense*', that require further appraisal with the application of molecular, morphological and experimental approaches. Two of the species and 10 of the lineages (arguably species) delineated in the datasets studied originate from central and northern Europe thus indicating a substantial unrecognized genetic diversity inferred from molecular evidence on *Diplostomum* spp. in Europe.

1. Introduction

The trematode genus, *Diplostomum* von Nordmann, 1832, represents a large group of widely distributed parasites with complex life-cycles involving freshwater lymnaeid snails and fish as intermediate hosts and fish-eating birds as definitive hosts. Metacercariae in the eyes of freshwater fish are considered major pathogens since heavy infections may be a source of substantial losses of wild and farmed fish; this has lead to intense field and experimental studies on this larval stage in Europe (reviewed in Shigin, 1986a; Chappell et al., 1994). However, a single species of uncertain taxonomic status named "*Diplostomum spathaceum*" has been used as a model system and much of the published data relies on parasite material collected in the field that may have been based on misidentified isolates (Shigin, 1986a, 1993; Chappell et al., 1994; Niewiadomska, 1996).

The taxonomy of the genus *Diplostomum* is still in a controversial state due to the presence of morphologically similar cryptic species, the simple morphology of the larval stages, and the fact that different stages of the life-cycle have been the focus of separate taxonomic treatments (Valtonen and Gibson, 1997). There are 41 nominal species of *Diplostomum* described within the Palaearctic, predominantly from Europe; of these 25 were considered valid in the latest taxonomic revision of the genus (Shigin,

☆ Note: Nucleotide sequence data reported in this paper are available in GenBank under accession numbers **JX986837**–**JX986858** (ITS1-5.8S-ITS2) and **JX986859**–**JX986909** (*cox*1). Note: Supplementary data associated with this article.

* Corresponding author at: Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Tel.: +420 38 5310351; fax: +420 38 5310388.
E-mail addresses: Aneta.Kostadinova@uv.es, kostadinova@paru.cas.cz (A. Kostadinova).

0020-7519/$36.00 © 2012 Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc.
http://dx.doi.org/10.1016/j.ijpara.2012.10.019

1993). A list of these species and their synonyms according to Shigin (1993) and Niewiadomska (2010) is provided in Supplementary Table S1. However, a combination of identification and taxonomic problems, have led to the biological paradox of a large number of *Diplostomum* spp. described and recorded in fish-eating birds whilst there is substantially lower species diversity in naturally infected intermediate fish and snail hosts. Thus, on a local European scale, recent checklist data for the Czech and Slovak Republics provide a ratio 9:3:2 species in birds, fish and snails, respectively (Moravec, 2001; Faltýnková, 2005; Faltýnková et al., 2007; Sitko et al., 2006). Whereas the low species richness recorded in snails reflects the scarcity of data, the numerous records available on *Diplostomum* spp. infections in fish indicate that both the host and spatial distribution of *D. spathaceum* have been overestimated (50 host species, 363 records) and that a large proportion of the species diversity was probably missed due to identification failure (unidentified metacercariae of *Diplostomum* recorded in 51 host species, 108 records, see Moravec, 2001). The problematic nature of species identification of the metacercariae, in particular, represents a major impediment for the assessment of their actual role in wild fish populations and the advancement of the knowledge of parasite biology and evolutionary aspects of host-parasite relationships of *Diplostomum* spp.

Species circumscription has benefited immensely from the incorporation of molecular data which facilitate unambiguous species identification, diagnoses of problematic taxa and discovery of cryptic species. DNA-based approaches can also produce a rapid acceleration in studies of host-parasite associations and life-cycles. Thus, for trematodes which utilise complex life-cycles, sequences of reliably identified adult stages may provide direct and efficient means of identifying larval stages and thus inferring complete life-cycles (Criscione et al., 2005; Pérez-Ponce de León and Nadler, 2010). Until recently this approach has exclusively been applied to marine trematodes and then predominantly using sequences of the internal transcribed spacer (ITS) regions of rDNA (reviewed by Nolan and Cribb, 2005). Although the application of DNA-based identification of *Diplostomum* spp. is in its infancy, ITS rDNA sequences are now available for eight named species: complete sequences of the ITS1-5.8S-ITS2 gene cluster for *Diplostomum huronense* (La Rue, 1927), *Diplostomum indistinctum* (Guberlet, 1923) and *Diplostomum baeri* Dubois, 1937 from fish and/or gulls collected in Canada (Galazzo et al., 2002; Locke et al., 2010a, b) and partial ITS1 sequences for *D. baeri*, *Diplostomum mergi* Dubois, 1932, *Diplostomum paracaudum* (Iles, 1959), *Diplostomum parviventosum* Dubois, 1932, *Diplostomum pseudospathaceum* Niewiadomska, 1984 and *D. spathaceum* (Rudolphi, 1819) from larval stages collected in Poland (Niewiadomska and Laskowski, 2002). Furthermore, ITS1-5.8S-ITS2 sequences for nine additional presumed species (unidentified isolates labelled as *Diplostomum* sp. 1–9) have been generated recently from fish metacercariae in Canada (Locke et al., 2010a, b) and Rellstab et al. (2011) published 82 partial ITS1 sequences from snail, fish and bird *Diplostomum* isolates from Finland which they assigned without morphological identification to the six European Diplostomum spp. sequenced by Niewiadomska and Laskowski (2002). However, the relatively low levels of divergence observed in the ITS region for sequenced *Diplostomum* spp. (as low as 0–0.35%; see Niewiadomska and Laskowski, 2002; Locke et al., 2010a) indicate that this region alone does not provide sufficient resolution to discriminate between all possible species combinations.

A better understanding of the genotypic and phenotypic complexity within the genus *Diplostomum* would benefit from the use of more variable genes and for a number of reasons it appears that the best candidate is the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) barcode region. The platyhelminth *cox*1 gene exhibits a much higher variation compared with the ITS regions and may thus serve as a more suitable marker for species detection (Vilas et al., 2005). The barcode region (first 650 nucleotides (nt) of the *cox*1 gene) has proven to be suitable for the identification of a large range of animal taxa and for recognition of cryptic species diversity (reviewed by Frézal and Leblois, 2008). Diplostomid-specific primers flanking the *cox*1 barcode region have recently been developed (Moszczynska et al., 2009) and used to build a large barcode library (306 *cox*1 barcode sequences linked to 58 ITS sequences from the same specimens) for the North American *Diplostomum* spp. (Locke et al., 2010a,b). The results of these first molecular prospecting studies (sensu Blouin (2002)) on metacercariae from fish in St. Lawrence River (Canada) have revealed much higher species diversity than previously estimated from morphology alone (Locke et al., 2010a, b). This is in sharp contrast with the scarcity of molecular data from Europe that hampers large-scale screening of natural *Diplostomum* infections in the fish and snail intermediate hosts.

We believe this study is the first attempt to address molecular prospecting for the diversity of the Palaearctic species of *Diplostomum* by generating a sequence database linking *cox*1 and ITS1 sequences for isolates from central Europe which were identified based on parasite morphology. The primary goal was to achieve reliable identification of *Diplostomum* spp. parasitising populations of *Salmo trutta fario* and the lymnaeid snails, *Radix auricularia*, *Lymnaea stagnalis* and *Stagnicola palustris*, required for the interpretation of ongoing ecological studies in the River Ruhr, Germany, drainage. A three-step approach to the identification of the metacercariae from fish eyes was adopted. First, the isolates collected in fish were assigned to species/morphs based on their morphology and location in fish eye; morphological identification for the adult isolates and most of the cercarial isolates was also achieved. Secondly, molecular prospecting of the isolates was attempted by generating partial *cox*1 sequences from randomly selected isolates of the metacercarial morphs and all adult and cercarial isolates. Thirdly, upon examination of the relationships between isolates of metacercariae, cercariae and adult *Diplostomum* spp. based on *cox*1 sequences, selective subsampling was carried out for ITS (ITS1-5.8S – ITS2 gene cluster) rDNA sequencing in order to achieve molecular identification of the isolates of the *cox*1-derived clades and to test the identification of *D. pseudospathaceum* and *D. spathaceum* based on the morphology of all life-cycle stages. Finally, an analysis restricted to the ITS1 rDNA region, which allowed the incorporation of existing data for six European and seven North American species of *Diplostomum*, corroborated the cryptic species diversity revealed by *cox*1 and depicted the existence of more genetically distinct lineages within the recognised European species of *Diplostomum*.

2. Materials and methods

2.1. Sample collection

The species/isolates, their hosts, localities and the accession numbers of the sequences are provided in Table 1. A majority of the isolates represented eye dwelling metacercariae from *Salmo trutta* collected by electrofishing in the River Ruhr (upstream and downstream from the Henne Reservoir; 51°20'57"N; 8°16'24"E) and the River Lenne (upstream and downstream from the Werdohl-Elverlingsen Power Plant; 51°16'31"N; 7°42'13"E) in autumn 2009 and in spring and autumn 2010 (see map in Fig. 1). Additional metacercarial isolates were obtained from the eyes of *Gobio gobio* (River Ruhr at Henne Reservoir) and *Gasterosteus aculeatus* (River Ruhr at Hengsteysee; 51°24'54"N; 7°28'33"E). Further, in order to match sequences from different life-cycle stages three known snail hosts of *Diplostomum* spp. were sampled and used as sources of