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AN INTEGRATIVE TAXONOMIC APPROACH TO THE STUDY OF TREMATODE DIVERSITY AND LIFE-CYCLES IN FRESHWATER ECOSYSTEMS

PhD Thesis

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ANNOTATION

This study applies an integrative approach to species delimitation within complexes of cryptic species within three major digenean families, Diplostomidae, Echinostomatidae and Plagiorchiidae. It is the first attempt to alleviate confusion associated with the taxonomy of two complex and widely distributed in the freshwater ecosystems digenean groups, the genus *Diplostomum* (Diplostomidae) and the '*revolutum*' species complex of *Echinostoma* (Echinostomatidae), in future molecular, morphological and ecological studies. Profiting from a large-scale sampling and fruitful collaborations, we have generated large sequence libraries for the European species of these groups, linking mitochondrial (*cox*1 or *nad*1) and nuclear (ITS or 28S rDNA) sequences for isolates from intermediate and definitive hosts that were identified based on parasite morphology and by assessing their usefulness for species discrimination. This study is also the first to use morphological and molecular data in conjunction to distinguish between morphologically similar larval stages of *Plagiorchis* spp. (Plagiorchiidae), *Tylodelphys* spp. (Diplostomidae) and *Petasiger* spp. (Echinostomatidae) and the first to apply *cox*1/*nad*1 'barcoding' to species prospecting within these groups in natural host populations. Hypothesis-testing to delimit species boundaries within the focal digenean species complexes was carried out *via* combining different lines of evidence, molecular, morphological and ecological. The results, including morphological descriptions and identification keys where possible, will advance the taxonomy and ensure consistent identification of the life-cycle stages and thus provide prerequisites for a better understanding of the diversity of these important parasites in the freshwater ecosystems.

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DECLARATION

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 10 December 2014 Simona Georgieva

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CANDIDATE'S CONTRIBUTION TO THE PAPERS

I Simona Georgieva participated in the sampling, parasite screening, identification and morphological characterisation, performed the sequencing and phylogenetic analyses, and drafted the manuscript. Overall contribution: *c.*80%

II Simona Georgieva contributed substantially to parasite screening and identification, obtained part of the sequences, carried out the morphometric characterisation and statistical analyses and wrote the respective parts of the manuscript, and helped with the preparation of the figures. Overall contribution: *c.*70%

III Simona Georgieva contributed substantially to parasite screening and identification, carried out the SEM study of the cercariae and the morphological characterisation of the metacercariae, and helped drafting the manuscript. Overall contribution: *c.*50%

IV Simona Georgieva participated in parasite screening and identification and morphological assessment, carried out the sequencing and phylogenetic analyses and prepared the first draft of the MS and figures. Overall contribution: *c.*80%

V Simona Georgieva obtained the morphometric data, supervised and contributed substantially to the sequencing, took part in the phylogenetic analyses and drafting the manuscript. Overall contribution: *c.*60%

VI Simona Georgieva took part in parasite screening and identification, carried out the sequencing and phylogenetic analysis and drafted the corresponding parts of the manuscript. Overall contribution: *c.*50%.

VII Simona Georgieva contributed substantially to the sampling, parasite screening, identification and morphological characterisation of the isolates, carried out the major part of the sequencing, performed the phylogenetic analyses and prepared the first draft of the manuscript. Overall contribution: *c.*80%

VIII Simona Georgieva contributed substantially to the sampling, parasite screening and identification, obtained the morphometric data and prepared the descriptions for the larval stages of two species, and helped drafting the manuscript. Overall contribution: *c.*80%

IX Simona Georgieva conceived the study, carried out parasite screening and identification, sequencing and phylogenetic analysis, and drafted the manuscript. Overall contribution: *c.*90%.

X Simona Georgieva carried out the sequencing and phylogenetic analyses and drafted the corresponding parts of the manuscript. Overall contribution: *c.*40%.

XI Simona Georgieva contributed substantially to the sampling, parasite screening and identification, performed part of the sequencing, supervised the phylogenetic and morphometric analyses and revised the first draft of the manuscript. Overall contribution: *c.*50%.

Agreement of the co-authors

The senior and corresponding authors of the manuscripts included in this thesis, hereby confirm that Simona Georgieva contributed significantly to these publications as detailed above:

Aneta Kostadinova Anna Faltýnková Ana Pérez-del-Olmo

Christian Selbach Jana Zikmundová

NOMENCLATURAL ACTS

I herewith declare that the nomenclatural acts in paper VIII of this thesis should be regarded as unpublished according to article 8.1 of the International Code of Zoological Nomenclature (ICZN), and will only become availabe after the publication of Volume 90, Issue 1 of *Systematic Parasitology* (due 15 January 2015).

TABLE OF CONTENTS

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1. GENERAL INTRODUCTION

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1.1. INTEGRATIVE TAXONOMY: A WHOLE GREATER THAN THE SUM OF ITS PARTS

Delimiting species, one of the two frequently stated empirical goals of systematic biology, i.e. discover monophyletic groups at higher levels and lineages (i.e. species) at lower levels, is important in the context of understanding many evolutionary mechanisms and processes (Sites & Marshall, 2003). Furthermore, several areas of research in community ecology are strongly linked to taxonomic work and correct recognition of the species, e.g. the development of realistic species richness estimators, quantifying global patterns of biodiversity based on delineating geographical ranges and regional occurrence patterns of species, assessment of the influence of global climate change on community structure and phylogenetic influences on community structure (Gotelli, 2004). For example, a taxonomic wish-list for community ecology includes (i) illustrated taxonomic keys for species-level identification based on morphological characters; (ii) comprehensive nomenclature including historical record of previously used nomenclature; (iii) species spatial and temporal records physically associated with specimens; (iv) resolved classifications and phylogenies (Gotelli, 2004).

However, the community of practicing taxonomists is steadily diminishing due to changes in priorities for funding and because expertise is usually lost when authorities retire. As a result, the taxonomic resources, human and otherwise, cannot meet the high demands of delimiting the units of and describing life's diversity focused on the question "How many species are there?" on local, regional and global scales. In contrast, another field offering a replacement DNA-based identification system for animals-at-large has flourished. Hebert et al. (2003a, b) proposed DNA barcoding as a tool for accurate species identification and delineation. It is based on the rapid rate of evolution of the mitochondrial DNA characteristic for most of the metazoans so that relatively short sequences generated in routine PCR reactions may be sufficient for species identification and delineation. To date, barcoding approaches have focused largely on the initial "Folmer" region of cytochrome *c* oxidase subunit 1 (*cox*1) gene.

Since its advent, DNA barcoding has initiated a heated debate on whether this strategy is an inevitable replacement of taxonomic research rather than an additional tool to taxonomy (see Dayrat et al., 2005; Will et al., 2005 and references therein). The debate generally revolved around the question as to whether morphology or molecular data should play a central role in taxonomy. In particular, the second and more controversial ambition of DNA barcoding. i.e. to enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible

morphology (Hebert et al., 2003a) has attracted strong criticisms. Critics made clear distinction between DNA-based identification and “DNA taxonomy”. Whereas the use of “barcode” data generated for described species as a diagnostic tool to aid identification is generally accepted as “*applied taxonomy* if done properly but bad if done alone or primarily”, the “DNA taxonomy”, i.e. the discovery and characterisation of species based on molecular data alone focusing on a small portion of the genome, is considered as an initiative to replace the current multi-character approach to taxonomy (e.g. Will et al., 2005; Boero, 2010; Santos & Faria, 2011) that does not qualify as taxonomy (Caira, 2011). The debate over barcoding is not DNA versus morphology, but rather concerns the use of a single-character system (i.e. single gene) and of a single, simple and most basic phylogenetic method available (Neighbour-Joining phenograms) in taxonomy and systematics (Rubinoff et al., 2006; Will et al., 2005). For example, in cases of rate variation of morphological and molecular divergence and emergence of isolating mechanisms, *a priori* criteria for species recognition such as predefined genetic thresholds, are vulnerable to error (Meyer & Paulay, 2005; Rubinoff et al., 2006). Therefore, even if the problems with delimiting species boundaries using molecular criteria alone are left aside, barcoding strategy may contribute to the question “How many?” but is ineffective in answering the question “Which ones?” (Caira, 2011).

Reconciliation has been offered by Dayrat (2005) and Will et al. (2005) who independently coined the term “integrative taxonomy” for the use of a range of data and methods (from different disciplines, e.g. comparative morphology, phylogeography, population genetics, ecology, development, behaviour etc.) for the discovery and species delineation synthetically. Dayrat (2005) suggested this as the best possible future for taxonomy solving two problems that otherwise would continue to grow, i.e. “the frustration of non-taxonomists with how traditional taxonomists describe species and create new species names, and the feeling shared by many taxonomists that their discipline is isolated from the rest of the life sciences“. Studies on cryptic species complexes suggest that molecular and morphological taxonomy are inseparably linked and, in concert with all sources of data, form a “whole greater than its parts” (Page et al., 2005 and references therein). Furthermore, a recent metaanalysis has shown that when multiple sources of data are used for analysing a taxonomic problem, the clearest result is agreement among disciplines (Schlick-Steiner et al., 2010). Will et al. (2005) stressed that the way forward is to do integrative taxonomy first so that after the establishment of a solid taxonomy the most useful characters, DNA sequences or morphological, can be used for species identification. Furthermore, morphological data can be used to establish links with existing taxonomy and draw nomenclatural consequences (e.g.

Schlick-Steiner et al., 2007; Carstens et al., 2013; see also Nolan & Cribb, 2005 for examples within the Digenea).

One of the important consequences of the application of molecular data to species delineation and large-scale exhaustive DNA barcoding surveys is the significant amount of previously unrecognised cryptic diversity revealed across the animal kingdom even among the best taxonomically studied groups of organisms (Bickford et al., 2007; April et al., 2011 and references therein). Two or more species are considered to be "cryptic" if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable (Bickford et al., 2007). Cryptic lineage complexes are widespread throughout the biosphere and continuously reported for diverse taxonomic groups and biomes (Bickford et al., 2007; Pfenninger & Schwenk, 2007; Tronelj & Fiser, 2009; Pérez-Ponce de León & Nadler, 2010). For most taxonomic groups the time of discovery of cryptic species has just begun so that low proportions of cryptic species may indicate that the routine use of molecular techniques as a tool for their discovery has been introduced relatively recently (Tronelj & Fiser, 2009).

Parasites account for a large part of known species diversity and are considered to have a high potential for sympatric speciation (McCoy, 2003) and are thus among the best candidates for the exploration of species boundaries with the aid of molecular methods. It is not surprising that new techniques, methodologies and data sources have been readily incorporated in parasitology research. The use of DNA for parasite identification goes back to the beginning of molecular systematics but has become widespread in recent years (reviewed in Nolan & Cribb, 2005; Olson & Tkach, 2005). The accumulation of sequences from adults provided direct and efficient means of identifying larval ontogenic stages and thus inferring complete life-cycles (Olson & Tkach 2005). The mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) and the cytochrome *c* oxidase subunit 1 (*cox*1) genes as well as the two internal transcribed spacers of the rRNA gene (ITS1 and ITS2) are the most widely used markers in the molecular identification, elucidation of life-cycles and prospecting for cryptic species within the Digenea (Nolan & Cribb, 2005; Olson & Tkach 2005; Vilas et al., 2005; Criscione et al., 2005). This has increased species discoveries and helped to document a large number of cryptic/sibling and morphologically similar digenean species (Pérez-Ponce de León & Nadler, 2010). Two important outcomes of these studies are that the molecular data support existing morphological species concepts (Nolan & Cribb, 2005) as, e.g. in cestodes of elasmobranches (see Caira, 2011), and that they reveal the existence of cryptic species, which were either unknown or only suspected (Nolan & Cribb, 2005; Olson & Tkach, 2005; Pérez-Ponce de León & Nadler, 2010).

Cryptic parasite species may differ in traits important to host-parasite interactions, such as host susceptibility, pathogenesis and epidemiology (Miura et al., 2005 and references therein). Therefore, recognition of cryptic species among digeneans has important implications not only for the accurate biodiversity assessments but also for the development of control measures in aquaculture, epidemiological studies and monitoring of potential zoonoses and detection of invasive species (Leung et al., 2009 and references therein). This is especially true for freshwater digeneans of medical, economical or ecological importance; these are also among the groups that have been more intensely investigated for cryptic species (Pérez-Ponce de León & Nadler, 2010). Parasite cryptic species have been recognised using molecular tools since the 1990s (Nadler, 1990) but the research along this line is still in its infancy (Pérez-Ponce de León & Nadler, 2010). In a recent analysis of published reports using molecular tools, Poulin (2011) revealed that more cryptic species of trematodes are found than in other helminth taxa and suggested that the current estimates (Poulin & Morand, 2004) of trematode diversity may need to be tripled, bringing it to approximately 75,000 extant species. However, most studies identifying cryptic species do not extend to a more detailed morphological characterisation that can serve as "reciprocal illumination" *sensu* Hennig (1966) or formal taxonomic revisions (Pérez-Ponce de León & Nadler, 2010). Therefore, the warning of Pérez-Ponce de León & Nadler (2010), i.e. "simply recognising potential cryptic species, without actually delimiting and describing them, will lead to increased taxonomic uncertainty that is counterproductive to research progress and synthesis in parasite systematics" is still valid.

Since the reviews of Nolan & Cribb (2005) and Olson & Tkach (2005) there has been a remarkable increase in taxonomic knowledge of digeneans through the use of DNA data and especially though the combined application of morphological and molecular methods. A quick survey in the Web of Science for two time periods (2000–2005 and 2005–2014) using "digenea* AND molecular" and "trematod* AND molecular" in the title revealed more than a three-fold increase of the number of papers during the second period (49 *vs* 15 records). To assess the application of integrative approaches, the search terms were changed to "digenea* AND morphological AND molecular" and "trematod* AND morphological AND molecular". This search has shown that the number of studies during 2005–2014 has increased over that during 2000–2005 by a factor of nearly five (24 *vs* 5 records). These data indicate that the concept of integrative taxonomy is being rapidly recognised in digenean research.

In the following sections the focus will be placed on the molecular approaches to the research on species diversity directly relevant to the taxonomic groups subject to analysis in the present study, i.e. species complexes of three digenean families (Diplostomidae,

Echinostomatidae and Plagiorchiidae). Although at an initial static state at the beginning of the PhD study, two of the groups were subject of intensive studies recently, thus both justifying our selection of the focal taxa and placing our research efforts into a wider context.

1.2. FAMILY DIPLOSTOMIDAE POIRIER, 1886

1.2.1. GENUS *DIPLOSTOMUM* NORDMANN, 1832

The family Diplostomidae Poirier, 1886 comprises a large group of parasites of numerous orders of birds and mammals with cosmopolitan distribution that utilise complex, typically tree-host (snail-fish-bird/mammal) life-cycles (Dubois, 1961, 1970; Niewiadomska, 2002). Diplostomid larvae (metacercariae) are found encysted, encapsulated in tissues or free in skin, eyes, musculature and central nervous system of fishes (Gibson, 1996). Both the infective dispersal stages (cercariae) and the metacercariae of diplostomids are important pathogens that are implicated in substantial impacts on both natural and aquacultured fish populations. Thus, migration of large numbers of infective post-cercarial stages towards the sites of infection cause haemorrhaging of capillaries and obstructed blood vessels primarily in the head and brain and may cause mortalities particularly in young fish (Szidat & Nani, 1951; Shigin, 1986a). At high densities the metacercariae can cause haemorrhaging in the musculature, eye cataracts or cranial distortion with disruption of the brain tissue that ultimately result in reduced host survival (Shigin, 1986a; Chappell, 1995; Sandland & Goater, 2001).

The type-genus *Diplostomum* Nordmann, 1832 represents the most species-rich group within the family of widely distributed across the Holarctic parasites with life-cycles involving freshwater lymnaeid snails and fish (occasionally amphibians) as intermediate hosts and fish-eating birds as definitive hosts. The metacercariae in the eyes are considered to be major fish pathogens causing losses in farmed fish and this has led to intensive field and experimental studies on this larval stage, predominantly in northern Europe (reviewed in Shigin, 1986a; Chappell et al., 1994; Chappell, 1995). However, the model systems used in these studies have been referred to as "*Diplostomum spathaceum*", a species with uncertain taxonomic status, 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). Although *Diplostomum spathaceum* (*sensu lato*) has been shown to include a cryptic species, *D. pseudospathaceum* Niewiadomska, 1984 [syn. *Diplostomum chromatophorum* (Brown, 1931)] (see Shigin, 1986a, b, 1993; Niewiadomska,

1984, 1986, 1989) this action has been largely ignored in many recent studies on *Diplostomum* spp. concerning species life-history strategies (Karvonen et al., 2004a, 2006a), life-cycle dynamics (Karvonen et al., 2006b), transmission and infectivity of the cercariae (Karvonen et al., 2003), snail-parasite interactions (Seppäla et al., 2008a), parasite interspecific interactions (Seppäla et al., 2009; Karvonen et al., 2009), fish resistance and avoidance behaviour (Karvonen et al., 2004b, c, 2005a, b), effects of metacercariae on fish growth (Karvonen & Seppäla, 2008), oxygen consumption and feeding (Voutilainen et al., 2008), parasite-induced changes in fish behaviour (Seppäla et al., 2004) and vulnerability to predation (Seppäla et al., 2008b). Unfortunately, even the most recent studies on the infectivity of *Diplostomum* spp. in farmed conditions and the occurrence of parasite-induced cataracts in natural fish populations still refer to either unidentified *Diplostomum* sp. (Voutilainen et al., 2009) or to a composite group of *Diplostomum* spp. (Seppäla et al., 2011). The lack of accurate species identification thus represents a major impediment in the assessment of transmission dynamics and pathogenicity that vary among species of *Diplostomum* in aquaculture conditions and of the effects of these parasites in natural fish populations, as well as in addressing broader questions related to geographical distribution and host-parasite association patterns.

The taxonomy of the genus *Diplostomum* is still in a controversial state due to (i) the presence of morphologically similar cryptic species; (ii) the slight morphological differences at all life-cycle stages and the similarities in the life-cycles; (iii) the phenotypic plasticity of the metacercariae and adults; (iv) the simple morphology of the larval stages; and (v) the difficulties in linking life-cycle stages that requires experimental approach. The situation is further complicated by the fact that different stages of the life-cycle have been the focus of separate taxonomic treatments that have rarely been related successfully and by the differences of opinion by authorities on the group (see Valtonen & Gibson, 1997 and references therein). Thus, of the 41 nominal species of *Diplostomum* described within the Palaearctic (predominantly in Europe), Shigin (1993) considered valid 25 in his taxonomic revision of the genus. However, there is agreement of opinion by Shigin (1993) and Niewiadomska (2010) in relation to the systematic status of four species and disagreement in relation to seven species (no comments by Niewiadomska on the species status were available for 23 species; for details see Supplementary Table S1 in Paper I below). The problematic identification of *Diplostomum* spp. is reflected in low species richness reported in relatively large-scale inventories in natural snail, fish and bird populations in central Europe, e.g. two, three and nine species in snails, fish and birds, respectively, were reported in the recent checklists for the Czech and Slovak Republics (Moravec, 2001; Faltýnková, 2005; Faltýnková

et al., 2007; Sitko et al., 2006). Notably, 363 records of metacercariae in 50 fish species refer to *D. spathaceum* and further 108 records in 51 fish species refer to unidentified material of *Diplostomum* spp.

The cryptic diversity in combination with the lack of unequivocal morphological criteria for species discrimination among *Diplostomum* spp. indicate that the application of DNA-based approaches may provide a promising independent method for assessment of species boundaries within the genus. The pioneer studies have focused on sequencing of the internal transcribed spacer 1 (ITS1) of the ribosomal rRNA gene cluster; this marker has been used in a few subsequent studies.

Niewiadomska & Laskowski (2002) obtained partial ITS1 sequences for six species [*D. baeri* Dubois, 1937, *D. mergi* Dubois, 1932, *D. paracaudum* (Iles, 1959), *D. parviventosum* Dubois, 1932, *D. pseudospathaceum* Niewiadomska, 1984 and *D. spathaceum* (Rudolphi, 1819)] from larval stages (predominantly cercariae) collected in Poland and revealed a generally low interspecific divergence (1.3–4.7%). Unfortunately, this first molecular study on *Diplostomum* spp. has created a problem since the authors reported identical ITS1 sequences for *D. spathaceum* and *D. parviventosum*, which they considered morphologically distinct. Nolan & Cribb (2005) suggested the possibility that the sequences of Niewiadomska & Laskowski (2002) for *D. spathaceum* were reported in error.

Galazzo et al. (2002) used ITS1-5.8S-ITS2 sequences from experimentally obtained adult worms and successfully distinguished three North American species: *D. huronense* (La Rue, 1927), *D. indistinctum* (Guberlet, 1923) and *D. baeri*. They found that the interspecific divergence over the entire region ranges from 1.7 to 4.4% but failed to distinguish a cryptic species of *D. indistintum* (*Diplostomum* sp. 1; see Locke et al., 2010a). Galazzo et al. (2002) also found unexpected differences (at 23 nt positions, uncorrected p-distance of *c*.4%) in the partial ITS1 sequences from isolates identified as *D. baeri* from Canada and Europe (isolate sequenced by Niewiadomska & Laskowski, 2002) indicating specific distinction. Phylogenetic analysis of partial ITS1 sequences indicated that the North American and European species sequenced by Niewiadomska & Laskowski (2002) represent divergent groups within the genus.

Cavaleiro et al. (2011) attempted morphological and molecular identification of two lens morphotypes of *Diplostomum* sp. from *Platichthys flesus* off Portugal. They found that the two morphotypes are genetically identical and exhibit fewer differences in the ITS1 with *D. paracaudum* in comparisons with the sequences for *Diplostomum* spp. available on GenBank. It is worth noting that although *D. paracaudum* has been considered a synonym of *D. spathaceum* (*sensu stricto*) by Shigin (1986a, 1993) its separate status has been maintained

by Niewiadomska (1987, 2010) although no data from natural infections in fish and birds exist.

Rellstab et al. (2011) published 82 partial ITS1 sequences from isolates of *Diplostomum* from snail, fish and bird hosts in Finland which they provisionally assigned without morphological identification to the five genetically distinct forms sequenced by Niewiadomska & Laskowski (2002). These authors detected 61 single nucleotide polymorphisms (SNPs) (of these, 19 with at least one interspecific allele) and developed pyrosequencing assays to analyse two regions in the ITS1 that included four interspecific SNPs to differentiate between the five species in naturally pooled DNA. Rellstab et al. (2011) reported high accuracy and repeatability of the SNP analysis and concluded that pyrosequencing may be a promising method for diversity assessment in multi-species natural communities of *Diplostomum* spp. However, SNP analysis cannot be used for detecting new species that have been missed in obtaining the genetic data for assay development (Rellstab et al., 2011).

Haarder et al. (2013) reported infections with *D. pseudospathaceum* in *L. stagnalis* and with *D. mergi* in *Radix balthica* in Lake Furesø (Denmark) diagnosed by the use of ITS1 sequence analysis.

Aiming at the application of more variable loci, Moszczynska et al. (2009) developed diplostomid-specific primers flanking the cytochrome oxidase *c* subunit 1 (*cox*1) barcode region. These were used by Locke et al. (2010a, b) to distinguish *Diplostomum* spp. in a large sample of metacercariae from 17 fish and one amphibian hosts and adults from three gull species. The findings based on *cox*1 data (79 sequences available on GenBank) were corroborated with sub-sampled sequences of the ITS1-5.8S-ITS2 region of rDNA (21 sequences available on GenBank) and revealed much higher species diversity than previously assessed from morphological data. Locke et al. (2010a, b) detected 12 species: three named (*D. baeri*, *D. huronense* and *D. indistinctum*) and nine unidentified species (*Diplostomum* spp. 1–9).

Recently, Behrmann-Godel (2013) used *cox*1 and ITS1-5.8S-ITS2 sequences to identify *Diplostomum* spp. in perch fry (*Perca fluviatilis*), other fish species and the snail *Radix auricularia* in Lake Constance (Germany) in order to study parasite succession and elucidate transmission pathways. She identified four species: *D. baeri*, *D. paracaudum*, *D. pseudospathaceum* and *D. spathaceum* (annotated as *D. mergi* in GenBank).

In summary, recent molecular studies on *Diplostomum* spp. have provided nuclear (ITS rDNA) and mitochondrial (*cox*1) sequences for eight named and nine unidentified species of *Diplostomum* (Table 1) These revealed relatively low levels of divergence in the

Table 1 Summary of the molecular evidence available for identification of *Diplostomum* spp. at the onset and during the course of the present study

Species	Host	Country	No. of sequences			Source[a]
			*cox*1	ITS[b]	ITS1 only	
D. spathaceum (Rudolphi, 1919)	*Radix ovata*	Poland			2	1
	Radix auricularia; *Rutilus rutilus*	Germany	3	1		8
D. baeri Dubois, 1937	*Perca fluviatilis*	Poland			1	1
	Perca fluviatilis	Germany	18	1		8
	Perca flavescens; *Larus delawarensis* (exp.)	Canada	10	1		2; 4; 5
D. cf. *baeri*	*Myxas glutinosa*; *Radix ovata*; *Coregonus albula*	Finland			7	7
D. huronense (La Rue, 1927)	*Ambloplites rupestris*; *Catostomus commersoni*; *Moxostoma anisurum*, *Notemigonus crysoleucas*; *Perca flavescens*; *Larus argentatus*; *L. delawarensis* (exp.)	Canada	10	3		2; 4–6
D. indistinctum (Guberlet, 1923)	*Catostomus commersoni*; *Neogobius melanostomus*; *Larus delawarensis* (exp.)	Canada	5	2		2; 4–6
D. mergi Dubois, 1932	*Radix ovata*	Poland			1	1
	Radix balthica	Denmark		2		9
D. cf. *mergi*	*Radix ovata*; *Alburnus alburnus*; *Rutilus rutilus*	Finland			5	7
D. paracaudum (Iles, 1959)	*Radix ovata*	Poland			1	1
	Radix auricularia, *Coregonus lavaretus*,	Germany	1	1		8
D. cf. *paracaudum*	*Gymnocephalus cernua*; *Leuciscus leuciscus*; *Oncorhynchus mykiss*; *Rutilus rutilus*; *Sander lucioperca*; *Larus fuscus*	Finland			33	7
D. parviventosum Dubois, 1932	*Radix ovata*	Poland			2	1
D. cf. *parviventosum*/ *spathaceum*	*Rutilus rutilus*	Finland			4	7
D. pseudospathaceum Niewiadomska, 1984	*Lymnaea stagnalis*	Poland			1	1
	Lymnaea stagnalis; *Radix labiata*; *Gymnocephalus cernua*	Germany	6	1		8
	Radix balthica	Denmark		1		9
D. cf. *pseudospathaceum*	*Lymnaea stagnalis*; *Myxas glutinosa*; *Abramis brama* × *Blicca bjoerkna*; *Alburnus alburnus*; *Coregonus albula*; *C. lavaretus*; *Gymnocephalus cernua*; *Perca fluviatilis*; *Rutilus rutilus*; *Oncorhynchus mykiss*; *Sander lucioperca*	Finland			33	7

Table 1 Continued

Species	Host	Country	No. of sequences			Source[a]
			*cox*1	ITS[b]	ITS1 only	
Diplostomum sp. 1	*Ambloplites rupestris*; *Catostomus commersoni*; *Etheostoma nigrum*; *Labidesthes sicculus*; *Lepomis gibbosus*; *Micropterus dolomieu*; *Moxostoma macrolepidotum*; *Notropis hudsonius*; *Larus argentatus*, *L. delawarensis* (exp.); *L. marinus*	Canada	23	7		4–6
Diplostomum sp. 2	*Notropis hudsonius*; *Pimephales notatus*	Canada	7	1		4–6
Diplostomum sp. 3	*Ambloplites rupestris*; *Lepomis gibbosus*; *Micropterus salmoides*; *Notemigonus crysoleucas*; *Pimephales notatus*; *Rana pipiens*	Canada	8	1		4–6
Diplostomum sp. 4	*Carpiodes cyprinus*; *Catostomus commersoni*; *Couesius plumbeus*; *Micropterus salmoides*; *Moxostoma macrolepidotum*; *Neogobius melanostomus*; *Notropis hudsonius*; *Percina caprodes*; *Perca flavescens*; *Pimephales notatus*; *Larus argentatus*; *L. delawarensis* (exp.)	Canada	19	5		4–6
Diplostomum sp. 5	*Perca flavescens*	Canada	1			5
Diplostomum sp. 6	*Pimephales notatus*	Canada	1			5
Diplostomum sp. 7	*Pimephales notatus*	Canada	1			5
Diplostomum sp. 8	*Rana pipiens*	Canada	1	1		5
Diplostomum sp. 9	*Percina caprodes*	Canada	1	1		5
Diplostomum sp.	*Platichthys flesus*	Portugal			1	3

[a]References numbered chronologically: 1, Niewiadomska & Laskowski (2002); 2, Galazzo et al. (2002); 3, Cavaleiro et al. (2009); 4, Moszczynska et al. (2009); 5, Locke et al. (2010a); 6, Locke et al. (2010b); 7, Rellstab et al. (2011); 8, Behrmann-Godel (2013); 9, Haarder et al. (2013)
[b]ITS1-5.8S-ITS2

ITS1 region and provided evidence that *cox*1 may serve as a more efficient marker in elucidating life-cycles and recognition of cryptic species diversity within *Diplostomum*. However, inspection of the molecular evidence available for species identification within the genus *Diplostomum* at the onset and early stages of the present study provided in Table 1 indicates geographical and taxonomic biases. Thus most of the *cox*1 and ITS1-5.8S-ITS2 sequences are based on materials from North America (Canada; 77% and 76% of the totals, respectively) whereas most of the ITS1 sequences (80%) are based on materials from northern Europe (Finland). Furthermore, the taxonomic coverage within each continental subset is rather uneven. Of the 12 species sequenced in Canada, five represent singletons and most of the sequences (48% and 55% for *cox*1 and ITS, respectively) belong to two species (*Diplostomum* sp. 1 and 4). Similarly, the ITS1 dataset from Europe is over dominated by sequences for two species (76%) (*D. paracaudum* and *D. pseudospathaceum*); however most of the isolates (68) are provisionally identified. Finally, morphological evidence useful for species identification of the sequenced isolates is provided in just two studies (Galazzo et al., 2002; Cavaleiro et al., 2011).

1.2.2. GENUS *TYLODELPHYS* DIESING, 1850

Tylodelphys Diesing, 1850 is a small genus comprising 15 nominal species described from adults in groups with scattered geographical distribution: six species in Europe [*T. clavata* von Nordmann, 1832 (type-species); *T. conifera* (Mehlis, 1846); *T. excavata* (Rudolphi, 1803); *T. glossoides* (Dubois, 1928); *T. podicipina* Kozicka & Niewiadomska, 1960 and *T. strigicola* Odening, 1962]; three in Asia (India) [*T. darteri* R. K. Mehra, 1962; *T. duboisilla* (R. K. Mehra, 1962) and *T. rauschi* K. S. Singh, 1956]; three in South America [*T. elongata* (Lutz, 1928); *T. americana* (Dubois, 1936) and *T. adulta* Lunaschi & Drago, 2004]; two in Africa [*T. aegiptica* El-Naffar, Khalifa & Salda, 1980 and *T. xenopi* (Nigrelli & Maraventano, 1944)]; and one in North America [*T. immer* Dubois, 1961]. Eight further 'species' have been described based on metacercarial stage only: six in South America (Argentina) [*T. argentinus* Quaggiotto & Valverde, 1992; *T. barilochensis* Quaggiotto & Valverde, 1992; *T. cardiophilus* Szidat, 1969; *T. crubensis* Quaggiotto & Valverde, 1992; *T. destructor* Szidat & Nani, 1951 and *T. jenynsiae* Szidat, 1969], one in North America [*T. scheuringi* (Hughes, 1929)] (see Dubois, 1970; Lunaschi & Drago, 2004 and references therein) and one in Africa [*T. grandis* Zhokhov, Morozova & Tessema, 2010] (see Zhokhov et al., 2010).

To date, only one species, has been reported from a continent different from that of the original description: *T. clavata* in Africa (Rwanda; see Dubois, 1970) but this record is likely

erroneous. Taxonomic studies on the species of *Tylodelphys* are scarce. In contrast with the wealth of data on the European species, very little is known of their natural history and the morphology of the life-cycle stages, especially in Africa where the number of records has expanded recently. However, almost all records in fishes from this continent refer to identified to the species level metacercariae.

Molecular data for *Tylodelphys* spp. were not available at the onset of the study. Recently, Moszczynska et al. (2009), Locke et al. (2010a, b) and Behrmann-Godel (2013) provided *cox*1 and ITS1-5.8S-ITS2 sequences for *T. scheuringi* from North America and *T. clavata* from Europe, respectively.

1.3. FAMILY ECHINOSTOMATIDAE LOOSS, 1899

1.3.1. GENUS *ECHINOSTOMA* RUDOLPHI, 1809

The digenean family Echinostomatidae Looss, 1899 is a diverse and complex group with long and complicated taxonomic history. It currently comprises 43 genera belonging to ten subfamilies with cosmopolitan distribution and broad range of hosts. As adults, echinostomatids are predominantly parasites of birds, but also infect mammals, including humans, and occasionally reptiles and fishes (Kostadinova, 2005). Echinostomatids typically utilise three hosts in their complex life-cycles and thus represent important components of both freshwater and marine ecosystems.

The type- and most diverse genus of the family, *Echinostoma* Rudolphi, 1809, contains more than 120 nominal morphologically similar species of parasites associated with the freshwater environment (Kostadinova & Gibson, 2000). However, this diversity may have been inflated due to a long history of inadequate descriptions and poor differential diagnoses.

Species of *Echinostoma* parasitise, as adults, a wide range of aquatic birds and mammals, including humans. They utilise freshwater pulmonate (Planorbidae, Lymnaeidae) and prosobranch (Viviparidae) gastropods as first intermediate hosts and a wide range of freshwater molluscs (Gastropoda, Bivalvia), planarians and tadpoles as second intermediate hosts (Kostadinova, 2005). Host-larval parasite interactions in nature have attracted significant research efforts recently in association with the presumed "emergence" of "echinostome" (a collective group including species of the genera *Echinostoma* and *Echinoparyphium*) infections in the amphibian populations in North America (see e.g. Johnson & McKenzie, 2009 for a review). These authors stressed that "echinostomes" are widespread parasites of amphibians (recorded in amphibians of 16 species from 25 states in

the USA) with infection intensities of up to nearly 2000 metacercariae per amphibian and thus having the potential to inhibit renal function and reduce host survival. “Echinostome” infections have been associated with population declines, extinctions and developmental deformities in frog populations and causes high mortality rates of up to 40% in the tadpoles due to compromised renal function. These observations have raised concerns about their importance in determining the survival of tadpoles and heir subsequent recruitment into frog populations, as well as one of the causes of the increasing frog extinctions (Holland et al., 2006). However, currently no protocols for the identification of the possible species involved exist. Another aspect of the increased interest in the systematics of *Echinostoma* spp. is associated with the existence of food-borne species of public health importance in the South East Asia.

However, the taxonomy and systematics of the species within this large genus is further complicated by an extensive synonymy and the loss, lack or inaccessibility of type-materials. It appears that separate systematic treatment of species groups, recognised based on the number of spines on collar and other morphological and life-cycle features may be a practical step towards a comprehensive revision of the genus. One such species group is the so-called ‘*revolutum*’ species complex encompassing the type-species of the genus, *Echinostoma revolutum* (Frölich, 1802), and the species with 37 collar spines which are among the most extensively studied echinostomatids. However, species within this group are characterised by high interspecific homogeneity of the morphological characters of the larval and adult stages used for species differentiation. Due to this, a large number of species have been described within the group for which no reliable morphological characters enabling species discrimination exist (see Kostadinova & Gibson, 2000 for a review of the approaches to species discrimination within the ‘*revolutum*’ species complex).

The ‘*revolutum*’ group has been revised twice. Beaver (1937) suggested that it consists of a single polymorphic species, i.e. *E. revolutum*. He synonymised with ***E. revolutum*** nine species and regarded 11 additional species as “***syn. inq***.” (i.e. possible synonymous but inadequately described or distinguished species). Nineteen additional species have been described between Beaver’s revision and the second attempt at “lumping” carried out by Kanev and colleagues (Kanev, 1985; Kanev, 1994; Kanev et al., 1994, 1995a, b) that reduced the 40 species to five: *E. revolutum* (with three synonyms), *E. trivolvis* (Cort, 1914) (with two synonyms), *E. caproni* Richard, 1964 (with three synonyms), *E. jurini* (Skvortsov, 1924) (with three synonyms) and *E. echinatum* (Zeder, 1803) (with five synonyms). Kanev (1985) listed further 48 species as *species inquirendae* (not all belonging to the ‘*revolutum*’ group). Kanev (1985, 1994) distinguished the species he considered valid broadly by the nature of

their mollusc (at a familial level) and final hosts (birds or mammals or both) and the geographical range on a global scale favouring the idea of allopatric speciation at a continental level with only two sympatric combinations: (i) *E. revolutum*, *E. echinatum* and *E. jurini* in Eurasia and (ii) *E. trivolvis*, *E. caproni* and *E. echinatum* in South America. Since the last revision three new species were described (*E. friedi* Toledo, Muños-Antolí & Esteban, 2000 in Europe; *E. deserticum* Kechemir, Jourdane & Mas-Coma, 2002 in Africa; and *E. luisreyi* Maldonado, Vieira & Lanfredi, 2003 in South America) and two species (*E. miyagawai* Ishii, 1932 and *E. paraensei*) were revalidated based on morphological and morphometric data for the life-cycle stages (Kostadinova et al., 2000a, b) and isoenzyme studies (Sloss et al., 1995), respectively.

Our knowledge of the diversity and distribution of the species within the '*revolutum*' group has advanced significantly as a result of the use of molecular characters (see Table 1 for a summary of the data available at the onset of the study). Morgan & Blair (1995) first used DNA sequence data to examine the relationships and species boundaries within the '*revolutum*' group. Based on rDNA sequences (ITS1-5.8S-ITS2 cluster) obtained from laboratory-maintained strains of five nominal species (*E. trivolvis*, *E. revolutum*, *E. caproni*, *E. liei* and *E. paraensei*) plus two African isolates (*Echinostoma* sp. I and *Echinostoma* sp. II), they distinguished five species, confirmed the distinct status of *E. paraensei* and the identity of three African isolates as strains of *E. caproni*. However, these authors detected very low sequence variation in the ITS region among *Echinostoma* spp. (1.1–3.7%) and could not resolve the position of the European strain identified by Kanev as *E. revolutum*. It is worth noting that Sorensen et al. (1998) found surprising intraspecific ITS sequence variation between three isolates of *E. revolutum* from North America (USA) and the German isolate studied by Morgan & Blair (1995).

In a follow up study Morgan & Blair (1998a) obtained partial sequences of the mitochondrial *cox*1 and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) genes for the same laboratory strains and assessed the relative merits of the ribosomal and mitochondrial genes for investigating phylogenetic relationships and distinguishing species within the '*revolutum*' group. They found that *nad*1 is diverging significantly faster than *cox*1 and ITS and exhibits greater pairwise divergence (average divergence of 14% *vs* 8% and 2.2%, respectively) and concluded that although all three regions successfully distinguished the nominal species sequenced, *nad*1 appears to be the most informative region for investigating relationships and a more suitable marker for detection of strains and species within the '*revolutum*' group.

Morgan & Blair (1998b) used partial *nad*1 sequences for seven echinostome species [*E. caproni*, *E. trivolvis*, *E. paraensei*, *E. revolutum*, *E. hortense* Asada, 1926, *Echinostoma* sp. I and *Echinostoma* sp. (Australia)] obtained earlier (Morgan & Blair (1998a), to match unidentified echinostome isolates (cercariae, metacercariae and adults) collected in Australia. Their analysis identified three isolates as strains of *E. revolutum*, one isolate as a strain of *E. paraensei*, plus at least three unidentified species with more than 37 collar spines. Based on these results, Morgan & Blair (1998b) reported the discovery of *E. revolutum* and *E. paraensei* in Australia.

However, Kostadinova (1999) and Kostadinova et al. (2000a, b) have shown that the material identified and described as *E. revolutum* by Kanev (1985, 1994) represents a mixture of at least two species of the '*revolutum*' group. This, coupled with the observed intraspecific variation in the ITS observed by Sorensen et al. (1998), indicates that the identification of the German isolate used in the molecular studies of Morgan & Blair (1995, 1998a, b) as *E. revolutum* is uncertain.

Kostadinova et al. (2003) carried the first integrated analysis focused on a morphological identification of the voucher specimens from Morgan & Blair's (1998b) study and by sequencing of experimentally obtained *E. revolutum* and natural isolates of six species of the closely related cosmopolitan genera *Echinoparyphium*, *Hypoderaeum* and *Isthmiophora* from Europe, identified on morphological grounds. Results demonstrated congruence between morphological and molecular identification of the species studied. Phylogenetic analyses of Kostadinova et al. (2003) clarified the affiliation to at least the generic level of all unidentified Australian isolates assigned to *Echinostoma* by Morgan & Blair (1998b) and morphological examination resulted in identification of three isolates as *Echinoparyphium ellisi* Johnston & Simpson, 1944, *E. hydromyos* Angel, 1967 and *Echinostoma* cf. *robustum*. Finally, the *nad*1 phylogeny of Kostadinova et al. (2003) revealed that the European and Australian isolates (plus the German isolate identified as *E. revolutum*) represent distinct species, thus providing explanation for the contradictory findings with respect to the reference material of *E. revolutum* in the studies of Morgan & Blair (1995, 1998a, b).

Recently, Detwiler et al. (2010) sequenced more than 150 isolates from snails (*Lymnaea elodes*, *Helisoma trivolvis* and *Biomphalaria glabrata*) at two mitochondrial genes (*nad*1 and *cox*1) and one nuclear gene (ITS) to determine whether cryptic species were present at five sites in North and South America. They demonstrated the presence of five cryptic *Echinostoma* spp. lineages, one *Hypoderaeum* spp. lineage, and three *Echinoparyphium* spp. lineages. Detwiler et al. (2010) observed cryptic life history patterns in

Table 2 Summary of the molecular evidence available for species identification within the '*revolutum*' species complex at the onset and during the course of the present study

Species	Host	Country	No. of sequences				Source[a]
			*nad*1	*cox*1	ITS	28S	
E. revolutum (Frölich, 1802)	Laboratory strain	"Germany"	1	1	1		1–3
	Austropeplea lessoni; *Glyptophysa* sp.	Australia	3				3
	Columba livia (exp.; source: *Radix peregra*); *Lymnaea stagnalis*	Bulgaria; Finland	4		1		7
	Lymnaea elodes	USA			1		5
	Lymnaea elodes	USA	36	19	3		11; 14
	Helisoma trivolvis						
	Laboratory strain (ex *Mesocricetus auratus*)	UK	1			1	8; 11
	"domestic ducks"	Thailand; Lao PDR		2			12
	"domestic ducks"	Thailand			1		13
E. caproni Richard, 1964 (syn. *E. liei* & *Echinostoma* sp. II of Morgan & Blair, 1995)	Laboratory strain	Cameroon; Madagascar; Egypt	3	2	3		1–3
	Rattus norvegicus	Egypt	1			1	9
	Laboratory strain	France	1		1	1	4
E. deserticum Kechemir, Jourdane & Mas-Coma, 2002 (syn. *Echinostoma* sp. I of Morgan & Blair, 1995; 1998a,b)	Laboratory strain	Niger	1	1	1		1–3
E. friedi Toledo, Muñoz-Antoli & Esteban, 2000	*Mesocricetus auratus* (exp.)	Spain	1		1	1	9
E. cf. *friedi*	*Radix peregra*	UK	1				7
E. paraensei Lie & Basch, 1967	Laboratory strain	Brazil	1	1	1		1; 2;3
	Glyptophysa sp.	Australia	1				3
	Biomphalaria glabrata; *Nectomys squamipes*	Brazil			2		6
	"hamster"	USA				1	10
E. robustum Yamaguti, 1935	*Lymnaea elodes*; *Biomphalaria glabrata*	USA; Brazil	3	3	2		11
E. trivolvis (Cort, 1914)	Laboratory strain	North America	1	1	1		1–3
	Helisoma trivolvis	USA			2		5
	Lymnaea elodes; *Helisoma trivolvis*; *Ondatra zibethicus*	USA	24	4	4		11; 14
Echinostoma NZ-Ad	*Branta canadensis*	New Zealand	1				3

[a]References numbered chronologically: 1, Morgan & Blair (1995); 2, Morgan & Blair (1998a); 3, Morgan & Blair (1998b); 4, Mollaret et al. (1997); 5, Sorensen et al. (1998); 6, Maldonado et al. (2001); 7, Kostadinova et al. (2003); 8, Olson et al. (2003); 9, Marcilla et al. (unpublished; 2003); 10, Lofty et al. (2008); 11, Detwiler et al. (2010); 12, Saijuntha et al. (2011a); 13, Saijuntha et al. (2011b); 14, Detwiler et al. (2012)

two species groups inferred to be with cosmopolitan distributions, *Echinostoma revolutum* and *Echinostoma robustum*. Detwiler et al. (2012) used *nad*1 sequences to search for the cryptic echinostomatid lineages in the definitive host, *Ondatra zibethicus*, from Virginia (USA). They revealed at least five genetic lineages with one, *Echinostoma trivolvis* Lineage b, being predominant in both prevalence and intensity of infection. Their study also provided additional evidence that *E. trivolvis* is a species complex comprised of three distinct lineages.

To summarise, the most recent molecular studies on *Echinostoma* spp. carried out in the North America indicated cryptic diversity within two of the seven named species of *Echinostoma* (Table 2; Appendix 2). At the onset of the study the molecular data on *Echinostoma* spp. were scarce (20 *nad*1, eight *cox*1, 11 ITS and five 28S sequences) and predominantly based on laboratory strains so that natural genetic variation has not been assessed. Recent findings of Detwiller et al. (2010, 2012) have shown higher genetic diversity in natural populations. However, in contrast with the large number of sequences obtained recently from North America, data from European natural populations of *Echinostoma* spp. are virtually lacking since only sequences for two species, *E. revolutum* and *E. friedi*, were available at the onset of this study.

1.3.2. GENUS *PETASIGER* DIETZ, 1909

Species of the genus *Petasiger* Dietz, 1909 represent a relatively large group (33 nominal species; of these 23 described from the Palaearctic; see Faltýnková et al., 2008) of parasites with cosmopolitan distribution parasitising fish-eating birds (Podicipedidae, Phalacrocoracidae, Anhingidae, Phoenicopteridae, occasionally Anatidae and Laridae). *Petasiger* spp. utilise a three-host life-cycle: cercariae develop in rediae in planorbid snails and metacercariae are found in oesophagus or pharynx of freshwater teleosts (Kostadinova, 2005). Faltýnková et al. (2008) revised the genus and presented a key to and list of the records and hosts of the 18 species they considered valid. Of these, only seven have been described or recorded in Europe: *P. exaeretus* Dietz, 1909; *P. grandivesicularis* Ishii, 1935; *P. islandicus* Kostadinova & Skírnisson, 2007; *P. megacanthus* (Kotlán, 1922), *P. neocomense* Fuhrmann, 1927; *P. phalacrocoracis* (Yamaguti, 1939); and *P. pungens* (Linstow, 1893).

However, although numerous records from bird hosts in Europe exist, the data on the occurrence of the parasite life history stages in their intermediate hosts are scarce due to the morphological similarities of the large-tailed cercariae belonging to the so-called "*magnacauda*" group (see Kostadinova & Chipev, 1992). The life-cycles of only two European species, *P. neocomense* and *P. grandivesicularis*, have been completed

experimentally (Karmanova, 1971; Kostadinova & Chipev, 1992) and six otherwise unidentified large-tailed cercariae have been described in Europe: *Cercaria thamesensis* Khan, 1960 and *Cercaria hamptonensis* Khan, 1960 ex *Planorbis planorbis* (L.) from the River Thames, UK (Khan, 1960), *Cercaria rashidi* Nasir, 1962 and *Cercaria titfordensis* Nasir, 1962 ex *Planorbis carinatus* Müller from lakes in Birmingham, UK (Nasir, 1962), *Petasiger* sp. of Ginetsinskaya & Dobrovolskij (1964) ex *P. planorbis* in the Volga Delta, Russia (Ginetsinskaya & Dobrovolskij, 1964) and *Petasiger* sp. of Kostadinova (1997) ex *P. planorbis* in Lake Durankulak, Bulgaria.

1.4. FAMILY PLAGIORCHIIDAE LÜHE, 1901

1.4.1. GENUS *PLAGIORCHIS* LÜHE, 1899

The family Plagiorchiidae Lühe, 1901 represents a very large digenean group parasitic in tetrapods worldwide. Plagiorchiids utilise a typical plagiorchiid life-cycle, with cercariae developing within sporocysts in pulmonate gastropods (first intermediate hosts); metacercariae in aquatic arthropods, predominantly insects, and also in molluscs and amphibians (second intermediate hosts); and adults parasitising in the digestive tract of amphibians, reptiles, birds and mammals (Tkach, 2008).

Plagiorchis Lühe, 1899, the type- and perhaps the most speciose genus of the family, includes parasites of birds and mammals, accidentally of amphibians and reptiles, with cosmopolitan distribution (Tkach, 2008). Species of *Plagiorchis* are characterised with high morphological variability and low levels of host-specificity. They utilise lymnaeid snails and aquatic insects and freshwater crustaceans as first and second intermediate hosts, respectively. Larval stages of *Plagiorchis* spp. are ubiquitous and ecologically important parasites in snail populations of freshwater ecosystems in Europe (e.g. Faltýnková et al., 2007; Soldánová et al., 2011). *Plagiorchis elegans* (Rudolphi, 1802) is among the most frequently recorded parasite of *Lymnaea stagnalis* (L.) in Europe (Väyrynen et al., 2000; Faltýnková, 2005; Faltýnková & Haas, 2006; Żbikowska et al., 2006; Żbikowska, 2007; Faltýnková et al., 2007) and has been recognised as "dominant", "most common" and "most frequent" trematode in these surveys. Recent studies of communities of larval trematodes have shown that *P. elegans* is one of the three species contributing substantially to the community structure and patterns of parasite flow (Soldánová et al., 2011) and as a species with the highest rates of colonisation of the snails populations in eutrophic fish ponds in Central Europe (Soldánová & Kostadinova, 2011). However, the numerous records of a single species should be considered

with caution since these may represent an underestimation of the parasite diversity because of difficulties in distinguishing the morphologically similar larval stages (cercariae) used for species identification of *Plagiorchis* spp. parasitising lymnaeid snails.

Tkach et al. (2000b) first applied DNA sequencing combined with a detailed morphological study of specimens from different geographical regions to achieve a reliable differentiation of *Plagiorchis vespertilionis* (Müller, 1780) and two other closely related *Plagiorchis* spp. and suggested that the ITS region of nuclear rDNA has proved to be very useful for species delimitation in digeneans. At the onset of the study there were four 28S and 11 ITS sequences available for five species of *Plagiorchis*: *P. elegans*, *P. koreanus* Ogata, 1938, *P. maculosus* (Rudolphi, 1802), *P. muelleri* (Tkach & Sharpilo, 1990) and *P. vespertilionis* (see Tkach et al., 1999; 2000a, b, 2001; Snyder & Tkach, 2001).

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2. AIM AND OBJECTIVES

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This study was conceived within the framework of the project "Trematode communities in molluscs as a model system to forecast the impact of climate change on freshwater ecosystems in Central Europe" funded by the Czech Science Foundation. Initially focused on the development of methods for rapid and accurate identification during the assessment of larval parasite diversity in key freshwater molluscs in Central Europe, the study logically expanded to involve all life-cycle stages (where possible) of the most abundant and widespread digenean groups in the freshwater environment, i.e. species complexes within the families Diplostomidae, Echinostomatidae and Plagiorchiidae. The geographic extent of the sampling was also widened through fruitful collaborations. Overall, this research profits from the examination of large samples of digenean larval stages from diverse hosts and freshwater ecosystems and from the assembly of a mass of critical taxonomic expertise within the project.

As indicated in the Introduction, all prerequisites justifying the application of an integrative approach to the species diversity in the digenean groups studied were met at the onset of the project, i.e. morphological similarity between closely related species, existence of complexes of cryptic sibling species, convoluted taxonomy, complex synonymy, difficulties and/or impossibility in identification of larval stages, disagreement between authorities, and virtual lack of molecular data. At the onset of this study there was scant infromation in the sequence databases for the European species of the groups studied. The sequences for *Diplostomum* spp. have been limited to the ITS1 region (8 eight isolates; six species); there were seven sequences for *Echinostoma* spp. (two ITS1-5.8S-ITS2, six *nad*1 and one 28S rDNA sequence; two species); 13 sequences for *Plagiorchis* spp. (nine ITS1-5.8S-ITS2 and four 28S rDNA sequences; five species) and no sequences were available for *Petasiger* spp. and *Tylodelphys* spp.

The large dataset gained using standardised protocols for obtaining multiple-marker data, morphological, molecular and ecological, afforded comparative assessment of morphological and molecular variation within species complexes of three digenean families flourishing in the freshwater environment addressing the following questions:

- How many species of the focal digenean groups are there in Europe? How many species globally?
- What are the levels of morphological and/or molecular variability within the complexes of closely related cryptic species of the focal digenean groups?
- Are the patterns of variation of morphological and genetic traits concordant?
- To what extent does morphological identification reflect species boundaries defined by molecular markers?

2.1. Aim

The aim of this study is to apply an integrative approach to species delimitation within complexes of cryptic species of three major digenean freshwater groups in order to advance the taxonomy and ensure consistent identification of the life-cycle stages and thus provide prerequisites for a better understanding of the diversity of these important parasites in the freshwater ecosystems.

2.2. Objectives

2.2.1. To undertake a molecular prospecting survey for the diversity of the European species of *Diplostomum*, *Echinostoma*, *Petasiger* and *Plagiorchis* and the African species of *Tylodelphys* by generating sequence databases linking mitochondrial and ribosomal DNA sequences for isolates identified based on parasite morphology.

2.2.2. To delimit species boundaries within the focal digenean species complexes *via* combining different lines of evidence (molecular, morphological and ecological).

2.2.3. To carry out a taxonomic revision of the '*revolutum*' species complex of *Echinostoma* (Echinostomatidae) through an integration of morphological, experimental and molecular evidence.

2.2.4. To provide morphological/morphometric characterisation of the parasites and construct (where possible) identification keys to the species level.

2.3. Outline of the results

The results of this study are presented in 11 sub-chapters, each representing a separate publication with co-authors (see statement on candidate's contribution) and structured into abstract, introduction, materials and methods, results, discussion and supplementary data (where applicable). Two appendices contain summarrised information that is illuminating the overall taxonomic and sequencing outputs from this study that are structured by family: Diplostomidae (Appendix 1) and Echinostomatidae (Appendix 2). Appendix 3 contains reprints of three additional papers published in peer-reviewed journals during the tenure of my PhD candidacy and Appendix 4 contains my CV.

3. RESEARCH PAPERS

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3.1.1. Paper I

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

Georgieva, S., Soldánová, M., Pérez-del-Olmo, A., Dangel, D. R., Sitko, J., Sures, B. & Kostadinova, A.

International Journal for Parasitology (2013) 43: 57–72

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

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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

☆ 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.

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3.1.2. Paper II

Fish pathogens near the Arctic Circle: molecular, morphological and ecological evidence for unexpected diversity of Diplostomum *(Digenea: Diplostomidae) in Iceland*

Blasco-Costa, I., Faltýnková, A., Georgieva, S., Skírnisson, K., Scholz, T. & Kostadinova, A.

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Fish pathogens near the Arctic Circle: molecular, morphological and ecological evidence for unexpected diversity of *Diplostomum* (Digenea: diplostomidae) in Iceland ☆

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ABSTRACT

Host–parasite systems at high latitudes are promising model systems for detecting and predicting the impact of accelerated environmental change. A major challenge is the lack of baselines for the diversity and distribution of parasites in Arctic wildlife, especially in the freshwater environment. Here we present the first known estimates of the species diversity and host associations of *Diplostomum* spp. in sub-Arctic freshwater ecosystems of the Palaearctic. Our analyses integrating different analytical approaches, phylogenies based on mitochondrial and nuclear DNA, estimates of genetic divergence, character-based barcoding, morphological examination, precise detection of microhabitat specialisation and host use, led to the discovery of one described and five putative new species that complete their life-cycles within a fairly narrow geographic area in Iceland. This increases the species richness of *Diplostomum* in Iceland by 200% and raises the number of molecularly characterised species from the Palaearctic to 17 species. Our results suggest that the diversity of *Diplostomum* spp. is underestimated globally in the high latitude ecosystems and call for a cautionary approach to pathogen identification in developing the much needed baselines of pathogen diversity that may help detect effects of climate change in the freshwater environment of the sub-Arctic.

1. Introduction

The ecosystems in the Earth's northern circumpolar regions, typically characterised as simple, low diversity systems with short trophic linkages, few pathogens and limited capacity for adaptation to environmental change (Hoberg et al., 2012), have been identified as a vital frontier for the exploration of emerging infectious diseases and the large-scale drivers that influence the distribution, host associations and evolution of pathogens in wildlife populations (Hoberg et al., 2008). Host-parasite systems at high latitudes are characterised by low diversity, structured by cycles of episodic dispersal/isolation and diversification in response to shifting climates (reviewed in Hoberg et al., 2012) and thus hold promise as model systems for detecting and predicting the impact of accelerated environmental change. This has called for an integrative approach in developing baselines for contemporary diversity and distribution of parasites and parasitic diseases in the northern wildlife (Hoberg et al., 2008, 2012).

Accurate identification is vital for our understanding of the diversity of northern parasites, host-parasite associations and the detection of disease emergence. The application of molecular prospecting for taxonomic diversity and combining morphological and molecular data for accurate parasite identification appear key to the recent detection of considerable unrecognised diversity among most major groups of macroparasites, with new species and genera being discovered in well-studied host groups (Hoberg et al., 2012 and references therein). However, in contrast to the wealth of knowledge gained recently in research focused on terrestrial host-parasite systems (reviewed in Hoberg et al., 2012), data on parasite diversity and/or distribution in freshwater systems are rather limited. Recent exploration of freshwater parasite diversity

☆ Nucleotide sequence data reported in this paper are available in GenBank under accession numbers KJ726508–KJ726542 (ITS1-5.8S-ITS2) and KJ726433–KJ726507 (*cox*1)

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3.1.3. Paper III

Diplostomum *von Nordmann, 1832 (Digenea: Diplostomidae) in the sub-Arctic: descriptions of the larval stages of six species discovered recently in Iceland*

Faltýnková, A., Georgieva, S., Kostadinova, A., Blasco-Costa, I., Scholz, T. & Skírnisson, K.

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Syst Parasitol (2014) 89:195–213
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Diplostomum von Nordmann, 1832 (Digenea: Diplostomidae) in the sub-Arctic: descriptions of the larval stages of six species discovered recently in Iceland

Anna Faltýnková · Simona Georgieva · Aneta Kostadinova · Isabel Blasco-Costa · Tomáš Scholz · Karl Skírnisson

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Abstract Frequent infections with *Diplostomum* spp. (Digenea: Diplostomidae) were found in the freshwater snail *Radix peregra* (Müller) and three fish species, the salmonids *Salmo trutta fario* L., *Salvelinus alpinus* (L.) and the gasterosteid *Gasterosteus aculeatus* L., collected in four lakes in south-western Iceland in 2012. Detailed analysis of the isolates integrating molecular, morphological and ecological data revealed that these belong to *Diplostomum spathaceum* (Rudolphi, 1819) and five putative new species (three infecting both snails and fish). This paper provides detailed descriptions of the metacercariae of the six species-level lineages of *Diplostomum* spp. and of the cercariae of three of the lineages discovered in Iceland with comments on the application of ITS1 rDNA for species distinction within *Diplostomum* von Nordmann, 1832 in the light of the novel data. We strongly suggest the use of molecular evidence based on *cox*1 gene sequences (in addition to ITS1-5.8S-ITS2 sequences) in association with detailed assessment of the morphology of the larval stages in future studies of *Diplostomum* spp. in fish and snails.

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Introduction

In a study on digenean parasites of the freshwater snail *Radix peregra* (Müller) and three fish species, the salmonids *Salmo trutta fario* L. and *Salvelinus alpinus* (L.) and the gasterosteid *Gasterosteus aculeatus* L., in four lakes in south-western Iceland, we found frequent infections with *Diplostomum* spp. Detailed analysis of the isolates integrating molecular, morphological and ecological data resulted in the discovery of one described [*Diplostomum spathaceum* (Rudolphi, 1819)] and five putative new species that complete their life-cycles within a narrow geographic area (Blasco-Costa et al., 2014). Formal naming of the new species awaits discovery of the adults *via* matching of larval and adult life-cycle stages with the aid of molecular markers. However, this may be severely delayed or deemed impossible due to ethical reasons and other obstacles in examination of the bird definitive hosts.

3.1.4. Paper IV

Molecular and morphological evidence for three species of Diplostomum *(Digenea: Diplostomidae), parasites of fishes and fish-eating birds in Spain*

Pérez-del-Olmo, A. Georgieva, S., Pula, H. & Kostadinova, A.

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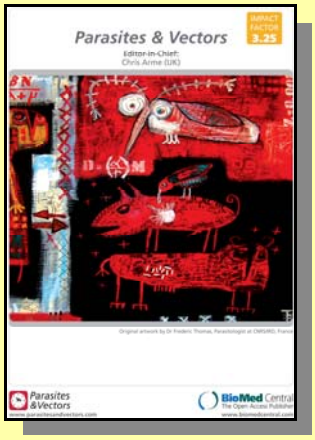

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Parasites & Vectors

RESEARCH Open Access

Molecular and morphological evidence for three species of *Diplostomum* (Digenea: Diplostomidae), parasites of fishes and fish-eating birds in Spain

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Abstract

Background: Recent molecular studies have revealed high species diversity of *Diplostomum* in central and northern Europe. However, our knowledge of the distribution of *Diplostomum* spp. in the southern distributional range in Europe of the snail intermediate hosts (*Lymnaea stagnalis* and *Radix* spp.) is rather limited. This study aims to fill this gap in our knowledge using molecular and morphological evidence.

Methods: Nineteen fish species and six fish-eating bird species were sampled opportunistically in three regions (Catalonia, Extremadura and Aragon) in Spain. All isolates of *Diplostomum* spp. were characterised morphologically and molecularly. Partial sequences of the barcode region of the *cox*1 mitochondrial gene and complete sequences of the ribosomal ITS1-5.8S-ITS2 gene cluster were used for molecular identification of the isolates.

Results: Integrated morphological and molecular analyses demonstrated the presence of three species among the larval and adult isolates of *Diplostomum* spp. sampled in Spain: *Diplostomum spathaceum* (in fish and birds), *D. pseudospathaceum* (in birds) and *Diplostomum* sp. (in fish) referred to as Clade Q *sensu* Georgieva *et al.* (Int J Parasitol, 43:57–72, 2013). We detected ten *cox*1 haplotypes among the isolates of *D. spathaceum* with only one haplotype shared with adult isolates from central and northern Europe. No specific geographic pattern of the distribution of the novel haplotypes was found.

Conclusion: This first molecular exploration of the diversity of *Diplostomum* spp. in southern Europe indicates much lower species richness compared with the northern regions of Europe.

Keywords: *Diplostomum spathaceum*, *Diplostomum pseudospathaceum*, Lens metacercariae, Freshwater fish, Gulls, Spain, *Cox*1, ITS1-5.8S-ITS2

Background

Diplostomum von Nordmann, 1832 is a relatively large genus of widely distributed digeneans with three-host life-cycles involving lymnaeid snails and fish as intermediate hosts and fish-eating birds (predominantly gulls) as definitive hosts. There are 41 nominal species described within the Palaearctic, mainly from Europe (see [1] for details). However, treatment of the data on the geographic and host ranges of *Diplostomum* spp. have long been hindered by taxonomic and identification problems concerning all life-cycle stages.

The use of molecular markers has proved to be valuable and more efficient than experimental approaches in elucidating parasite life-cycles by linking larvae with adults, e.g. [1-5]. The mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) barcode region was found to be suitable for this goal as well as for the identification and recognition of cryptic species diversity within *Diplostomum* [1,6,7].

Recent molecular studies linking *cox*1 and ITS1-5.8S-ITS2 sequences for larval and adult isolates, which were identified based on parasite morphology, have revealed high species diversity of *Diplostomum* in central and northern Europe [1,7]. However, our knowledge of the distribution of *Diplostomum* spp. in the southern

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distributional range in Europe of the snail intermediate hosts (*Lymnaea stagnalis* and *Radix* spp.) is rather limited. Virtually no data exist for infections with *Diplostomum* spp. in the intermediate and definitive hosts in southern Europe. In Spain, two species have been recorded in populations of the gull definitive hosts. *Diplostomum spathaceum* was reported in four out of 324 yellow-legged gulls referred to as "*Larus cachinnans*" [8] and "*Larus michahellis*" [9] in Galicia and *D. pseudospathaceum* was recorded in one of 122 "*L. cachinnans*" from Medes Islands [10,11]. Similarly, there is a lack of data from the intermediate fish hosts; only unidentified metacercariae of *Diplostomum* sp. were reported in *Anguilla anguilla* in the Rivers Ulla and Tea in Galicia [12].

In this study, we used the molecular framework and the recently generated genetic datasets for Nearctic and Palaearctic species of the genus [1,6,13] to investigate species diversity of *Diplostomum* in birds and fishes sampled opportunistically in three regions in the northern and southern Spain. We provide the first molecular evidence associated with descriptions of the hologenophores *sensu* Pleijel *et al*. [14] for three species of *Diplostomum*.

Methods

Sample collection and processing

An opportunistic sampling strategy was adopted for this study, which was focused on examination of a diverse array of hosts rather than large samples of a single host species. Table 1 provides a list of the fish hosts and localities in different regions in Spain. Fish were obtained in collaboration with the regional governments of Extremadura, Aragón and Catalunya. A total of 230 fish belonging to 19 species and 10 families was examined in 2012 for the presence of eye dwelling metacercariae. The samples of *Pseudochondrostoma willkommi* and *Salmo trutta* collected in Villafranco del Guadiana and Jerte were obtained from aquiculture centres of the regional government of Extremadura whereas the remaining fish species/samples were collected in rivers. The largest number of individuals and species was collected in the Ebro Delta. The aquaculture system in Villafranco del Guadiana Aquaculture Centre comprises a central octagonal pool (depth 1 m; surface c.100 m^2) surrounded by a group of pentagonal pools (depth <1 m; surface c.100 m^2) (Figure 1A). The central pool is used for culturing mature breeders of *P. willkommi* of different ages whereas the peripheral pools are used for fish fry for up to two seasons; the latter are transferred to the central pool after reaching maturity. All pools are covered with nets to decrease predation by fish-eating birds and have an open water circulation system with a steady flow of 20 L/min. Although efforts are made to keep the water quality within the accepted ranges, the degree of eutrophication is high. Pools have been completely dried on various occasions but soon afterwards were repopulated by freshwater snails.

A total of 31 fish eating birds were obtained from bird recovery centres in Catalunya (Spain) in 2012 in order to obtain adult specimens of *Diplostomum* (Table 2). Six species of birds of four families were examined: (i) Laridae [*Larus ridibundus* L., *Larus argentatus michahellis* Naumann]; (ii) Sternidae [*Sterna sandvicensis* Latham]; (iii) Ardeidae [*Ardea cinerea* L. and *Ixobrychus minutus* (L.)]; (iv) Phalacrocoracidae [*Phalacrocorax aristotelis* (L.)]. The largest number of birds was obtained from the Ebro Delta.

All metacercariae were dissected out from fresh fish, fixed in hot saline solution and preserved in molecular biology grade ethanol whereas all adult worms were collected from birds found dead and frozen until necropsy; these were also preserved in molecular grade ethanol. The morphology of the larval and adult stages of *Diplostomum* spp. was studied on live and fixed material from series of photomicrographs made for each isolate with a digital camera of an Olympus BX51 microscope prior to sequencing; measurements were taken from the digital images with the aid of Quick Photo Camera 2.3 image analysis software. The structure of the secondary excretory system was reconstructed from serial microphotographs and the number of excretory concretions was counted.

All measurements in the descriptions and tables are in micrometres and are presented as the range followed by the mean in parentheses.

Sequence generation

Total genomic DNA was isolated from single ethanol-fixed adult individuals using the Chelex method (see [15] for details). Partial fragments of the barcode region of the *cox*1 mitochondrial gene [16] were obtained by polymerase chain reaction (PCR) amplifications using Ready-To-Go PCR beads (GE Healthcare, UK) and the diplostomid-specific PCR primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′) and Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′) designed by Moszczynska *et al*. [16] (see [1] for details). PCR amplifications of the ITS1-5.8S-ITS2 gene cluster were performed as above using the primers D1 (forward: 5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2 (reverse: 5′-CGT TAC TGA GGG AAT CCT GGT-3′) and thermocycling conditions of Galazzo *et al*. [17].

PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen Ltd, UK) and sequenced directly from both strands using the PCR primers (*cox*1) and the primers from [18]: BD1 (forward: 5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2: (reverse: 5′-TAT GCT TAA ATT CAG CGG GT-3′) (ITS1-5.8S-ITS2) with ABI BigDye chemistry (ABI Perkin-Elmer, UK), alcohol-precipitated, and run on an ABI Prism 3130 x 1 automated sequencer. Contiguous sequences were assembled with MEGA v5 [19]

Table 1 Summary data for the fish species examined/infected with *Diplostomum* spp.

Fish species	Fish family	Locality	Date of collection	No. examined (infected)	Total length (range, mm)
**Carassius auratus* (L.)	Cyprinidae	Ebro Delta[a]	18.ii.2012	2	121 – 248
**Cyprinus carpio* L.	Cyprinidae			13 (1)	290 – 379
**Silurus glanis* L.	Siluridae			2	440 – 460
**Pseudorasbora parva* (Temminck & Schlegel)	Cyprinidae			15	45 – 103
**Lepomis gibbosus* (L.)	Centrarchidae			1	52
Liza ramada (Risso) juv.	Mugilidae			10	90 – 183
**Misgurnus anguillicaudatus* (Cantor)	Cobitidae			15 (1)	50 – 128
Anguilla anguilla (L.)	Anguillidae	Ebro Delta[a]	17.v.2012	5	158 – 255
Atherina boyeri Risso	Atherinidae			10	34 – 44
**Cyprinus carpio* L.	Cyprinidae			1	192
**Gambusia holbrooki* Girard	Poeciliidae			18	24 – 50
Liza ramada (Risso) juv.	Mugilidae			1	58
**Lepomis gibbosus* (L.)	Centrarchidae			14	43 – 65
**Misgurnus anguillicaudatus* (Cantor)	Cobitidae			16 (2)	52 – 122
Pomatoschistus microps (Krøyer)	Gobiidae			1	32
**Pseudorasbora parva* (Temminck & Schlegel)	Cyprinidae			27	49 – 79
**Silurus glanis* L.	Siluridae			1 (1)	409
Tropidophoxinellus alburnoides (Steindachner)	Cyprinidae	River Albarragena[b]	21.ii.2012	4	57 – 89
Tropidophoxinellus alburnoides (Steindachner)	Cyprinidae	River Luorianilla[b]	06.vi.2012	8	55 – 75
Pseudochondrostoma willkommii (Steindachner)	Cyprinidae	Villafranco del Guadiana[b]	06.iii.2012	10 (10)	235 – 262
Salmo trutta L.	Salmonidae	Jerte[c]	07.iii.2012	3	262 – 291
Parachondrostoma miegii (Steindachner)	Cyprinidae	River Piedra[d]	24.ix.2012	5	139 – 177
Oncorhynchus mykiss (Walbaum)	Salmonidae			2	170 – 195
Squalius pyrenaicus (Günther)	Cyprinidae			10	84 – 135
Salmo trutta L.	Salmonidae	Lake Espejo[d]	24.ix.2012	2	490 – 497
Luciobarbus graellsii (Steindachner)	Cyprinidae			3	236 – 405
Oncorhynchus mykiss (Walbaum)	Salmonidae			1	441
Salmo trutta L.	Salmonidae	River Aragon[d]	25.ix.2012	12	70 – 188
Salmo trutta L.	Salmonidae	River Ara[e]	25.ix.2012	12	68 – 146
Gobio lozanoi Doadrio & Madeira	Cyprinidae	River Cinca[e]	25.ix.2012	1	53
**Gambusia holbrooki* Girard	Poeciliidae			5	21 – 29

*Invasive species are marked with a star; [a]Tarragona; [b]Badajoz; [c]Caceres; [d]Zaragoza; [e]Huesca.

and submitted to GenBank (details and accession numbers are shown in Table 3).

Alignments and data analysis

The newly-generated and published sequences were aligned together with MUSCLE implemented in MEGA v5; *cox*1 sequences were aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code [20]. The *cox*1 alignment (410 nt; 46 sequences) comprised the 18 newly-generated (Table 3) and 28 published sequences, the latter including 1 – 5 representative sequences per species/lineage identified in previous studies in Europe [1,13]; see Table 4 for details. The ITS1-5.8S-ITS2 alignment (997 nt; 35 sequences) comprised seven new sequences for Spanish isolates sub-sampled within the *cox*1-derived clades and 29 published sequences, representative for the species/lineages sequenced in Europe [1,13] and Canada [6,17] (for details see Table 4). Sequences for *Tylodelphys clavata* were used as outgroups.

Distance-based [neighbour-joining (NJ)] and model-based [maximum likelihood (ML) and Bayesian inference (BI)] algorithms were used for tree reconstruction. Prior to analyses the best-fit nucleotide substitution models were selected in jModelTest 2.1.1 [21,22] using the Akaike Information Criterion (AIC). These were the Hasegawa-Kishino-Yano model including estimates of invariant sites

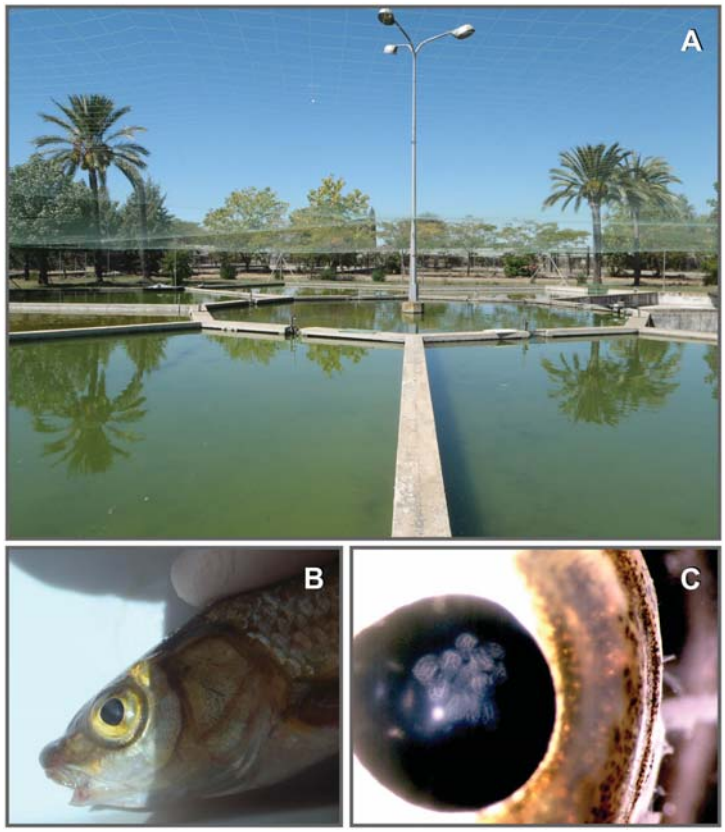

Figure 1 Focus of infection with *D. spathaceum* at the Aquaculture Centre in Villafranco del Guadiana. A, Pool system for culturing *Pseudochondrostoma willkommi* at the Aquaculture Centre in Villafranco del Guadiana; **B**, *P. willkommii* infected with large numbers of lens-dwelling metacercariae of *Diplostomum spathaceum*; **C**, Eye of *P. willkommii* with lens capsule close to rupture due to the large numbers of metacercariae of *D. spathaceum*.

and among-site rate heterogeneity (HKY + I + G) for the *cox*1 dataset and the Hasegawa-Kishino-Yano model including estimates of among-site rate heterogeneity (HKY + G) for the ITS dataset. ML analyses were performed in PhyML 3.0 [23] with a non-parametric bootstrap validation based on 1,000 replicates. BI analyses were carried out in MrBayes 3.2 [24] using Markov Chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains during 10^7 generations, sampling trees every 10^3 generations. The first 25% of the sampled trees were discarded as "burn-in" for each data set and the consensus tree topology and the nodal support were estimated from the remaining samples as posterior probability values [25]. Distance matrices (p-distance model, i.e. the percentage of pairwise character differences with pairwise deletion of gaps) were also calculated and explored with MEGA v5.

Results

Diplostomum spp. infections in fish and birds

Of the 230 fish of 19 species studied, only 15 were infected with *Diplostomum* spp.: one *Cyprinus carpio* (Cyprinidae), one *Silurus glanis* (Siluridae), three *Misgurnus anguillicaudatus* (Cobitidae) and ten *Pseudochondrostoma willkommii* (Cyprinidae). All infected fishes were collected in the Ebro Delta (Tarragona, Spain) with the exception of *P. willkomii* originating from the aquaculture centre of Villafranco del Guadiana (Badajoz, Spain) (Table 1). It is worth noting that infections with metacercariae of *Diplostomum* spp. were detected in some (*C. carpio* and *M. anguillicaudatus*) and not in other relatively well-sampled species (*Pseudorasbora parva*, *Gambusia holbrooki* and *Lepomis gibbosus*) in the Ebro Delta but also in one of the three *S. glanis* sampled in this locality. All infections with *Diplostomum* spp. in the fish from Ebro Delta were of low intensity (1 to 4 metacercariae).

All *P. willkommii* (n = 10) examined from the aquaculture centre in Villafranco de Guadiana were infected with 95–139 metacercariae. Due to the high parasite load, infections were detectable by visual examination especially in older mature fish (Figure 1B,C). The overall prevalence of infection is estimated as 60–65% with a trend of increase with fish age: 0–25% in fish during the

Table 2 Summary data for the bird species examined/ infected with *Diplostomum* spp.

Bird species	Collection site	No. examined (infected)
Larus argentatus michahellis Naumann	Ebro Delta (Tarragona)	6 (2)
Larus argentatus michahellis Naumann	Barcelona	2
Larus argentatus michahellis Naumann	Alella (Barcelona)	1
Larus argentatus michahellis Naumann	Sabadell (Barcelona)	1
Larus argentatus michahellis Naumann	Empuria Brava (Girona)	1
Larus argentatus michahellis Naumann	Figueres (Girona)	1
Larus argentatus michahellis Naumann	Roses (Girona)	2
Larus argentatus michahellis Naumann	Tarragona	1
Larus argentatus michahellis Naumann	Cambrils (Tarragona)	1
Larus ridibundus L.	Ebro Delta (Tarragona)	5 (3)
Larus ridibundus L.	Cunit (Tarragona)	1 (1)
Sterna sandvicensis (Latham)	Roda de Bará (Tarragona)	1
Phalacrocorax aristotelis (L.)	Tarragona	1
Ardea cinerea L.	Ebro Delta (Tarragona)	5
Ixobrychus minutus (L.)	Ebro Delta (Tarragona)	2

first year; 25–50% during the second year; 50–75% during the third year; up to 90% during the fourth year.

A total of 31 fish-eating birds belonging to six species was examined (Table 2). Of these, only six gulls were infected with *Diplostomum* spp.: two *Larus argentatus michahellis* and three *L. ridibundus* originating from Ebro Delta (Tarragona) and one *L. ridibundus* from Cunit (Tarragona). Representative adult specimens of the two *Diplostomum* spp. identified in the material from gulls based on morphology, i.e. *D. spathaceum* and *D. pseudospathaceum*, and all metacercariae recovered from fish were selected for sequencing.

Molecular identification

Partial *cox*1 sequences were obtained for seven adult isolates collected from two gull hosts (*Larus ridibundus* and *L. cachinnans*) and 11 metacercarial isolates collected from the lenses of four fish hosts (*Cyprinus carpio*, *Misgurnus anguillicaudatus*, *Pseudochondrostoma willkommii* and *Silurus glanis*). Similar to a previous study on *Diplostomum* spp. in Europe [1], phylogenetic analyses of the *cox*1 dataset (410 nt) recovered eight species/lineages comprising *D. spathaceum, D. pseudospathaceum*, *D. spathaceum*/*parviventosum* referred to as Clade Q *sensu* Georgieva *et al*. [1], '*D. mergi*' complex (including three putative species) and '*D. baeri*' complex (representing two sibling species) (Figure 2). The analyses provided robust evidence that most of the isolates are conspecific with *D. spathaceum sensu* Georgieva *et al*. [1] (Figure 2). These represented five adult isolates ex *L. ridibundus* and *L. argentatus michahellis* from Ebro Delta, one adult isolate ex *L. ridibundus* from Cunit, seven metacercarial isolates ex *P. willkommii* from Villafranco del Guadiana, two metacercarial isolates ex *M. anguillicaudatus* and a single isolate ex *S. glanis*, the last two fish species both collected from Ebro Delta.

The intraspecific divergence within the *D. spathaceum* clade ranged between 0 and 1.5%, i.e. within the known range of intraspecific variation for *Diplostomum* spp. [1]. The material collected in Spain was represented by a total of 10 haplotypes (Table 3) with only one haplotype shared with adult isolates from central and northern Europe (haplotype 2, isolate ex *M. anguillicaudatus* and JX986892). There was no specific geographic pattern of the distribution of the novel haplotypes. Thus isolates from *Pseudochondrostoma willkommii* from the population of Villafranco del Guadiana were represented by six haplotypes with only one shared and there were shared haplotypes among isolates from geographically distant host samples, e.g. among larval isolates from Villafranco del Guadiana and adult isolates from Ebro Delta and Cunit (haplotypes 1, 3 and 4) (see Table 3 for details).

Numerous attempts were made to obtain sequences for isolates of adult *D. pseudospathaceum* identified based on morphology but only one was successful; this may be due to the fact that the infected birds were collected long after their death. The sequence for the single isolate ex *Larus ridibundus* clustered within the strongly supported clade (Figure 2) representing sequences for adult isolates of *D. pseudospathaceum* identified based on morphology [1]. The Spanish isolate was represented by a unique haplotype which differed by 1.2-1.7% from the remaining three haplotypes within the *D. pseudospathaceum* clade.

Finally, a sequence from a single metacercaria ex *Cyprinus carpio* from the Ebro Delta clustered together with sequences for one cercarial isolate ex *Radix auricularia* (RA97) and two metacercarial isolates ex *R. rutilus* (RR43 and RR45) from Lake Constance, all reported as *D. spathaceum* [13] but labelled as *D. mergi* in GenBank (see Clade Q in Figure 2).

A total of seven ITS1-5.8S-ITS2 sequences was generated after a selective sub-sampling of the Spanish isolates within the three *cox*1 clades of *Diplostomum* spp. The analysis of the ITS data (997 nt positions) resulted in molecular identification of these isolates concordant with that based on the *cox*1 gene trees with strong support (Figure 3). The intraspecific divergence within the *D. spathaceum* clade ranged between 0 and 0.4%. The five representative isolates from the *cox*1 dataset corresponded to four genotypes (with one genotype shared

Table 3 Summary data for the isolates of *Diplostomum* spp. from fishes and birds collected in Spain and used for generation of the *cox*1 and ITS1-5.8S-ITS2 sequences

Species	Life-cycle stage[a]	Isolate	Haplotype	Host	Locality	GenBank accession numbers	
						*cox*1	ITS1-5.8S-ITS2
Diplostomum sp. (Clade Q)	M	CCED	–	*Cyprinus carpio*	Ebro Delta	KP025770	KP025788
Diplostomum pseudospathaceum	A	LRED1	–	*Larus ridibundus*	Ebro Delta	KP025771	JX986854[b]
Diplostomum spathaceum	A	LCED1	1	*Larus argentatus michahellis*	Ebro Delta	KP025772	–
Diplostomum spathaceum	A	LCED2	6	*Larus argentatus michahellis*	Ebro Delta	KP025773	–
Diplostomum spathaceum	A	LCED3	4	*Larus argentatus michahellis*	Ebro Delta	KP025774	–
Diplostomum spathaceum	A	LRC	3	*Larus ridibundus*	Cunit	KP025775	KP025789
Diplostomum spathaceum	A	LRED2	10	*Larus ridibundus*	Ebro Delta	KP025776	–
Diplostomum spathaceum	A	LRED3	8	*Larus ridibundus*	Ebro Delta	KP025777	–
Diplostomum spathaceum	M	MAED1	4	*Misgurnus anguillicaudatus*	Ebro Delta	KP025778	KP025790
Diplostomum spathaceum	M	MAED2	2	*Misgurnus anguillicaudatus*	Ebro Delta	KP025779	KP025791
Diplostomum spathaceum	M	PWVG1	5	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025780	–
Diplostomum spathaceum	M	PWVG2	4	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025781	KP025792
Diplostomum spathaceum	M	PWVG3	7	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025782	KP025793
Diplostomum spathaceum	M	PWVG4	1	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025783	–
Diplostomum spathaceum	M	PWVG5	9	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025784	–
Diplostomum spathaceum	M	PWVG6	3	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025785	–
Diplostomum spathaceum	M	PWVG7	7	*Pseudochondrostoma willkommii*	Villafranco del Guadiana	KP025786	–
Diplostomum spathaceum	M	SGED	6	*Silurus glanis*	Ebro Delta	KP025787	–

[a]M, metacercaria, A, adult; [b]ITS sequence identical with JX986854 of Georgieva *et al.* [1].

between an isolate ex *M. anguillicaudatus* from Ebro Delta and one ex *L. ridibundus* from Cunit).

The sequence from the single adult isolate identified as *D. pseudospathaceum* based on morphology and *cox*1 phylogeny was identical with six sequences of Georgieva *et al*. [1] based on larval and adult isolates from the Czech Republic and Germany and one sequence of Berhrmann-Godel [13]; all these sequences formed a strongly supported clade representing *D. pseudospathaceum* (Figure 3) which also included *Diplostomum* sp. 3 of Locke *et al*. [6] as in previous studies [1,7].

As in the *cox*1 solution, the sequence for the metacercarial isolate ex *C. carpio* clustered together with a sequence labelled in GenBank as "*D. mergi*" for a cercarial isolate (RA97) ex *Radix auricularia* from Lake Constance [13] within the Clade Q *sensu* Georgieva *et al*. [1]. The divergence between the two sequences was 0.8%.

Descriptions of the molecular voucher material

***Diplostomum spathaceum* (Rudolphi, 1819) (adult)**

***Hosts*:** *Larus argentatus michahellis* Naumann; *L. ridibundus* L.

***Localities*:** Ebro Delta, Cunit (Tarragona, Spain).

***Site in host*:** Small intestine.

[Based on five frozen specimens (hologenophores) preserved in ethanol (molecular biology grade)]. Body 1,971 – 2,189 (2,085) long (Figure 4). Forebody oval, dorso-ventrally flattened, 782 – 1,155 long [40 – 43 (42)% of total body length], with maximum width 504 – 726 (592) at level of holdfast organ. Hindbody, elongate-oval, narrower anteriorly, 1,252 – 1,368 (1,285) long, with maximum width 387 – 575 (477) at level of anterior testis.

Oral sucker ventro-subterminal, subspherical, 71 – 93 × 70 – 92 (81 × 78). Pseudosuckers well developed, 109 – 155 × 44 – 62 (139 × 56). Ventral sucker subglobular, 65 – 95 × 80 – 99 (83 × 89), similar in size to oral sucker, located just anterior to mid-forebody. Holdfast organ large, subglobular, 150 – 236 × 202 – 288 (215 × 224), fairly close to or contiguous with ventral sucker. Prepharynx short or absent; pharynx elongate-oval, 55 – 89 × 45 – 59 (73 × 52); oesophagus indistinct; caeca narrow.

Testes 2, large, in posterior half of hindbody; anterior testis transversely elongate, asymmetrical, 171 – 203 × 154 – 224 (183 × 191); posterior testis transversely elongate, symmetrical, horseshoe-shaped, 190 – 317 × 240 – 399 (247 × 334). Seminal vesicle voluminous. Gentital pore dorso-subterminal. Ovary small, dextral, pretesticular, subglobular, 87 × 83, contiguous with anterior testis.

Table 4 Summary data for the isolates of *Diplostomum* spp. retrieved from GenBank

Trematode species	Isolate	Life-cycle stage[a]	Host species	Locality	Accession No. (*cox*1)	Accession No. (ITS1-5.8S-ITS2)	Reference
'*Diplostomum baeri*' 1	STR3	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986862	JX986837	Georgieva *et al.* [1]
'*Diplostomum baeri*' 1	STL1	M	*Salmo trutta fario*	Germany: River Lenne	JX986863	-	Georgieva *et al.* [1]
'*Diplostomum baeri*' 1	STR4	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986864		Georgieva *et al.* [1]
'*Diplostomum baeri*' 1	STL2	M	*Salmo trutta fario*	Germany: River Lenne	JX986865	-	Georgieva *et al.* [1]
'*Diplostomum baeri*' 1	STR7	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986869	-	Georgieva *et al.* [1]
Diplostomum baeri	PF5D3	M	*Perca fluviatilis*	Germany: Lake Constance	JQ639195	-	Behrmann-Godel [13]
Diplostomum baeri	PF15D9	M	*Perca fluviatilis*	Germany: Lake Constance	JQ639193	-	Behrmann-Godel [13]
Diplostomum baeri	PF15D4	M	*Perca fluviatilis*	Germany: Lake Constance	JQ639187	-	Behrmann-Godel [13]
Diplostomum baeri	PF8D7	M	*Perca fluviatilis*	Germany: Lake Constance	JQ639191	-	Behrmann-Godel [13]
Diplostomum baeri	PF6D3	M	*Perca fluviatilis*	Germany: Lake Constance	JQ639189	-	Behrmann-Godel [13]
Diplostomum baeri	–	A	*Larus delawarensis* (exp.)[b]	Canada	-	AY123042	Galazzo *et al.* [17]
Diplostomum huronense	–	A	*Larus delawarensis* (exp.)[b]	Canada	-	AY123044	Galazzo *et al.* [17]
Diplostomum huronense	D.LL.IVT.Cc.3 F.1	M	*Catostomus commersoni*	Canada	-	GQ292513	Locke *et al.* [6]
Diplostomum indistinctum	D.RL.D.Cc.1.2	M	*Catostomus commersoni*	Canada		GQ292508	Locke *et al.* [6]
'*Diplostomum mergi*' 1	RAH1	C	*Radix auricularia*	Germany: Hengsteysee	JX986873	JX986838	Georgieva *et al.* [1]
'*Diplostomum mergi*' 2	RAH2	C	*Radix auricularia*	Germany: Hengsteysee	JX986874	-	Georgieva *et al.* [1]
'*Diplostomum mergi*' 2	RAH3	C	*Radix auricularia*	Germany: Hengsteysee	JX986875	JX986839	Georgieva *et al.* [1]
'*Diplostomum mergi*' 2	RAH4	C	*Radix auricularia*	Germany: Hengsteysee	JX986876	-	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	GGR2	M	*Gobio gobio*	Germany: River Ruhr (Henne)	JX986877	JX986840	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	STR10	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986878	JX986841	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	STR11	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986879	-	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	STR12	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986880	-	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	GGR3	M	*Gobio gobio*	Germany: River Ruhr (Henne)	-	JX986842	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	GGR4	M	*Gobio gobio*	Germany: River Ruhr (Henne)	-	JX986843	Georgieva *et al.* [1]
'*Diplostomum mergi*' 3	STR15	M	*Salmo trutta fario*	Germany: River Ruhr (Henne)	JX986886	-	Georgieva *et al.* [1]
Diplostomum mergi	RR45	M	*Rutilus rutilus*	Germany: Lake Constance	JQ639178	-	Behrmann-Godel [13]
Diplostomum mergi	RR43	M	*Rutilus rutilus*	Germany: Lake Constance	JQ639177	-	Behrmann-Godel [13]
Diplostomum mergi	RA97	C	*Radix auricularia*	Germany: Lake Constance	JQ639179	JQ665458	Behrmann-Godel [13]
Diplostomum paracaudum	CL100	M	*Coregonus lavaretus*	Germany: Lake Constance	-	JQ665457	Behrmann-Godel [13]
Diplostomum pseudospathaceum	LCT3	A	*Larus cachinnans*	Czech Republic: near Tovačov	JX986896	JX986849	Georgieva *et al.* [1]
Diplostomum pseudospathaceum	LSB2	C	*Lymnaea stagnalis*	Germany: Baldeneysee	-	JX986850	Georgieva *et al.* [1]
Diplostomum pseudospathaceum	LSH1	C	*Lymnaea stagnalis*	Germany: Harkortsee	-	JX986851	Georgieva *et al.* [1]

Table 4 Summary data for the isolates of *Diplostomum* spp. retrieved from GenBank *(Continued)*

Diplostomum pseudospathaceum	GAH6	M	*Gasterosteus aculeatus*	Germany: Hengsteysee	-	JX986852	Georgieva *et al.* [1]
Diplostomum pseudospathaceum	LAG2	A	*Larus argentatus*	Poland: near Gdańsk	JX986904	JX986853	Georgieva *et al.* [1]
Diplostomum pseudospathaceum	LCT4	A	*Larus cachinnans*	Czech Republic: near Tovačov	JX986905	JX986854	Georgieva *et al.* [1]
Diplostomum pseudospathaceum	GC87	M	*Gymnocephalus cernuus*	Germany: Lake Constance	-	JQ665456	Behrmann-Godel [13]
Diplostomum spathaceum	LCT1	A	*Larus cachinnans*	Czech Republic: near Tovačov	JX986887	JX986844	Georgieva *et al.* [1]
Diplostomum spathaceum	RAH6	C	*Radix auricularia*	Germany: Hengsteysee		JX986846	Georgieva *et al.* [1]
Diplostomum spathaceum	RAH5	C	*Radix auricularia*	Germany: Hengsteysee		JX986845	Georgieva *et al.* [1]
Diplostomum spathaceum	LAG1	A	*Larus argentatus*	Poland: near Gdańsk	JX986892	JX986847	Georgieva *et al.* [1]
Diplostomum spathaceum	LCT2	A	*Larus cachinnans*	Czech Republic: near Tovačov	JX986895	JX986848	Georgieva *et al.* [1]
Diplostomum sp. 1 SAL-2008	D.IN.SSO.Ld.2 F.6	A	*Larus delawarensis* (exp.)[b]	Canada	-	GQ292519	Locke *et al.* [6]
Diplostomum sp. 2 SAL-2008	D.BR.S.B.20.1	M	*Pimephales notatus*	Canada	-	GQ292505	Locke *et al.* [6]
Diplostomum sp. 3 SAL-2008	D.RL.B08.Ms.1 F.1	M	*Micropterus salmoides*	Canada	-	GQ292511	Locke *et al.* [6]
Diplostomum sp. 4 SAL-2008	D.IN.SSO.Ld.2 F.10	A	*Larus delawarensis*	Canada	-	GQ292520	Locke *et al.* [6]
Tylodelphys clavata	PFL1	M	*Perca fluviatilis*	Germany: River Lippe	JX986909	-	Georgieva *et al.* [1]
Tylodelphys clavata	CL91	M	*Coregonus lavaretus*	Germany: Lake Constance	-	JQ665459	Behrmann-Godel [13]

[a]C, cercaria, M, metacercaria; A, adult; [b]raised in experimental infection.

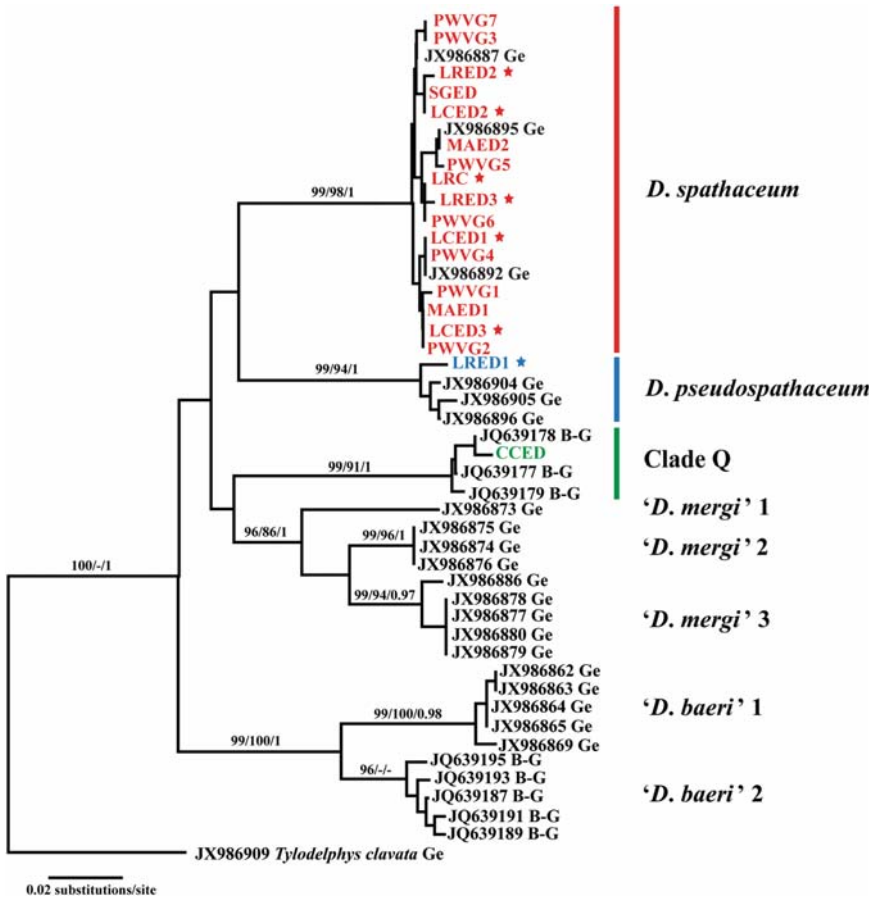

Figure 2 Neighbour-joining (NJ) phylogram reconstructed using the newly-generated and retrieved from GenBank *cox*1 sequences for *Diplostomum* spp. Nodal support from Maximum Likelihood (ML) and Bayesian Inference (BI) analyses indicated as NJ/ML/BI. Outgroup: *Tylodelphys clavata*. The scale-bar indicates the expected number of substitutions per site. Isolates from Spain are coded as in Table 3; stars indicate adult isolates from gulls.

Vitellarium follicular, follicles numerous, small, arranged in four lateral bands surrounding holdfast organ in forebody; bands reach to mid-level of holdfast organ, converge close to posterior margin of forebody, posteriorly to holdfast organ; vitelline follicles in hindbody in two wide, not well-delimited lateral bands, converging medially at level of testes, reaching fairly close to posterior extremity of body. Eggs few, 89 – 99 × 61 – 66 (95 × 63).

***Diplostomum spathaceum* (Rudolphi, 1819) (metacercaria)**
Hosts: *Pseudochondrostoma willkommii* (Steindachner); *Misgurnus anguillicaudatus* (Cantor); *Silurus glanis* L.
Localities: Villafranco del Guadiana (*P. willkommii*) and Ebro Delta (*M. anguillicaudatus* and *S. glanis*), Spain.
Site in host: Eye lens.

[Based on 10 metacercariae (hologenophores) fixed in hot saline solution and preserved in ethanol (molecular biology grade)]. Body elongate-oval, flattened, 277 – 453 × 198 – 295 (376 × 248); primordial hindbody 10 – 26 (16) long (Figure 5). Oral sucker elongate-oval, 40 – 57 × 36 – 41 (45 × 39). Ventral sucker transversely oval, 30 – 43 × 33 – 48 (38 × 43). Two contractile lappets (pseudosuckers) present on each side of oral sucker, 44 – 55 (48) long, with maximum width 22 – 30 (26). Prepharynx very short; pharynx elongate-oval, 29 – 43 × 19 – 26 (37 × 23); oesophagus short; caeca long, wide, reach posterior to holdfast organ. Holdfast organ large, elongate-oval, 63 – 89 × 59 – 90 (75 × 80). Reserve excretory system with numerous, relatively large excretory granules (170 – 184 in number), distributed in a median and two lateral fields.

***Diplostomum pseudospathaceum* Niewiadomska, 1984 (adult)**
***Host*:** *Larus ridibundus* L.
***Locality*:** Ebro Delta (Tarragona, Spain).
***Site in host*:** Small intestine.

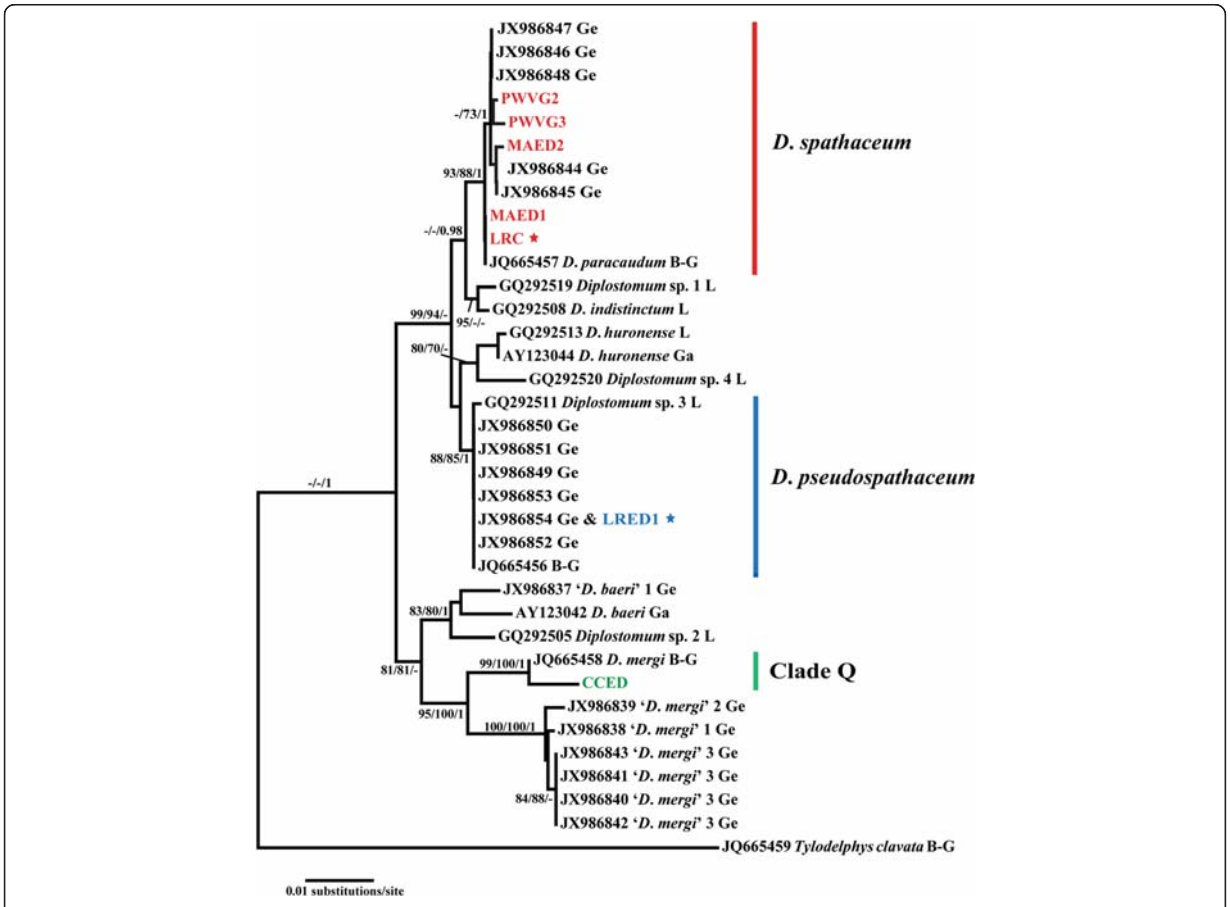

Figure 3 Neighbour-joining (NJ) phylogram reconstructed using the newly-generated and retrieved from GenBank ITS1-5.8S-ITS2 sequences for *Diplostomum* spp. Nodal support from Maximum Likelihood (ML) and Bayesian Inference (BI) analyses indicated as NJ/ML/BI. Outgroup: *Tylodelphys clavata*. The scale-bar indicates the expected number of substitutions per site. Isolates from Spain are coded as in Table 3; stars indicate adult isolates from gulls.

[Based on a single frozen specimen (hologenophore) preserved in ethanol (molecular biology grade)]. Body 2,884 long (Figure 6). Forebody elongate-oval, narrow, dorso-ventrally flattened, tapering anteriorly, 1,075 long (37% of total body length), with maximum width at level of ventral sucker, 526. Hindbody, elongate, sub-cylindrical, narrower anterior to ovary, 1,891 long, with maximum width at level of posterior testis, 163.

Oral sucker ventro-subterminal, subspherical, 69 × 73. Pseudosuckers well developed, 128 × 49. Ventral sucker transversely oval, 67 × 85, slightly larger than oral sucker, located just posterior to mid-forebody. Holdfast organ subglobular, 126 × 118, located well posterior to ventral sucker (at a distance >2 ventral sucker diameters). Prepharynx fairly short; pharynx elongate-oval, 53 × 35; oesophagus short; caeca narrow.

Testes 2, large, in posterior half of hindbody; anterior testis transversely elongate, asymmetrical, 132 × 75; posterior testis larger, transversely elongate, symmetrical, horseshoe-shaped, 237 × 315. Seminal vesicle voluminous. Gentital pore dorso-subterminal. Ovary small, submedian, pretesticular, subglobular, 79 × 78, nearly contiguous with anterior testis. Vitellarium follicular, follicles numerous, small, arranged in two median inter-caecal and four lateral extra-caecal bands in forebody, reaching to the posterior margin of ventral sucker anteriorly; bands, converge close to posterior margin of forebody, posteriorly to holdfast organ; vitelline follicles in hindbody in two wide, dense lateral bands, converging medially at level of gonads, reach fairly close to posterior extremity of body. Eggs few, 96 – 110 × 58 – 63.

***Diplostomum sp.* (metacercaria)**

***Host*:** *Cyprinus carpio* L.

***Locality*:** Ebro Delta (Tarragona, Spain).

***Site in host*:** Eye lens.

[Based on a single metacercaria (hologenophore) fixed and preserved in ethanol (molecular biology grade).]

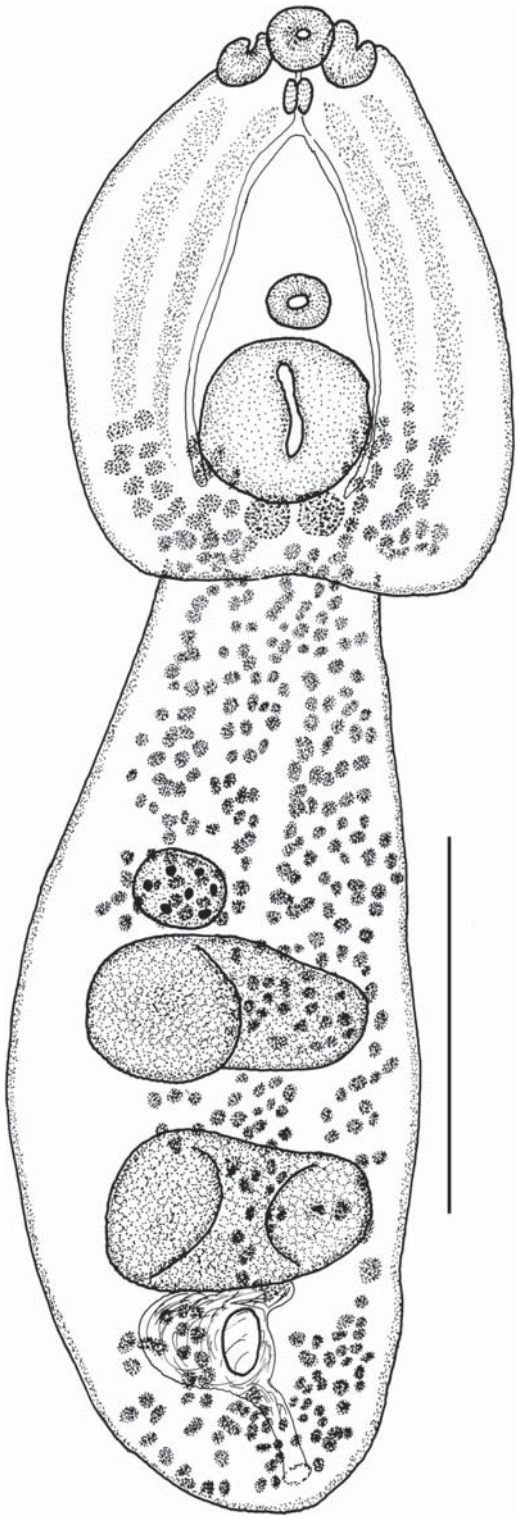

Figure 4 *Diplostomum spathaceum*. Adult ex *Larus argentatus michahellis* (hologenophore). Scale-bar: 500 μm.

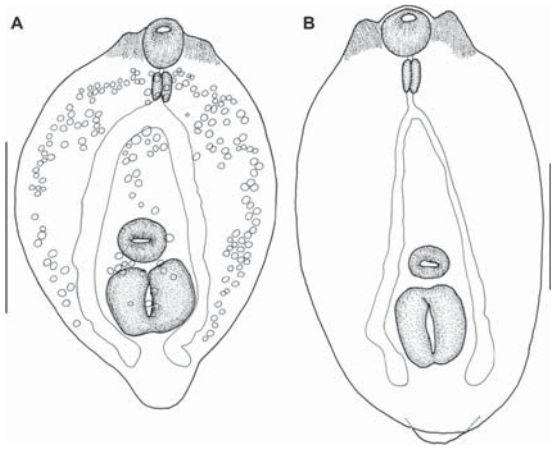

Figure 5 Metacercariae of *Diplostomum spathaceum* ex *Pseudochondrostoma willkommii*. A, Live metacercaria (hologenophore); **B**, Fixed metacercariae (hologenophore). Scale-bars: **A**, 200 μm; **B**, 100 μm.

Body elongate-oval, flattened, 229 × 180; primordial hindbody not evident (Figure 7). Oral sucker spherical, 29 × 29. Ventral sucker subspherical, 37 × 42. Two small contractile lappets (pseudosuckers) present on each side of oral sucker, 31 – 32 long, with maximum width 15 – 16. Prepharynx absent; pharynx subspherical, 24 × 23; oesophagus very short; caeca long, narrow, reach posterior to holdfast organ. Holdfast organ large, transversely elongate, 50 × 84. Reserve excretory system with numerous, dispersed, relatively large excretory granules (c. 215 in number).

Discussion

This first molecular exploration of the diversity of *Diplostomum* spp. in southern Europe indicates much lower species richness compared with the northern regions of Europe (3 *vs* 12 species). Of the six species of fish-eating birds studied in the north of Spain only two gull species were found to host adult *Diplostomum* spp.; however, sample sizes were rather small. The detection of metacercariae in fish also might have been influenced by the differential sample sizes. However, we found infections in an under-sampled fish host as well in some but not in other hosts with relatively large sample sizes. Notably, metacercariae of *Diplostomum* spp. were recovered in three out of the seven invasive fish species examined (*C. carpio*, *M. anguillicaudatus* and *S. glanis*; Table 1) thus indicating that these hosts may have a considerable contribution to the transmission of *Diplostomum* spp. in the Ebro Delta and elsewhere. *M. anguillicaudatus* and *S. glanis* are new host records for *D. spathaceum*.

Another important finding is the high prevalence and abundance of infection with *D. spathaceum* in *P. willkommii*,

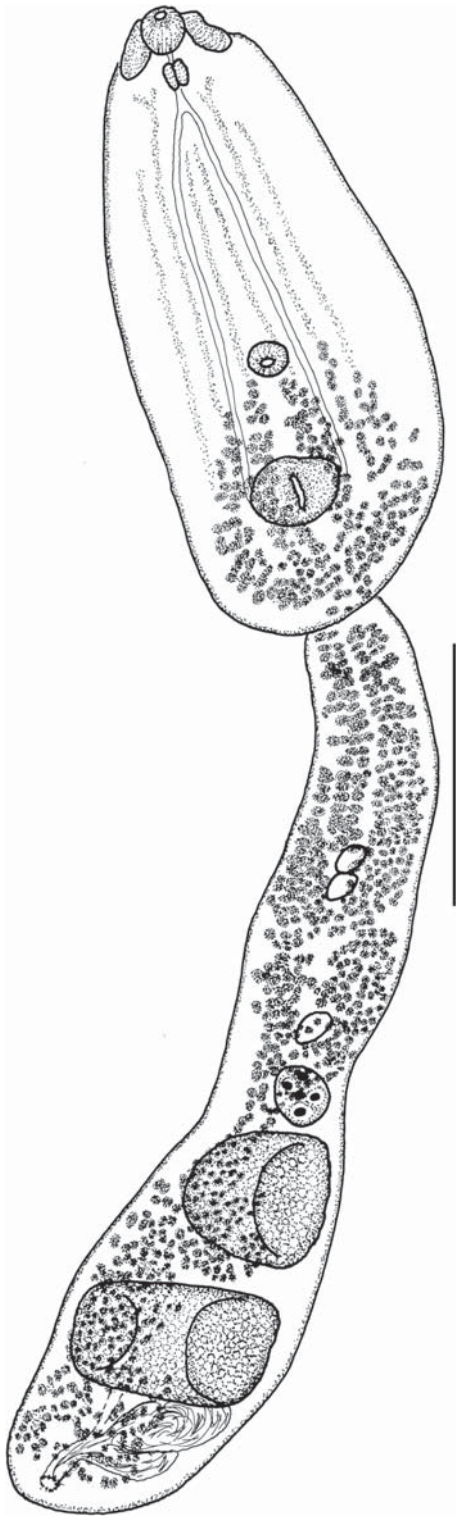

Figure 6 *Diplostomum pseudospathaceum*. Adult ex *Larus ridibundus* (hologenophore). Scale-bar: 500 μm.

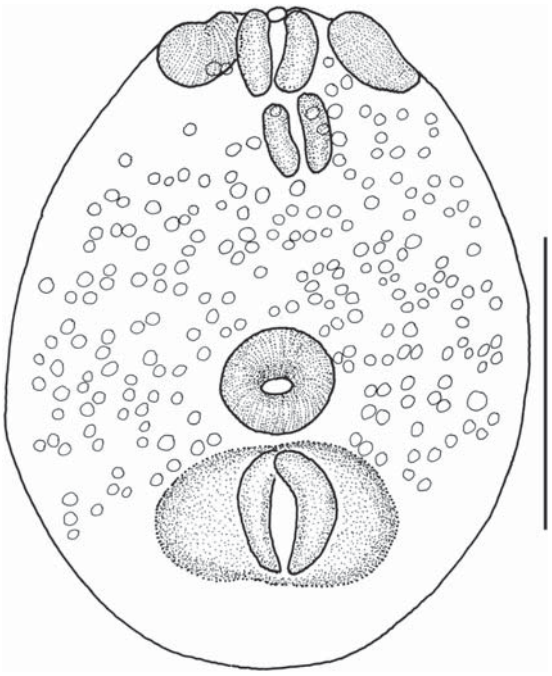

Figure 7 *Diplostomum* sp. ex *Cyprinus carpio*. Fixed metacercaria (hologenophore). Scale-bar: 100 μm.

a native vulnerable species [26] with distribution restricted to the southern Iberian Peninsula in Spain and Portugal. The high levels of infections in the aquaculture centre in Villafranco de Guadiana, where mature breeders from natural populations are being added yearly to the cultured population, reveal a further threat upon this fish species in both natural and fish farming conditions. The shallow, open nature of the pools probably contributes significantly to the establishment of a focus of infection with *D. spathaceum*.

To the best of our knowledge, this study is the first to provide detailed morphometric data and morphological description of the isolates of *Diplostomum* spp. in association with the molecular data used for identification. The morphology of the adult specimens of *D. spathaceum* and *D. pseudospathaceum* used for sequence generation agrees well with the descriptions of *D. spathaceum sensu stricto* and *D. pseudospathaceum* of Niewiadomska [27], respectively. The material of *D. spathaceum* ex *Larus* spp. from Ebro Delta is characterised by lower values (outside the lower range for the material ex *Larus fuscus* L. and *L. ridibundus* from Poland studied by Niewiadomska [27] for the size of the hindbody, holdfast organ, ovary and testes (Table 5). Similarly, the specimen of *D. pseudospathaceum* ex *L. ridibundus* from Ebro Delta had smaller holdfast organ, ovary and testes and much narrower hindbody and longer pseudosuckers compared with the

Table 5 Comparative metrical data for adults of *Diplostomum spathaceum* and *D. pseudospathaceum*

Species	*Dipostomum spathaceum*		*Dipostomum pseudospathaceum*	
Host	*Larus fuscus* L., *Larus ridibundus* L.	*Larus argentatus michahellis* Naumann; *Larus ridibundus* L.	*Larus ridibundus* L.	*Larus ridibundus* L.
Locality	Lake Mamry (Poland)	Ebro Delta (Spain)	Lake Mamry (Poland)	Ebro Delta (Spain)
Source	Niewiadomska [27]	Present study	Niewiadomska [27]	Present study
TL	up to 4,000	1,971 – 2,189	up to 3,600	2,884
FBL	1,110 – 1,480	782 – 1,155	1,030 – 1,720	1,075
FBW	590 – 850	504 – 726	400 – 680	526
HBL	1,560 – 2,920	1,252 – 1,368	960 – 2,190	1,891
HBW	560 – 660	387 – 575	420 – 720	163
OSL	57 – 95	71 – 93	67 – 78	69
OSW	74 – 102	70 – 92	68 – 95	73
PSL	102 – 153	109 – 155	51 – 115	128
PSW	–	44 – 62	–	49
VSL	78 – 95	65 – 95	68 – 103	67
VSW	89 – 102	80 – 99	62 – 119	85
HOL	238 – 374	150 – 236	153 – 335	126
HOW	259 – 399	202 – 288	163 – 388	118
PHL	59 – 74	55 – 89	44 – 74	53
PHW	51 – 74	45 – 59	47 – 66	35
ATL	185 – 540	171 – 203	188 – 503	132
ATW	421 – 629	154 – 224	296 – 629	75
PTL	348 – 592	190 – 317	255 – 666	237
PTW	466 – 658	240 – 399	370 – 666	315
OVL	138 – 222	87	111 – 187	79
OVW	163 – 236	83	142 – 238	78
FO/BL (%)	31 – 48	40 – 43	41 – 58	37
Egg-length	–	89 – 99	–	96 – 110
Egg-width	–	61 – 66	–	58 – 63

Abbreviations: *TL* total body length, *FBL* forebody length, *FBW* forebody width, *HBL* hindbody length, *HBW* hindbody width, *OSL* oral sucker length, *OSW* oral sucker width, *PSL* pseudosucker length, *PSW* pseudosucker width, *VSL* ventral sucker length, *VSW* ventral sucker width, *HOL* holdfast organ length, *HOW* holdfast organ width, *PHL* pharynx length, *PHW* pharynx width, *ATL* anterior testis length, *ATW* anterior testis width, *PTL* posterior testis length, *PTW* posterior testis width, *OVL* ovary length, *OVW* ovary width, *FO/BL (%)* forebody as a percentage of body length.

specimens from the same host studied in Poland (Table 5). These data indicate much higher geographic variation in the morphometric features in both *Diplostomum* spp.

The dimensions of the metacercariae from the three fish hosts identified molecularly as *D. spathaceum* varied within the range provided by Niewiadomska [28] for the metacercariae of this species raised experimentally in *C. carpio*. However, the mean values for the length of body and the size of suckers were lower in the specimens obtained in Spain (Table 6). The metacercaria of *Diplostomum* sp. that was found to be conspecific with the isolates of Clade Q *sensu* Georgieva *et al*. [1] had distinctly smaller oral sucker and shorter holdfast organ compared with both Spanish and Polish isolates of *D. spathaceum* (Table 6). Finally, the metacercariae of both *Diplostomum* spp. examined in Spain had distinctly lower number of excretory granules in the secondary excretory system than the experimentally raised metacercariae ex *C. carpio* (see [28]; Table 6).

Although the molecular and morphological identification of the larval and adult isolates of *D. spathaceum* and *D. pseudospathaceum* were straightforward, we failed to identify one isolate recovered in *C. carpio*. The analysis of both *cox*1 and ITS1-5.8S-ITS2 sequences placed this isolate within the Clade Q (i.e. questionable), a label used by Georgieva *et al*. [1] to indicate five identical ITS1 sequences from Europe: two for cercariae ex *R. ovata* identified as *D. spathaceum* and one for cercariae ex *R. ovata* identified as *D. parviventosum* by Niewiadomska & Laskowski [29] in Poland; one for a metacercaria ex

Table 6 Comparative metrical data for the metacercariae of *Diplostomum spathaceum* and *Diplostomum* sp. (Clade Q)

Species	*Dipostomum spathaceum*				*Diplostomum* sp. (Clade Q)
Host	*Cyprinus carpio* L.		*Pseudochondrostoma willkommii* (Steindachner); *Misgurnus anguillicaudatus* (Cantor); *Silurus glanis* L.		*Cyprinus carpio*
Locality	Experimental infection		Villafranco del Guadiana and Ebro Delta (Spain)		
Source	Niewiadomska [28]		Present study		Present study
	Range	Mean	Range	Mean	n =1
BL	340 – 451	398	277 – 453	376	229
BW	170 – 296	217	198 – 295	248	180
HL	–	–	10 – 26	16	0
OSL	42 – 54	48	40 – 57	45	29
OSW	42 – 52	45	36 – 41	39	29
PSL	–	–	44 – 55	48	31 – 32
PSW	–	–	22 – 30	26	15 – 16
VSL	39 – 56	46	30 – 43	38	37
VSW	42 – 59	53	33 – 48	43	42
PHL	25 – 39	31	29 – 43	37	24
PHW	12 – 25	20	19 – 26	23	23
HOL	68 – 93	77	63 – 89	75	50
HOW	62 – 102	85	59 – 90	80	84
No. of excretory granules	c. 300	–	170 – 184	178	c. 215

Abbreviations: *BL* body length, *BW* body width, *HL* primordial hindbody length, *OSL* oral sucker length, *OSW* oral sucker width, *PSL* pseudosucker length, *PSW* pseudosucker width, *VSL* ventral sucker length, *VSW* ventral sucker width, *HOL* holdfast organ length, *HOW* holdfast organ width, *PHL* pharynx length, *PHW* pharynx width.

R. rutilus from Finland submitted to GenBank as *D.* cf. *parviventosum*/*spathaceum* by Rellstab *et al.* [30]; and one for cercariae ex *R. auricularia* (isolate RA97) from Lake Constance [13]; the latter was designated as *D. spathaceum* but submitted to GenBank as *D. mergi*. Using the sequences of Behrmann-Godel [13] for both *cox*1 and ITS1-5.8S-ITS2, we found that this clade, incorporating our sequence for the metacercaria ex *C. carpio*, is strongly supported and reconstructed as sister to the species-level lineages of the '*Diplostomum mergi*' species complex *sensu* Georgieva *et al.* [1]. Unfortunately, no identification to the species level can be attempted for the isolates within this clade since all represent larval stages for which, with the exception of the present data, no morphological evidence has been provided. The congruent morphological and molecular identification of the adult isolates of *D. spathaceum* achieved here, supports the suggestion of Georgieva *et al.* [1] that isolates in Clade Q may represent *D. parviventosum*. Further molecular and morphological evidence is required, preferably based on adult isolates, in order to solve the species-level identification of this clade.

Conclusion

This first molecular exploration of the diversity of *Diplostomum* spp. in southern Europe indicates much lower species richness compared with the northern regions of Europe.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
APO conceived and designed the study, obtained the samples, undertook the morphological characterisation and helped draft the MS. HJP obtained samples, discussed the results and took part in the preparation of the MS and figures. SG carried out the sequencing and phylogenetic analyses, took part in the morphological assessment, and prepared the first draft of the MS and figures. AK coordinated the project and helped draft the MS. All authors read and approved the final manuscript.

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3.1.5. Paper V

A first insight into the barcodes for African diplostomids (Digenea: Diplostomidae): Brain parasites in Clarias gariepinus *(Siluriformes: Clariidae)*

Chibwana, F. D., Blasco-Costa, I., Georgieva, S., Hosea, K. M., Nkwengulila, G., Scholz, T. & Kostadinova, A.

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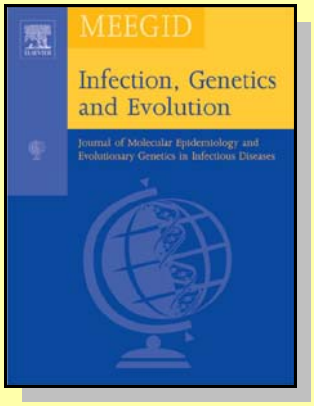

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Tylodelphys spp.
Diplostomum spp.
Clarias gariepinus
Synodontis nigrita
Tanzania
Africa

ABSTRACT

Diplostomid trematodes comprise a large and diverse group of widespread digeneans whose larval stages are important parasitic pathogens that may exert serious impacts in wild and cultured freshwater fish. However, our understanding of their diversity remains incomplete especially in the tropics. Our study is the first application of a DNA-based approach to diplostomid diversity in the African continent by generating a database linking sequences for the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) barcode region and ITS1-5.8S-ITS2 rRNA gene cluster for brain-infecting diplostomid metacercariae from the catfish *Clarias gariepinus*. Analyses of newly-generated partial *cox*1 sequences for 34 larval isolates of *Tylodelphys* spp. from Tanzania and *Diplostomum* spp. from Tanzania and Nigeria revealed three strongly supported reciprocally monophyletic lineages of *Tylodelphys* spp. and one of an unknown species of *Diplostomum*. The average intraspecific divergence for the *cox*1 sequences for each recognised novel lineage was distinctly lower compared with interspecific divergence (0.46–0.75% vs 11.7–14.8%). The phylogenetic hypotheses estimated from Bayesian inference and maximum likelihood analyses of ITS1-5.8S-ITS2 data exhibited congruent strong support for the *cox*1-derived lineages. Our study thus provides molecular-based evidence for the existence of three distinct brain-infecting species co-occurring in natural populations of *C. gariepinus*. Based on phylogenetic analyses, we re-allocated *Diplostomum mashonense* Beverley-Burton (1963) to the genus *Tylodelphys* as a new combination. We also generated *cox*1 and ITS1-5.8S-ITS2 sequences for an unknown species of *Diplostomum* from another African fish host, *Synodontis nigrita*.

1. Introduction

Diplostomid trematodes (Digenea) comprise a large and diverse group of widespread digeneans that parasitize, as adults, a wide range of piscivorous birds and occasionally mammals. They utilise a three-host life-cycle with freshwater snails acting as first intermediate hosts and freshwater fishes (occasionally amphibians) as second intermediate hosts. In freshwater fishes diplostomid larvae (metacercariae) are found encysted, encapsulated in tissues or free in skin, eyes, musculature and central nervous system (Gibson, 1996). Diplostomid larval stages are important pathogens that may exert serious impacts on both natural and aquacultured fish populations. The migration of large numbers of infective post-cercarial stages towards the specific sites of infection may cause fish mortalities particularly in young individuals due to haemorrhaging of capillaries and obstructed blood vessels primarily in the head and brain (Szidat and Nani, 1951; Shigin, 1986). Metacercariae can cause, at high densities, haemorrhaging in the musculature, eye cataracts or cranial distortion with disruption of the brain tissue that ultimately result in reduced host survival (Shigin, 1986; Chappell, 1995; Sandland and Goater, 2001).

However, our understanding of the diversity of diplostomids remains incomplete especially in the tropics where sampling effort has been low related to diversity and many species are yet to be discovered. Furthermore, identification of these parasites is problematic due to (i) the presence of morphologically similar species; (ii) the phenotypic plasticity of the adults and metacercariae; (iii) the simple larval morphology; and (iv) the difficulties in linking

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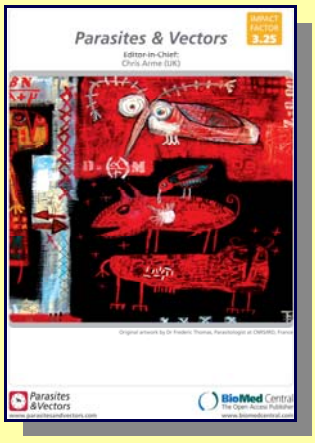
3.2.1. Paper VI

New cryptic species of the ‘revolutum’ *group of* Echinostoma *(Digenea: Echinostomatidae) revealed by molecular and morphological data*

Georgieva, S., Selbach, C., Faltýnková, A., Soldánová, M., Sures, B., Skírnisson, K. & Kostadinova, A.

Parasites & Vectors (2013) 6: 64



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Parasites & Vectors

RESEARCH Open Access

New cryptic species of the '*revolutum*' group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data

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Abstract

Background: The digenean species of *Echinostoma* (Echinostomatidae) with 37 collar spines that comprise the so-called '*revolutum*' species complex, qualify as cryptic due to the interspecific homogeneity of characters used to differentiate species. Only five species were considered valid in the most recent revision of the group but recent molecular studies have demonstrated a higher diversity within the group. In a study of the digeneans parasitising molluscs in central and northern Europe we found that *Radix auricularia*, *R. peregra* and *Stagnicola palustris* were infected with larval stages of two cryptic species of the '*revolutum*' complex, one resembling *E. revolutum* and one undescribed species, *Echinostoma* sp. IG. This paper provides morphological and molecular evidence for their delimitation.

Methods: Totals of 2,030 *R. auricularia,* 357 *R. peregra* and 577 *S. palustris* were collected in seven reservoirs of the River Ruhr catchment area in Germany and a total of 573 *R. peregra* was collected in five lakes in Iceland. Cercariae were examined and identified live and fixed in molecular grade ethanol for DNA isolation and in hot/cold 4% formaldehyde solution for obtaining measurements from fixed materials. Partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were amplified for 14 isolates.

Results: Detailed examination of cercarial morphology allowed us to differentiate the cercariae of the two *Echinostoma* spp. of the '*revolutum*' species complex. A total of 14 partial *nad*1 sequences was generated and aligned with selected published sequences for eight species of the '*revolutum*' species complex. Both NJ and BI analyses resulted in consensus trees with similar topologies in which the isolates from Europe formed strongly supported reciprocally monophyletic lineages. The analyses also provided evidence that North American isolates identified as *E. revolutum* represent another cryptic species of the '*revolutum*' species complex.

Conclusion: Our findings highlight the need for further analyses of patterns of interspecific variation based on molecular and morphological evidence to enhance the re-evaluation of the species and advance our understanding of the relationships within the '*revolutum*' group of *Echinostoma*.

Keywords: *Radix auricularia*, *Radix peregra*, *Stagnicola palustris*, *Echinostoma*, Cryptic species, Europe

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Background

The digenean species of *Echinostoma* Rudolphi, 1809 (Echinostomatidae) with 37 collar spines that comprise the so-called *Echinostoma* '*revolutum*' complex, qualify as cryptic (*sensu* Bickford *et al.* [1]; see also Pérez-Ponce de León and Nadler [2] for a recent review) due to the interspecific homogeneity of characters used to differentiate species. Only five species, the Eurasian *Echinostoma revolutum* (Frölich, 1802), *E. echinatum* (Zeder, 1803) and *E. jurini* (Skvortsov, 1924), the North American *E. trivolvis* (Cort, 1914) and the African *E. caproni* Richard, 1964, were considered valid in the most recent revision of the group using for species delimitation a single morphological feature of the larval stages (the number of pores of the para-oesophageal gland-cells in the cercaria), the specificity towards the first intermediate host (at the familial level), the ability to infect avian or mammalian hosts (or both) and geographical range on a global scale (continents) [3-5] (but see Kostadinova and Gibson [6] for a critical review). It is worth noting that *E. echinatum* has not been formally described and justified in a taxonomic publication and is not recognised as valid [see 6 for details]. However, recent molecular studies have demonstrated a higher diversity within the '*revolutum*' species complex. Thus one African species, *Echinostoma deserticum* Kechemir et al., 2002, and a yet unidentified species from New Zealand were distinguished based on molecular data [7] (see also [8]), and *E. trivolvis* was found to represent a species complex [9]. Additional data on the geographical distribution of the *Echinostoma* spp. have also been obtained. *E. revolutum* was recorded in Australia [7] and North America [10,11], *Echinostoma paraensei* Lie & Basch, 1967 in Australia and South America [7], and *E.* cf. *robustum* in North and South America [11].

The pioneer molecular studies, predominantly based on laboratory strains, have revealed that the mitochondrial *nad*1 gene provides a better resolution for investiga-

Table 1 List of species/isolates of the '*revolutum*' species complex used in this study, their hosts, localities and GenBank accession numbers

Species	Host	Locality	Accession no.	Reference
***Echinostoma* sp. IG**	*Radix peregra* (isolate RPI1)	Nordic House (Iceland)	KC618448	Present study
***Echinostoma* sp. IG**	*Radix auricularia* (isolate RAG1)	Hengsteysee (Germany)	KC618449	Present study
***Echinostoma* sp. IG**	*Radix auricularia* (isolate RAG2)	Hengsteysee (Germany)	KC618450	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI2)	Lake Myvatn (Iceland)	KC618451	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI3)	Lake Myvatn (Iceland)	KC618452	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPI4)	Lake Myvatn (Iceland)	KC618453	Present study
Echinostoma revolutum	*Stagnicola palustris* (isolate SPG1)	Hengsteysee (Germany)	KC618454	Present study
Echinostoma revolutum	*Radix auricularia* (isolate RAG3)	Hennetalsperre (Germany)	KC618455	Present study
Echinostoma revolutum	*Radix auricularia* (isolate RAG4)	Hennetalsperre (Germany)	KC618461	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG1)	Hennetalsperre (Germany)	KC618456	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG2)	Hennetalsperre (Germany)	KC618457	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG3)	Hennetalsperre (Germany)	KC618458	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG4)	Hennetalsperre (Germany)	KC618460	Present study
Echinostoma revolutum	*Radix peregra* (isolate RPG5)	Hennetalsperre (Germany)	KC618459	Present study
Echinostoma caproni	na	Cameroon	AF025838	Morgan & Blair [7,13]
Echinostoma caproni	na	Madagascar, Egypt	AF025837	Morgan & Blair [7,13]
Echinostoma caproni	*Rattus norvegicus*	Cairo (Egypt)	AJ564378	Marcilla et al. (unpublished)
E. deserticum*	na	Niger	AF025836	Morgan & Blair [7,13]
Echinostoma* cf. *friedi	*Planorbis* sp.	Wales (UK)	AY168937	Kostadinova *et al.* [14]
Echinostoma friedi	*Mesocricetus auratus* (exp.)	Pons, Valencia (Spain)	AJ564379	Marcilla *et al.* (unpublished)
Echinostoma paraensei	na	Brazil	AF025834	Morgan & Blair [7,13]
Echinostoma revolutum	*Radix peregra/Columba livia* (exp.)	Bulgaria	AY168933	Kostadinova *et al.* [14]
Echinostoma revolutum	*Lymnaea elodes/Gallus gallus* (exp.)	Shock Lake, Indiana (USA)	GQ463082	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463088	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463090	Detwiler *et al.* [11]
Echinostoma revolutum	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463086	Detwiler *et al.* [11]

Table 1 List of species/isolates of the '*revolutum*' species complex used in this study, their hosts, localities and GenBank accession numbers *(Continued)*

Echinostoma revolutum	*Lymnaea elodes*	Shock Lake, Indiana (USA)	GQ463084	Detwiler *et al.* [11]
Echinostoma revolutum	*Ondatra zibethicus*	Virginia (USA)	JQ670862	Detwiler *et al.* [11]
Echinostoma revolutum	na	"Germany, Europe"	AF025832	Morgan & Blair [7,13]
Echinostoma robustum **	*Lymnaea elodes*	Minnesota (USA)	GQ463054	Detwiler *et al.* [11]
Echinostoma robustum **	*Biomphalaria glabrata/G. gallus* (exp.)	Brazil	GQ463055	Detwiler *et al.* [11]
Echinostoma robustum **	*Lymnaea elodes*	Pond A, Indiana (USA)	GQ463053	Detwiler *et al.* [11]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670860	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670852	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670854	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670858	Detwiler *et al.* [9]
Echinostoma trivolvis	*Ondatra zibethicus*	Virginia (USA)	JQ670856	Detwiler *et al.* [9]
Echinoparyphium recurvatum	*Radix peregra*	Wales (UK)	AY168944	Kostadinova *et al.* [14]
Echinoparyphium aconiatum	*Lymnaea stagnalis*	Finland	AY168945	Kostadinova *et al.* [14]

* Syn. *Echinostoma* sp. I Africa of Morgan and Blair [17,13]; ** *sensu* Detwiler *et al.* [11].

ting relationships within the problematic *Echinostoma* '*revolutum*' species complex in comparison with the nuclear rRNA spacers and the mitochondrial *cox*1 gene [12,13]. The subsequent DNA-based studies [7,9-11,14] have provided a framework for investigating genetic variation in natural *Echinostoma* spp. populations and revealed novel data on the cryptic variation, identification and geographical distribution of the species of the '*revolutum*' complex.

However, in contrast with the wealth of sequences gathered recently from North America, which have revealed high diversity (six cryptic lineages) within the '*revolutum*' complex of *Echinostoma* [9,11], data from European natural populations are virtually lacking. Thus, of the eight species described and/or recorded from Europe, *i.e. E. revolutum*, *E. paraulum* Dietz, 1909, *E. jurini* (Skvortsov, 1924), *E. miyagawai* Ishii, 1932, *E. robustum* Yamaguti, 1935, *E. bolschewense* (Kotova, 1939), *E. nordiana* (Baschkirova, 1941), *E. friedi* Toledo et al., 2000 [3,5,15-22], sequence data are available only for *E. revolutum* [7,12-14] and *E. friedi* (GenBank AJ564379).

In a study of the digeneans parasitising molluscs in central and northern Europe we found that *Radix auricularia* (Linnaeus, 1758), *Radix peregra* (Müller, 1774) and *Stagnicola palustris* (Müller, 1774) were infected with larval stages of two species of the *Echinostoma* '*revolutum*' complex of cryptic species, one resembling *E. revolutum* sensu stricto (s.s.) and one undescribed species (see also [23]). Here we describe the cercariae of these two species and provide morphological and molecular evidence for their delimitation. Further, we extend the approaches of Morgan and Blair [7,13], Kostadinova *et al.* [14] and Detwiler *et al.* [11] to the relationships within the '*revolutum*' species complex inferred from the *nad*1 gene with the newly-generated sequence data from natural infections in snails in Europe. Phylogenetic analyses revealed the presence of

Table 2 Prevalence of *Echinostoma* spp. from natural infections in *Radix* spp. and *Stagnicola palustris* in Germany and Iceland

Species	Host	Locality	Prevalence (%)
Echinostoma revolutum	*Radix peregra*	Lake Myvatn (Iceland)	2.31
	Radix auricularia	Hennetalsperre (Germany)	1.92 - 10.00
	Radix peregra	Hennetalsperre (Germany)	37.50[a]
	Stagnicola palustris	Hengsteysee (Germany)	0.74
***Echinostoma* sp. IG**	*Radix peregra*	Nordic House (Iceland)	0.94
	Radix auricularia	Baldeneysee (Germany)	1.32 (2009)[b]
	Radix auricularia	Hengsteysee (Germany)	2.00 - 2.90 (2009)[b]
	Radix auricularia	Hengsteysee (Germany)	1.56 (2011)[b]

[a] Sample size small (n = 16); [b] Year indicated for different surveys of the same snail host.
Values are calculated for homogenous distinct samples only.

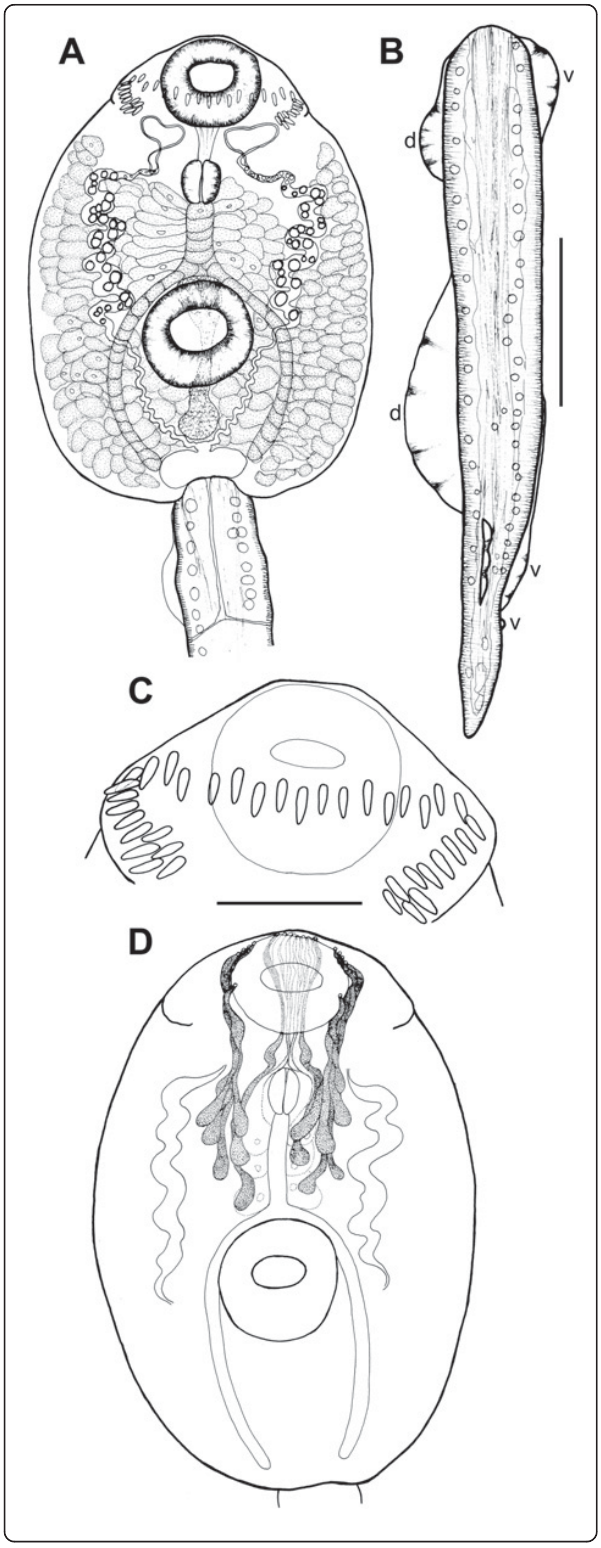

Figure 1 *Echinostoma* **sp. IG, drawings of live cercaria. A**. Body, ventral view. **B**. Tail, lateral view (note that only one of the two ventro-lateral fin-folds is illustrated). **C**. Head collar. **D**. Schematic illustration of the para-oesophageal gland-cells. *Abbreviations*: d, dorsal fin-fold; v, ventral fin-fold. *Scale-bars*: **A**, **B**, 100 μm; **C**, 50 μm.

additional cryptic lineages of the *Echinostoma* '*revolutum*' species complex.

Methods

Sample collection

Totals of 2,030 *R. auricularia,* 357 *R. peregra* and 577 *S. palustris* were collected during 2009–2012 in seven reservoirs of the River Ruhr catchment area (North Rhine-Westphalia, Germany): Baldeneysee (51°24′20.08"N, 7°2′22.47"E); Harkortsee (51°23′40.56"N, 7°24′8.27"E); Hengsteysee (51°24′52.17"N, 7°27′42.55"E); Hennetalsperre (51°19′50.97"N, 8°15′46.82"E); Kemnader See (51°25′19.05"N, 7°15′43.07"E); Sorpetalperre (51°20′ 15.01"N, 7°56′46.18"E); and Versetalsperre (51°10′55.71"N, 7°40′57.12"E). Seven distinct samples of *R. peregra* (a total of 573 snails) were collected in five localities in Iceland: Lakes Family Park (64°08′15"N, 21°52′03"W) and Nordic House (64°08′19"N, 21°56′45"W) in Reykjavik; Opnur (63°58′43"N, 21°10′37"W); Raudavatn (64°05′35"N, 21°47′14"W); and Helgavogur, Lake Myvatn (65°38′04"N, 16°55′28"W) in May and August 2012. Snails were collected randomly with a strainer or picked by hand from stones and floating vegetation along the shore at several sampling sites at each reservoir. In the laboratory, snails were labelled and placed individually into beakers with a small amount of lake water, and kept under a light source for up to 5 days to stimulate emergence of cercariae. Thereafter, snails were measured, dissected and examined for prepatent infections.

Morphological data

Cercariae were examined and identified live using the data from the keys of Faltýnková *et al.* [24,25] and other relevant primary sources [3,18-22]. Digital photographs of live cercariae (and rediae) were taken with a digital camera of an Olympus BX51 microscope. Vital stains (Neutral Red and Nile Blue sulphate) were used for visualisation of the para-oesophageal gland-cells of the cercariae. Measurements (in micrometres) were taken from the digital images with the aid of QuickPHOTO CAMERA 2.3 image analysis software or the program ImageJ [26]. Upon preliminary identification, two samples of cercariae (rediae) per isolate were fixed: (i) in molecular grade ethanol for DNA isolation and sequencing; and (ii) in hot/cold 4% formaldehyde solution for obtaining measurements from fixed materials. Snails

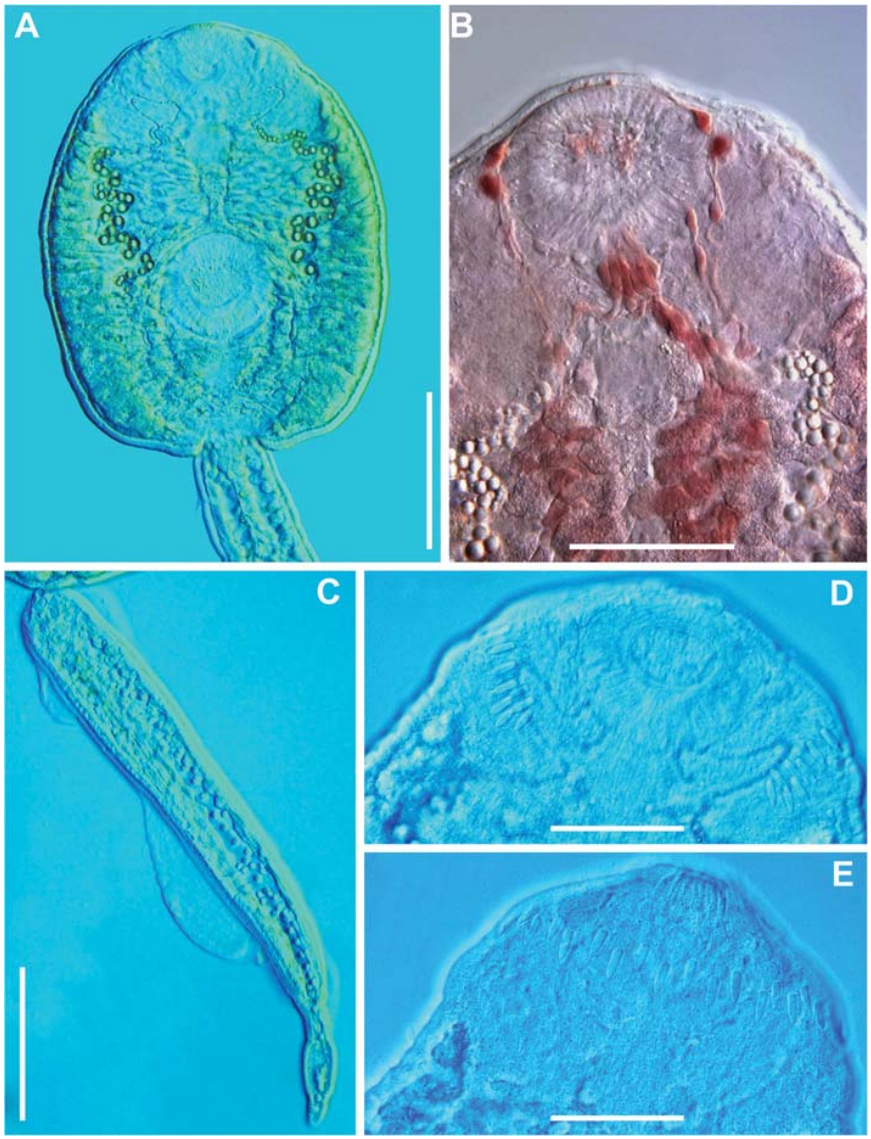

Figure 2 *Echinostoma* sp. IG, microphotographs of live cercaria. A. Body, ventral view. **B**. Dorsal view showing para-oesophageal gland-cells and outlets (staining with Neutral Red) **C**. Tail, lateral view. **D**. Head collar, ventral view showing angle and lateral spines. **E**. Head collar, dorsal view, showing dorsal collar spines. *Scale-bars*: **A**, **C**, 100 μm; **B**, **D**, **E**, 50 μm.

were identified using Glöer [27]. Although *R. peregra* and *R. ovata* (Draparnaud, 1805) have recently been treated as junior synonyms of *R. balthica* (Linnaeus, 1758) we used the name *R. peregra* following the molecular studies of Bargues *et al.* [28] and Huňová *et al.* [29] which provide sequences for snails sampled in both central Europe and Iceland.

Molecular data

Total genomic DNA was isolated from ethanol-fixed single rediae and/or 10–50 pooled cercariae obtained from a single snail individual by placing the samples in 200 μL of a 5% suspension of deionised water and Chelex® containing 0.1 mg/mL proteinase K, followed by incubation at 56°C for 3 h, boiling at 90°C for 8 min, and centrifugation at 14,000 g for 10 min. Polymerase chain reaction (PCR) amplifications of partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were performed in 25 μl reactions using Ready-To-Go-PCR Beads (GE Healthcare, UK) containing ~2.5 units of puReTaq DNA polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl,

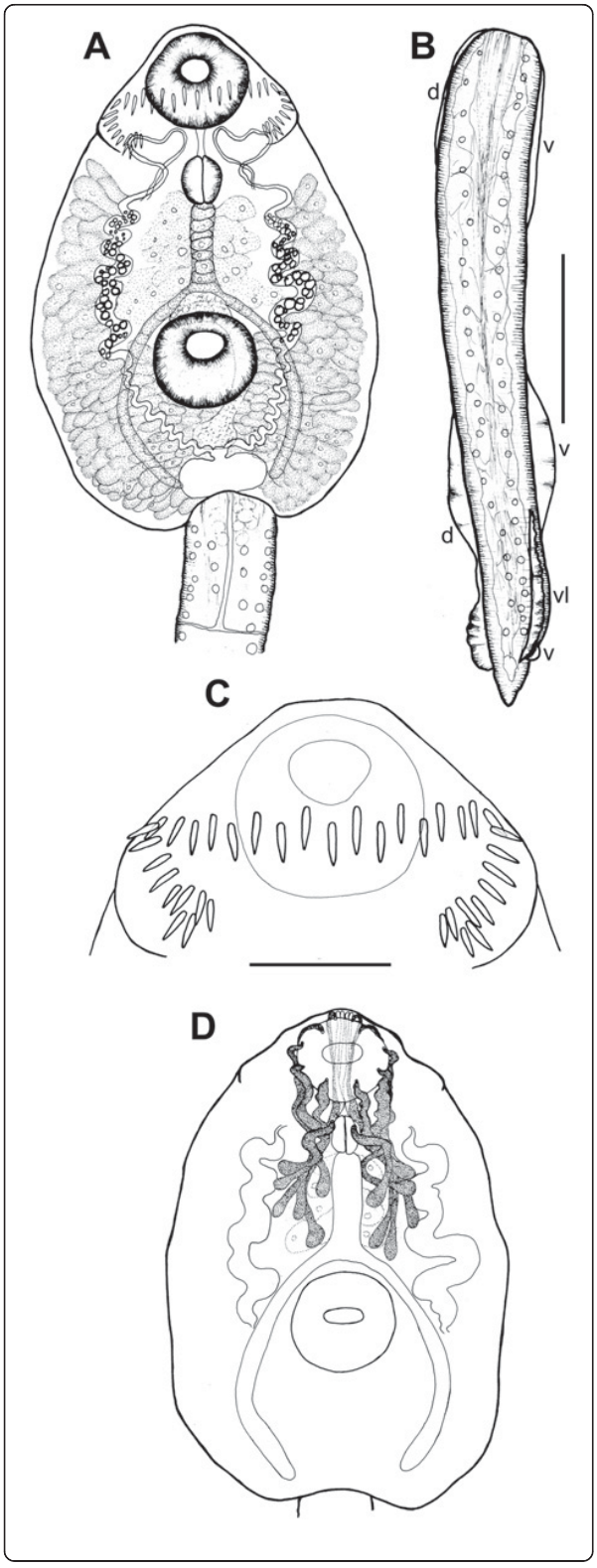

Figure 3 *Echinostoma revolutum*, drawings of live cercaria. A. Body, ventral view. **B**. Tail, lateral view (note that only one of the two ventro-lateral fin-folds is illustrated). **C**. Head collar. **D**. Schematic illustration of the para-oesophageal gland-cells. *Abbreviations*: d, dorsal fin-fold; v, ventral fin-fold; vl, ventro-lateral fin-fold. *Scale-bars*: **A**, **B**, 100 μm; **C**, 50 μm.

1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilisers including BSA, 10 mM of each PCR primer, and 50 ng of template DNA. The following PCR primers were used: forward NDJ11 (equivalent to JB11 in [13]) 5′-AGA TTC GTA AGG GGC CTA ATA-3′ and reverse NDJ2a: 5′-CTT CAG CCT CAG CAT AAT-3′ [14]. The thermocycling profile comprised initial denaturation at 95°C for 5 min, followed by 35 cycles with 30 s denaturation at 94°C, 20 s primer annealing at 48°C, and 45 s at 72°C for primer extension, with a final extension step of 4 min at 72°C.

PCR amplicons were purified using Qiagen QIAquick™ PCR Purification Kit (Qiagen Ltd, UK) and sequenced directly for both strands using the PCR primers. Sequencing was performed on an ABI Prism 3130xl automated sequencer using ABI Big Dye chemistry (ABI Perkin-Elmer, UK) according to the manufacturer's protocol. Contiguous sequences were assembled and edited using MEGA v5 [30] and submitted to GenBank (accession numbers shown in Table 1).

Newly-generated and published *nad*1 sequences for *Echinostoma* spp. (see Table 1 for details) were aligned using Clustal W implemented in MEGA v5 with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code [31]. Species boundaries were assessed via neighbour-joining (NJ) analyses of Kimura-2-parameter distances using MEGA v5 (nodal support estimated using 1,000 bootstrap resamplings) and Bayesian inference (BI) analysis using MrBayes 3.2 [32,33]. The best-fitting model of nucleotide substitution estimated prior to BI analysis using jModelTest 2.1 [34] was the Hasegawa-Kishino-Yano model including estimates of invariant sites and among-site rate heterogeneity (HKY + I + G).

BI log-likelihoods were estimated with default prior probabilities and likelihood model settings (nst = 2; rates = invgamma; ngammacat = 4) over 10^6 generations via 4 simultaneous Markov Chain Monte Carlo chains (nchains = 4) with a sampling frequency of 100. The first 25% of the samples were discarded (sump burnin = 2500) as determined by the stationarity of lnL assessed with Tracer v. 1.4 [35]; the remaining trees were used to construct the 50% majority-rule consensus tree and to estimate the nodal support as posterior probability values [36]. Genetic distances (uncorrected p-distance) were calculated with MEGA v5.

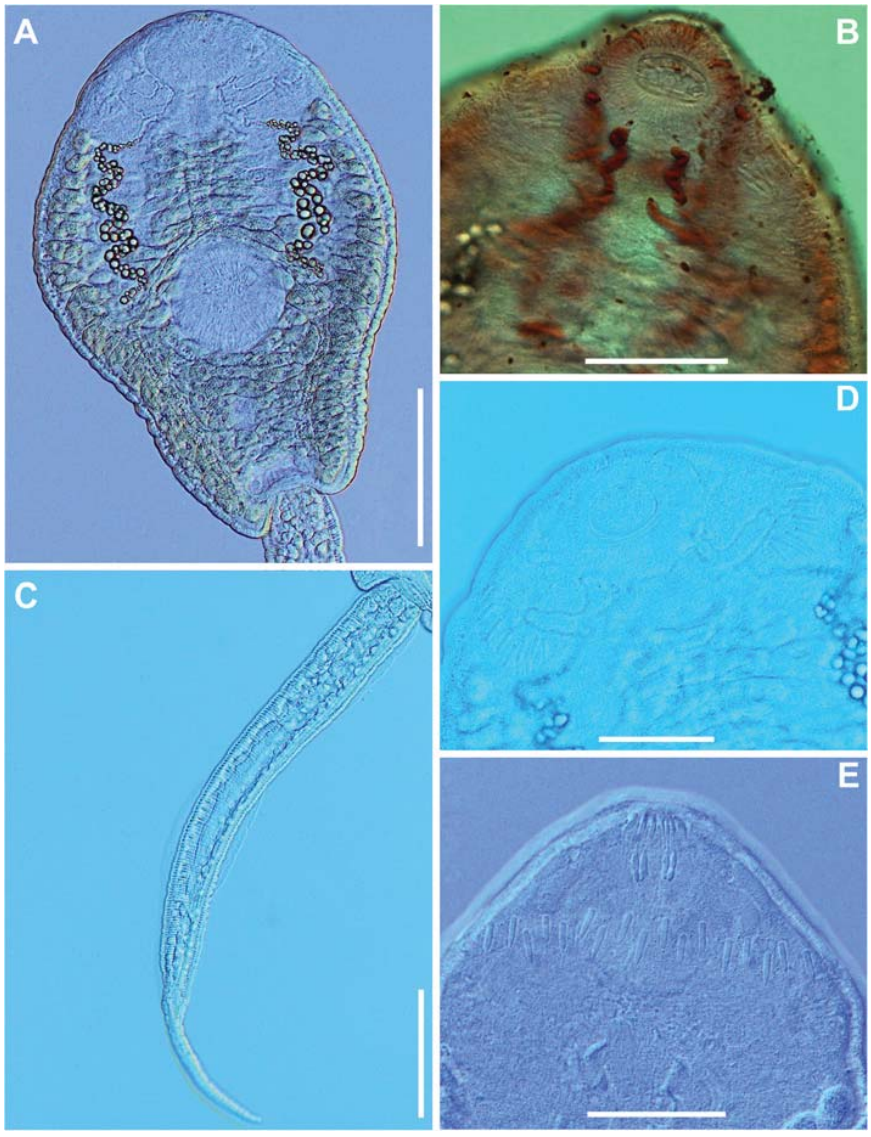

Figure 4 *Echinostoma revolutum*, microphotographs of live cercaria. A. Body, ventral view. **B**. Ventral view showing outlets of para-oesophageal gland-cells (staining with Neutral Red). **C**. Tail, lateral view. **D**. Head collar, ventral view showing angle and lateral spines. **E**. Head collar, dorsal view showing dorsal collar spines. *Scale-bars*: **A**, **C**, 100 μm; **B**, **D**, **E**, 50 μm.

Results

Morphological identification of infections in natural snail populations

We found larval stages of *Echinostoma* spp. in the snail populations sampled in three of the seven reservoirs in the River Ruhr drainage in Germany and in two of the five lakes in Iceland (see Table 2 for details on hosts and localities). Three lymnaeid snail species acted as first intermediate hosts of *Echinostoma* spp. of the '*revolutum*' species complex in the areas studied: *R. peregra* in the lakes in Iceland and *R. auricularia*, *R. peregra* and *S. palustris* in the reservoirs in Germany. Prevalences were usually low (typically 1-3%) but occasionally higher values were registered (Table 2).

Detailed examination of cercarial morphology allowed us to identify two types of echinostomatid cercariae among the isolates sampled in Iceland and Germany (Figures 1, 2, 3, 4, 5). Both types belong to the '*revolutum*' species complex of *Echinostoma* which is characterised by the following features of the cercariae: (i) 37 collar spines with an arrangement 5-6-15-6-5 (5 angle and 6 lateral spines on each side and 15 dorsal spines in a double row; Figures 1C, 2D,E, 3C, 4D,E); (ii) tail with a tip forming a highly contractile attenuated process and seven prominent

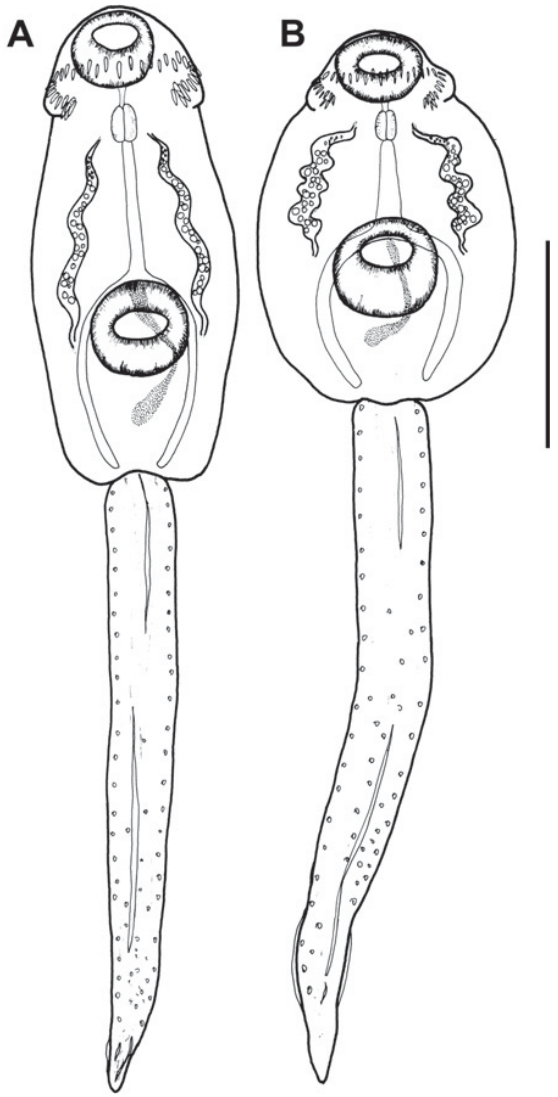

Figure 5 Cercariae fixed in 4% formaldehyde solution. A. *Echinostoma* sp. IG. **B**. *Echinostoma revolutum*. *scale-bar*: 100 μm.

tegumental fin-folds (2 dorsal, 3 ventral and 2 ventro-lateral; Figures 1B, 2C, 3B, 4C); and (iii) a flame-cell formula 2[(3 + 3 + 3) + (3 + 3 + 3)] = 36.

Eleven isolates (three ex *R. peregra* from Iceland, plus two ex *R. auricularia*, five ex *R. peregra* and one ex *S. palustris* from Germany) were identified as *E. revolutum* based on cercarial morphology and especially the presence of 12 small para-oesophageal gland-cells with long ducts, located between pharynx and ventral sucker [24] (Figures 2B, 4B). However, seven isolates of cercariae, one ex *R. peregra* from Iceland and six ex *R. auricularia* from Germany, further referred to as *Echinostoma* sp. IG (indicating the origin of the isolates *i.e.* Iceland and Germany) exhibited slight differences from the isolates identified as *E. revolutum* as follows: (i) collar spines with blunt (Figures 1C, 2D,E) *vs* sharp (Figures 3C, 4D,E) tips; (ii) para-oesophageal gland-cell outlets opening at the margin of the oral sucker only (one dorsal pair, four dorsolateral pairs, and one ventro-lateral pair; see Figures 1D, 2B) *vs* openings present on the ventral surface of the body (one pair at the level of pharynx; the remaining *i.e.* one dorsal pair, one dorsolateral pair, and three ventro-lateral pairs opening at the margin of the oral sucker, see Figures 3D, 4B); and (iii) distal dorsal tail fin-fold large *vs* less prominent (length 40-60% of tail length *vs* 20-38%; width c.70% of tail width *vs* 20-30%; compare Figures 1B, 2C and 3B, 4C; Table 3). Comparison of the metrical data obtained for live cercariae revealed that *Echinostoma* sp. IG had a shorter tail, with distinctly larger distal dorsal fin-fold and shorter distal ventral fin-fold (Table 3). Furthermore, although it was difficult to observe the fin-folds in fixed material thus rendering differentiation difficult, the cercariae of *Echinostoma* sp. IG were characterised by a distinctly more elongate, narrower body and a shorter tail (Figure 5; Table 3); this represents another distinguishing feature for the two European species studied by us.

Molecular analysis

A total of 14 partial *nad*1 sequences was generated (11 for *E. revolutum* and 3 for *Echinostoma* sp. IG; Table 1). These sequences were aligned with selected published sequences representing the data available for eight species of the '*revolutum*' species complex of *Echinostoma* generated from both laboratory strains [13] and natural isolates [9,11,14]; two otherwise unpublished sequences were also retrieved from GenBank (see Table 1 for details). The aligned dataset included 39 sequences and was comprised of 472 nt positions after trimming the ends to match the shortest aligned sequences. Sequences for *Echinoparyphium* spp. of Kostadinova *et al.* [14] were used as outgroups (Table 1).

Both NJ and BI analyses resulted in consensus trees with similar topologies (see Figure 6 for a phylogeny inferred from genetic distances and BI). The newly-generated sequences for *E. revolutum* formed a strongly supported clade which included a sequence for *E. revolutum* (s.s.) of Kostadinova *et al.* [14] (see also [6]). On the other hand, the sequences for the isolates identified as *Echinostoma* sp. IG formed a strongly supported reciprocally monophyletic lineage, basal to *Echinostoma* spp., which also incorporated the sequence for an isolate from Wales (UK) provisionally identified as *Echinostoma* cf. *friedi* by Kostadinova *et al.* [14]. The isolates comprising this lineage also exhibited the highest levels of divergence from the isolates of *Echinostoma* spp. analysed (p-distance range 17.2-21.6%; divergence from *E. friedi* (AJ564379) (p-distance range 18.9-19.1%).

Table 3 Comparative metrical data (in μm) for live and fixed cercariae of *Echinostoma* sp. IG and *E. revolutum* from natural infections in *Radix* spp. and *Stagnicola palustris* in Germany and Iceland

Species	*Echinostoma* sp. IG			*E. revolutum*		
	Live material	Fixed material		Live material	Fixed material	
	Range	Range	Mean	Range	Range	Mean
Body length	260 – 362	228 – 292	254	303 – 427	159 – 234	188
Body width (max.)	184 – 249	90 – 97	94	193 – 251	107 – 125	112
Oral sucker length	45 – 63	36 – 46	42	56 – 71	38 – 52	45
Oral sucker width	50 – 66	37 – 45	42	53 – 68	37 – 49	42
Ventral sucker length	54 – 72	43 – 54	48	63 – 83	47 – 66	55
Ventral sucker width	57 – 81	44 – 47	46	58 – 83	48 – 60	54
Pharynx length	25 – 29	16 – 25	20	27 – 36	20 – 24	21
Pharynx width	22 – 26	12 – 19	15	25 – 29	13 – 14	13
Oesophagus length	56 – 89	61 – 96	78	54 – 103	30 – 55	40
Tail length	334 – 353	296 – 378	344	364 – 417	316 – 405	367
Tail width (at base)	44 – 49	30 – 34	32	39 – 52	20 – 36	27
Tail-tip length	67 – 83	–	–	35 – 93	–	–
Proximal dorsal fin-fold length	49 – 63	50	–	41 – 153	–	–
Proximal dorsal fin-fold width	14 – 15	–	–	5 – 13	8 – 11	9
Distal dorsal fin-fold length	147 – 212	106 – 154	120	72 – 159	–	–
Distal dorsal fin-fold width	30 – 35	14 – 21	16	7 – 16	–	–
Proximal ventral fin-fold length	47 – 90	73	–	51 – 116	85	–
Proximal ventral fin-fold width	12 – 15	–	–	4 – 6	5	–
Distal ventral fin-fold length	44 – 64	41	–	74 – 202	99 – 157	125
Distal ventral fin-fold length	6 – 18	8	–	7 – 14	–	–

Unexpectedly, the European isolates of *E. revolutum* and those obtained from natural infections in *Lymnaea elodes* and *Ondatra zibethicus* (L.) in North America by Detwiler *et al.* [11] formed two strongly supported sister lineages. This solution (both NJ and BI analyses) was consistent with the distinctly higher inter-lineage divergence (p-distance; 4.9–6.8%) compared with intra-lineage divergence (p-distance range, European isolates: 0–2.1%, North American isolates: 0.4–1.1%). These data indicate that the North American isolates represent another cryptic species of the '*revolutum*' species complex.

Another unexpected result was that the sequence for *Echinostoma revolutum* of Morgan and Blair [7,13] (AF025832; isolate from Europe) exhibited a strong association with the sequence for *Echinostoma friedi* of Marcilla *et al.* (unpublished, GenBank AJ564379) based on an isolate of this species recently described by these authors [22] from Spain (p-distance 0.8%; divergence from nearest neighbours, *i.e. Echinostoma robustum* sensu Detwiler *et al.* [11], of 4.9-9.1%. The clade comprising the former two European isolates and those of *E. robustum* from North America exhibited a complex structure suggesting the existence of at least three species (subclade support indicated in Figure 6).

Discussion

The combined morphological and DNA-based approaches in this first intensive screening of *Radix* spp. for infections with *Echinostoma* spp. allowed us to delineate two cryptic species of the '*revolutum*' complex in central and northern Europe. Furthermore, comparative sequence analyses depicted three additional cryptic lineages in North America.

Both distance- and model-based phylogenies provided high support for reciprocal monophyly of *Echinostoma* sp. IG. The isolates of this lineage, that evidently represents a new species, awaiting further formal description after a discovery of the adult parasite stage, were found to be clearly distinguishable among the European isolates by using both morphological and molecular evidence. Although the identification of the European isolates of *Echinostoma* spp. followed the standard taxonomic practice, the detection of the new cryptic species required substantial taxonomic expertise. This involved detailed knowledge on the variation of the features used for species delimitation based on thorough morphological examination of a large number of cercariae from each isolate. The corroboration of our hypothesis for the distinct species status of the two species of *Echinostoma*

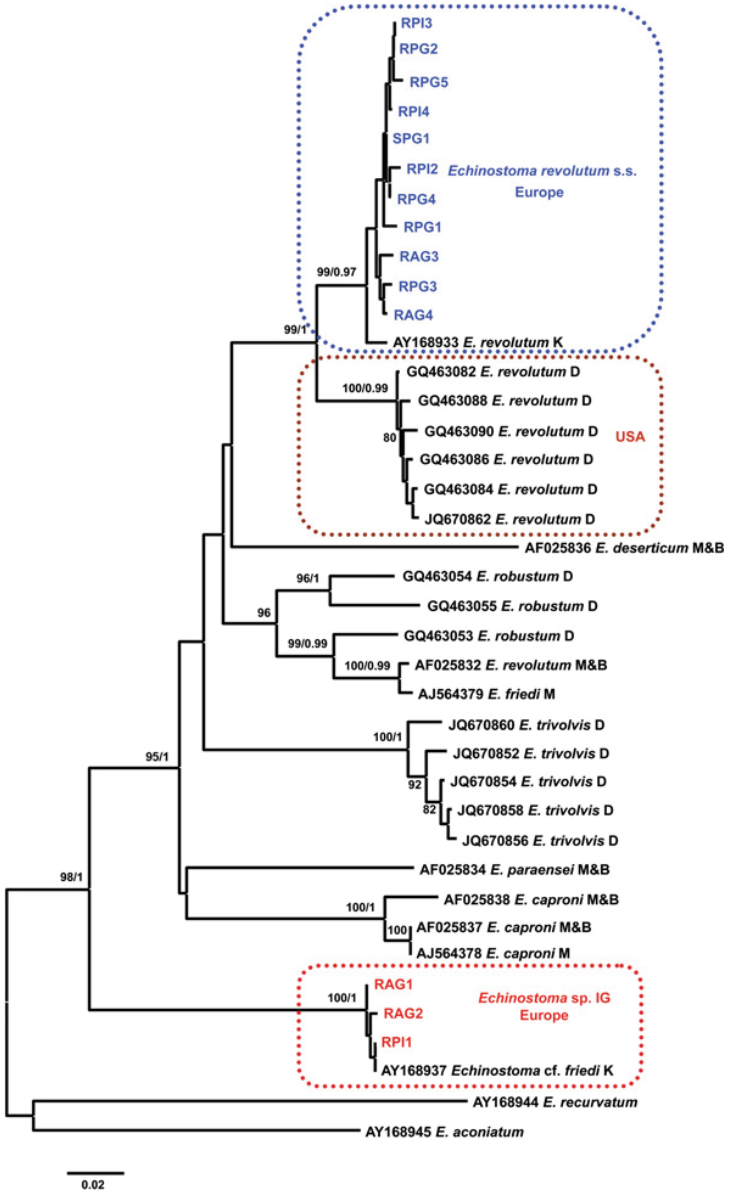

Figure 6 Neighbour-joining (NJ) phylogram reconstructed using the newly-generated and retrieved from GenBank *nad1* sequences (472 nt positions) for *Echinostoma* spp. of the '*revolutum*' species complex. Outgroup: *Echinoparyphium* spp. Nodal support (bootstrap values > 70% shown only) inferred from 1,000 replicates; these are followed by posterior probabilities from BI analysis. The scale-bar indicates expected number of substitutions per site. Sequence identification as in GenBank followed by a letter: D, Detwiler et al. [9,11]; K, Kostadinova *et al.* [14]; M, Marcilla *et al.* (GenBank); M & B, Morgan & Blair [7,13].

parasitising snail populations in Germany and Iceland on the basis of molecular data thus may appear secondary.

However, the distinguishing features are difficult to detect and/or subject to variation (reviewed in Kostadinova and Gibson [6]). For example, Kanev [3] described 16 ducts and pores of para-oesophageal gland-cells in the cercariae of *E. revolutum* ex *Lymnaea stagnalis*; of these, 12 were located on the oral sucker and four on the ventral surface. On the other hand, we detected only 12 small para-oesophageal gland-cells in the cercariae of *E. revolutum* ex *Radix* spp.; Faltýnková *et al.* [24] also provided this number for *E. revolutum* ex *L. stagnalis*. It is worth noting that recent field studies indicate that *E. revolutum* most commonly occurs in *L. stagnalis* in Europe [23,24], infections with this species have occasionally been reported in the past from *R. auricularia*, *R. peregra* and *R.*

ovata (Draparnaud, 1805) [22,37-45]. Further molecular study would reveal whether *Echinostoma* spp. of the '*revolutum*' species complex parasitising *L. stagnalis* and *Radix* spp. are conspecific or represent as yet undiscovered cryptic species. We believe that 'reciprocal illumination' *sensu* Hennig [46] of morphological characters upon a molecular-based species delimitation has a strong potential for delineating species boundaries within the '*revolutum*' complex of cryptic species.

Echinostoma sp. IG was found to be conspecific with an isolate from Wales (UK) provisionally identified as *Echinostoma* cf. *friedi* by Kostadinova *et al.* [14]. The lineage comprising this and the newly-sequenced isolates occupied a basal position (as in Kostadinova *et al.* [14]) and this is in sharp contrast with the phylogenetic solution based on *nad*1 gene of Detwiler *et al.* [11]. These authors wrote that "A comparison of samples identified as *E. robustum* (U58102) and *E. friedi* (AY168937) reveals that they are found within the same monophyletic clade and thus do not qualify as distinct species according to a phylogenetic definition. Additionally, they are genetically similar (0.009 genetic divergence, ND1 ..." and concluded that "the sample tentatively identified as *E. friedi* in Kostadinova *et al.* (2003) is genetically very similar to *E. robustum*". Our results clearly indicate that the sequence for *E. friedi* from its type-locality in Spain (AJ564379; Marcilla *et al.* unpublished sequence in GenBank) and for the European isolate labelled as *E. revolutum* (AF025832) of Morgan and Blair [7,12,13] represent conspecific isolates; the genetic divergence between these two isolates was 0.8%, *i.e.* substantially lower than that (*i.e.* 18.9-19.1%) between the lineage containing *E.* cf. *friedi* (AY168937) of Kostadinova *et al.* [14] and the European isolate labelled as *E. revolutum* (*nad*1 sequence AF025832; ITS sequence U58102) by Morgan and Blair [7,12,13]. We believe, therefore, that Detwiler *et al.* [11] have in fact used the otherwise unpublished sequence for *E. friedi* of Marcilla *et al.* (AJ564379) but have mislabelled it (as AY168937).

Kostadinova *et al.* [14] indicated a tentative affiliation to *E. robustum* of the isolates of the 'Australian-German' clade of *Echinostoma* spp. of Morgan and Blair [7], but suggested that this specific identification is pending a redescription of both larval and adult stages. The present results indicate that suggesting synonymy for the European isolate studied by Morgan and Blair [7,12,13] and *E. friedi* should await examination of a larger number of molecularly characterised natural isolates of the European species of the '*revolutum*' complex since our knowledge on cryptic diversity in this group is still limited. This suggestion is supported by the discovery of two genetically distinct, geographically separated lineages of *E. revolutum*: *E. revolutum* s.s. from Europe and *E. revolutum* of Detwiter *et al.* [11] from North America, thus demonstrating that the suggestion for the cosmopolitan distribution of this species [11] appears to be a result of cryptic variation. Indeed, these authors noted that their results of network analyses indicate gene flow and population expansion within North America but not on a global scale. The taxonomy of the North American species can be further scrutinised using the morphological data available for cercariae and/or experimentally developed adults [11,47].

Conclusion

The results of our study suggest that further analyses of patterns of interspecific variation based on a combination of molecular and well-documented morphological data would enhance the re-evaluation of the species and advance our understanding of the relationships within the '*revolutum*' group of *Echinostoma*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CS, MS and KS obtained the samples. CS, AF, MS and SG undertook the morphological study. SG carried out the sequencing and phylogenetic analysis. CS, SG, AF and MS prepared the first draft of the MS. KS, BS and AK conceived and coordinated the study and helped to draft the MS. All authors read and approved the final manuscript.

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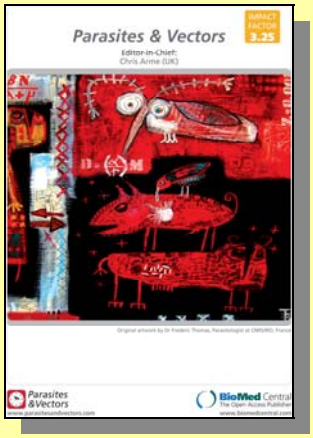
3.2.2. Paper VII

Echinostoma ‘revolutum’ *(Digenea: Echinostomatidae) species complex revisited: species delimitation based on novel molecular and morphological data gathered in Europe*

Georgieva, S., Faltýnková, A., Brown, R., Blasco-Costa, I., Soldánová, M., Sitko, J., Scholz, T. & Kostadinova, A.

Parasites & Vectors (2014) 7: 520



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Echinostoma '*revolutum*' (Digenea: Echinostomatidae) species complex revisited: species delimitation based on novel molecular and morphological data gathered in Europe

Simona Georgieva[1,2], Anna Faltýnková[1], Rebecca Brown[1,3], Isabel Blasco-Costa[1,4], Miroslava Soldánová[1], Jiljí Sitko[5], Tomáš Scholz[1] and Aneta Kostadinova[1*]

Abstract

Background: The systematics of echinostomes within the so-called '*revolutum*' group of the genus *Echinostoma*, which encompasses the type-species *E. revolutum* and a number of morphologically similar species, has long been controversial. Recent molecular studies indicate the existence of more species than previously considered valid, thus stressing the need for wider taxon sampling from natural host populations. This is especially true for Europe where morphological evidence indicates higher species diversity than previously thought, but where molecular data are virtually lacking. This gap in our knowledge was addressed in the present study through an integration of morphological and molecular approaches in the investigation of a dataset with larger taxonomic and geographical coverage.

Methods: More than 20,000 freshwater snails belonging to 16 species were collected during 1998–2012 from various localities in eight countries in Europe. Snail screening provided representative larval isolates for five species of the '*revolutum*' group, identified by their morphology. Adult isolates for four species recovered from natural and experimental infections were also identified. Partial fragments of the mitochondrial *nad*1 and 28S rRNA genes were amplified for 74 and 16 isolates, respectively; these were analysed together with the sequences of *Echinostoma* spp. available on GenBank.

Results: Delineation of the European *Echinostoma* spp. was carried out based on molecular, morphological and ecological data. The large-scale screening revealed infections with five *Echinostoma* spp., including one new species: *E. revolutum* (*sensu stricto*), *E. miyagawai*, *E. paraulum*, *E. bolschewense* and *Echinostoma* n. sp. The newly-generated *nad*1 sequences from Europe fall into six distinct, well-supported, reciprocally monophyletic lineages corresponding to the species identifications based on morphology; this was corroborated by the 28S rDNA sequences. The analyses of the total *nad*1 dataset provided evidence for 12 monophyletic groups and five singletons, which represent seven described/named species and ten cryptic species-level lineages of *Echinostoma*.

Conclusion: We conclude that *nad*1 should be the first choice for large-scale barcode-based identification of the species of the '*revolutum*' group. Our study provides a comprehensive reference library for precisely identified isolates of the European species and highlights the importance of an integrative approach for species identification linking molecular, morphological and biological data.

Keywords: *Echinostoma* '*revolutum*' species complex, Molecular and morphological data, *nad*1, 28S rDNA, Europe

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Background

The systematics of the echinostomes (Digenea: Echinostomatidae) within the so-called '*revolutum*' group of the genus *Echinostoma* Rudolphi, 1809, which encompasses the type-species *E. revolutum* (Frölich, 1802) and a number of morphologically similar species possessing 37 collar spines, has long been controversial. Problems in defining the species status within this complex include substantial interspecific homogeneity of the morphological characters of both larval and adult stages, inadequate descriptions, poor differential diagnoses and questionable synonymy [1,2] (see Kostadinova & Gibson [3] for a detailed review).

The '*revolutum*' group has been revised twice. Beaver [4] considered only *E. revolutum* valid, and placed nine species (*Distoma echinatum* Zeder, 1803, *Echinostoma miyagawai* Ishii, 1932, *E. cinetorchis* Ando & Ozaki, 1923, *E. armigerum* Barker & Irvine in Barker, 1915, *E. coalitum* Barker & Beaver in Barker, 1915, *E. mendax* Dietz, 1909, *E. paraulum* Dietz, 1909, *E. columbae* Zunker, 1925 and *E. limicoli* Johnson, 1920) in synonymy and listed additional 11 species as "*syn. inq.*". Kanev and colleagues [5-7] enlarged the '*revolutum*' group to five species, i.e. *E. revolutum* (syns *E. audyi* Lie & Umathevy, 1965, *E. ivaniosi* Mohandas, 1973, *E. paraulum* Dietz, 1909 and *E. revolutum* of Kosupko [8-11]), *E. trivolvis* (Cort, 1914) (syns *E. revolutum* of Beaver [4] and *E. rodriguesi* Hsu, Lie & Basch, 1968), *E. caproni* Richard, 1964 (syns *E. liei* Jeyarasasingam et al., 1972, *E. togoensis* Jourdan & Kulo, 1981 and *E. paraensei* Lie & Basch, 1967), *E. jurini* (Skvortsov, 1924) (syns *E. sisjakowi* Skvortzov, 1934, *E. orlovi* Romashov, 1966 and *E. bolschewense* (Kotova, 1939)) and *E. echinatum* (Zeder, 1803) (syns *Cercaria spinifera* La Valette, 1855, *E. lindoense* Sandground & Bonne, 1940, *E. barbosai* Lie & Basch, 1966, *E. miyagawai* of Kosupko [8-11] and *E. revolutum* of Našincová [12]).

These authors distinguished the five species based mainly on a single morphological feature of their larval stages (the number of outlets of the paraoesophageal gland-cells in the cercaria), the specificity towards the snail first intermediate host (at the familial level), their ability to infect avian or mammalian hosts (or both) and their geographical range on a global scale (continents) (see Kostadinova *et al.* [1] and Kostadinova & Gibson [3] for detailed comments). However, *E. echinatum* cannot be considered valid since this species has not been justified in a taxonomic publication. Further, the re-examination of the voucher specimens from Kanev's experimental studies used in his delimitation of *E. revolutum* and *E. echinatum* revealed a number of erroneous identifications including members of the genera *Hypoderaeum* Dietz, 1909 and *Echinoparyphium* Dietz, 1909, and a species of *Echinostoma* with 47 collar spines [1,13].

Kanev [5] favoured the idea of allopatric speciation at a continental scale with only two sympatric combinations: (i) *E. revolutum* and *E. echinatum* in Europe and Asia; and (ii) *E. trivolvis* (recorded as its synonym *E. rodriguesi* Hsu, Lie & Basch, 1968), *E. caproni* (recorded as its synonym *E. paraensei* Lie & Basch, 1967) and *E. echinatum* (recorded as its synonym *E. lindoense*) in South America. This simplistic scheme for the '*revolutum*' group has changed since. Based on molecular data, *E. revolutum* was recorded in Australia [14] and North America [15-17], *E. paraensei* was re-validated and recorded in Australia and South America [14,18], and as yet unidentified species/cryptic lineages of the group were distinguished in New Zealand, North America and Europe [14-17,19]. Furthermore, a number of species within the group have been described and/or redescribed based on experimental completion of the life-cycles. These include *E. bolschewense*; *E. friedi* Toledo, Muñoz-Antolí & Esteban, 2000; *E. spiniferum* (La Valette, 1855) *sensu* Našincová [20] and *E. miyagawai* Ishii, 1932 in Europe [1,2,20-22], *E. deserticum* Kechemir, Jourdane & Mas-Coma, 2002 in Africa and *E. luisreyi* Maldonado, Vieira & Lanfredi, 2003 in South America [23,24].

The first molecular study on the problematic '*revolutum*' group found very low levels (1.1–3.7%) of interspecific sequence variation for the nuclear rDNA ITS sequences from isolates of *Echinostoma* spp. maintained in the laboratory [25]. Morgan & Blair [26] obtained sequences of the mitochondrial *cox*1 and *nad*1 genes of these isolates and revealed that the *nad*1 gene provides a better resolution for investigating relationships within this group in comparison with both ITS and *cox*1. These authors used *nad*1 sequences to identify different larval stages of natural echinostome isolates from Australia and New Zealand and reported on the presence of isolates of *E. revolutum* and *E. paraensei* in Australia, plus five additional unidentified species (with more or less than 37 spines), all referred to as "*Echinostoma*" and an unknown species closely related to *E. revolutum* in New Zealand [14]. However, there appeared to be a problem with the identification of the German isolate of *E. revolutum* used by Morgan & Blair [14,25,26] (see Sorensen *et al.* [27] and Kostadinova *et al.* [1,2,28]). Kostadinova *et al.* [28] completed the life-cycle of *E. revolutum* in the laboratory and conducted a molecular study using this Bulgarian isolate and a number of European isolates from species of the genera closely related to *Echinostoma*. These authors provided evidence that the Australian material from Morgan and Blair's study [14] contained species from different genera (*Isthmiophora* Lühe, 1909, *Hypoderaeum* and *Echinoparyphium*; all referred to as "*Echinostoma*" in GenBank) and that the German and Bulgarian isolates of *E. revolutum* represent different species [3,28].

Recent molecular studies conducted by Detwiler and colleagues in North America suggested the existence of more than ten species of the genera *Echinostoma*,

Echinoparyphium and *Hypoderaeum* in natural host populations in the USA. These studies confirmed the presence of two species, identified as "*E. revolutum*" and "*E. robustum/friedi*", and flagged as potentially cryptic taxa divergent lineages for two species, *E. trivolvis* and "*E. robustum/friedi*" the USA [16,17]. Recently, Georgieva *et al*. [19] have shown that the North American isolates of "*E. revolutum*" studied by Detwiler *et al*. [16] represent another cryptic species of the '*revolutum*' species complex and provided molecular and morphological evidence for an as yet undescribed species of *Echinostoma* infecting *Radix* spp. in Germany and Iceland.

In summary, although some of the problems within the '*revolutum*' species complex have been tackled, the results of the recent molecular studies stress the need for (i) a wider taxon sampling from natural host populations, especially in Europe where morphological evidence indicates higher species diversity than previously thought, but where molecular data are virtually lacking, and (ii) an integration of molecular, morphological and biological data and taxonomic expertise as a way forward to achieving high resolution and consistency of the identification of *Echinostoma* spp.

This gap in our knowledge was addressed in the present study through an integration of morphological and molecular approaches in investigation of a dataset with larger taxonomic and geographical coverage. We carried out molecular prospecting (*sensu* Blouin [29]) for the diversity of the European species of *Echinostoma* by generating a sequence database linking *nad*1 and 28S rDNA sequences for larval and adult (experimentally raised and from naturally infected definitive hosts) isolates of *Echinostoma* spp. These were collected in an extensive sampling programme in eight countries in Europe and identified based on parasite morphology. The inclusion of reliably identified species from Europe in the substantially enlarged *nad*1 database and the phylogenetic and distance-based approaches to species delineation applied here further expand the molecular framework for the diversity and distribution of the '*revolutum*' group developed by Morgan & Blair and Detwiler and colleagues that will accelerate the taxonomic revision of this complex of morphologically similar species. Our results considerably enhance the consistency of the identification within this group of cryptic species based on molecular data and thus have implications for both monitoring the diversity and host-parasite relationships of *Echinostoma* spp. and detecting important pathogens in wild host populations and humans.

Methods

Sample collection

More than 20,000 freshwater snails belonging to 16 species [*Lymnaea stagnalis* (L.), *Radix auricularia* (L.), *R. peregra* (Müller), *Stagnicola palustris* (Müller), *Planorbis planorbis* (L.), *P. carinatus* Müller, *Planorbarius corneus* (L.), *Anisus leucostoma* (Millet), *A. vortex* (L.), *Bathyomphalus contortus* (L.), *Gyraulus albus* (Müller), *G. acronicus* (Férussac), *G. crista* (L.), *Segmentina nitida* (Müller), *Ancylus fluviatilis* Müller and *Viviparus acerosus* (Bourguignat)] were collected in an extensive sampling programme during 1998–2012 from various localities in eight countries in Europe: Austria, Bulgaria, Czech Republic, Finland, Germany, Hungary, Poland and Slovak Republic. Snails were screened for trematode infections and representative samples of each cercarial isolate (i.e. a group of identical individuals collected from a single host at one point in time [14]) of *Echinostoma* spp. were examined live and fixed in hot 4% formaldehyde solution for obtaining metrical data, and in molecular grade ethanol for DNA isolation (see Table 1 for a list of isolates, their hosts, localities and the accession numbers of the sequences). Cercariae were examined live and identified using the data from the relevant primary sources (e.g. Kosupko [9-11]; Našincová [12,21]; Kostadinova *et al*. [1,2]; Toledo *et al*. [22] and the keys in Faltýnková *et al*. [30,31].

Experimental completion of the life-cycle was carried out for two species (*E. revolutum* sampled in Bulgaria and *E. paraulum* sampled in Germany) and adult worms were available for morphological identification from the experiments of Našincová [12,20,21] for *E. bolschewense* and *Echinostoma* n. sp. Sequences were also generated from adult isolates of *E. revolutum*, *E. miyagawai* and *E. paraulum* recovered from bird definitive hosts in the wild: *Anas platyrhynchos* (L.) and *Aythya fuligula* (L.) collected in Poland (vicinities of Gdańsk) and the Czech Republic (vicinities of Tovačov), respectively (see Table 1 for details). All adults were identified prior to sequencing on morphological grounds following Kostadinova *et al*. [1,2,28].

Sequence generation

Total genomic DNA was isolated from alcohol-fixed isolates of cercariae or adult worms (posterior fifth of body, the remainder of the worm kept as voucher) using the protocols of Tkach & Pawlowski [32] or Georgieva *et al*. [19]. Polymerase chain reaction (PCR) amplifications were performed in 25 μl reactions using illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) containing ~2.5 units of puReTaq DNA polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and stabilisers including BSA, 10 pmol of each PCR primer, and 50 ng of genomic DNA.

Partial fragments of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) gene were amplified using the primers NDJ11 (forward; 5'-AGA TTC GTA AGG GGC CTA ATA-3' [26]) and NDJ2A (reverse; 5'-CTT CAGCCT CAG CAT AAT-3' [28]). The PCR thermocycling profile comprised initial denaturation at 95°C for 5 min, followed by 35 cycles (30 s denaturation

Table 1 Summary data for the isolates of *Echinostoma* spp. used for generation of the new *nad*1 and 28S rDNA sequences

Species	Isolate	Life-cycle stage	Host species	Collection site	*nad*1 haplotype ID	GenBank accession number	
						*nad*1	28S rDNA
E. bolschewense	EBG1	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065608	
E. bolschewense	EBG2	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065609	
E. bolschewense	EBG3	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065610	
E. bolschewense	EBG4	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065611	
E. bolschewense	EBG5	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065612	
E. bolschewense	EBG6	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065613	
E. bolschewense	EBG7	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065614	
E. bolschewense	EBG8	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065615	
E. bolschewense	EBG9	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065616	
E. bolschewense	EBG10	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065617	
E. bolschewense	EBG11	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065618	
E. bolschewense	EBG12	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065619	
E. bolschewense	EBG13	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	1	KP065620	KP065591
E. bolschewense	EBG14	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	2	KP065621	KP065592
E. bolschewense	EBG15	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	2	KP065622	
E. bolschewense	EBG16	C	*Viviparus acerosus*	Danube at Gabčíkovo (Slovakia)	2	KP065623	
E. miyagawai	EMGD1	A	*Anas platyrhynchos*	Vicinities of Gdańsk (Poland)	1	KP065624	
E. miyagawai	EMT1	A	*Aythya fuligula*	Vicinities of Tovačov (Czech Republic)	1	KP065625	
E. miyagawai	EML1	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	2	KP065626	
E. miyagawai	EML2	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	2	KP065627	
E. miyagawai	EML3	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	3	KP065628	
E. miyagawai	EML4	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	3	KP065629	
E. miyagawai	EML5	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	4	KP065630	
E. miyagawai	EML6	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	4	KP065631	
E. miyagawai	EML7	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	5	KP065632	
E. miyagawai	EML8	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	6	KP065633	
E. miyagawai	EML9	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	7	KP065634	
E. miyagawai	EML10	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	8	KP065635	
E. miyagawai	EML11	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	9	KP065636	
E. miyagawai	EML12	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	10	KP065637	
E. miyagawai	EML13	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	11	KP065638	
E. miyagawai	EMGD2	A	*Anas platyrhynchos*	Vicinities of Gdańsk (Poland)	12	KP065639	
E. miyagawai	EMT2	A	*Aythya fuligula*	Vicinities of Tovačov (Czech Republic)	13	KP065640	KP065593
E. miyagawai	EML14	C	*Planorbis planorbis*	Pond Loužek (Czech Republic)	14	KP065641	
***E. revolutum* (*s. str.*)**	ERBO1	C	*Lymnaea stagnalis*	Lake Bodensee (Germany)	1	KP065642	
***E. revolutum* (*s. str.*)**	ERBA1	C	*Lymnaea stagnalis*	Pond Bartoňovský (Czech Republic)	1	KP065643	KP065594
***E. revolutum* (*s. str.*)**	ERVD1	C	*Lymnaea stagnalis*	Pond Velký Dvorecký (Czech Republic)	1	KP065644	KP065595
***E. revolutum* (*s. str.*)**	ERHH1	C	*Lymnaea stagnalis*	Pond Hluboký u Hamru (Czech Republic)	1	KP065645	
***E. revolutum* (*s. str.*)**	ERV1	C	*Lymnaea stagnalis*	Pond Vlkovský (Czech Republic)	1	KP065646	
***E. revolutum* (*s. str.*)**	ERV2	C	*Lymnaea stagnalis*	Pond Vlkovský (Czech Republic)	1	KP065647	
***E. revolutum* (*s. str.*)**	ERPL1	C	*Radix auricularia*	Pond near Tomislawice (Poland)	1	KP065648	
***E. revolutum* (*s. str.*)**	ERBAL1	C	*Lymnaea stagnalis*	Lake Baldeneysee (Germany)	2	KP065649	

Table 1 Summary data for the isolates of *Echinostoma* spp. used for generation of the new *nad*1 and 28S rDNA sequences *(Continued)*

***E. revolutum* (*s. str.*)**	ERV3	C	*Lymnaea stagnalis*	Pond Vlkovský (Czech Republic)	3	KP065650	
***E. revolutum* (*s. str.*)**	ERBAL2	C	*Lymnaea stagnalis*	Lake Baldeneysee (Germany)	4	KP065651	
***E. revolutum* (*s. str.*)**	ERH1	C	*Lymnaea stagnalis*	Lake Hengsteysee (Germany)	5	KP065652	
***E. revolutum* (*s. str.*)**	ERT1	A	*Aythya fuligula*	Vicinities of Tovačov (Czech Republic)	6	KP065653	KP065596
***E. revolutum* (*s. str.*)**	ERHU1	C	*Lymnaea stagnalis*	Lake Huumojärvi, Oulu (Finland)	7	KP065654	
***E. revolutum* (*s. str.*)**	ERHU2	C	*Lymnaea stagnalis*	Lake Huumojärvi, Oulu (Finland)	8	KP065655	
***E. revolutum* (*s. str.*)**	ERK1	C	*Lymnaea stagnalis*	Pond near Krausenbechhofen (Germany)	9	KP065656	
***E. revolutum* (*s. str.*)**	ERHH2	C	*Lymnaea stagnalis*	Pond Hluboký u Hamru (Czech Republic)	10	KP065657	KP065597
***E. revolutum* (*s. str.*)**	ERHH3	C	*Lymnaea stagnalis*	Pond Hluboký u Hamru (Czech Republic)	11	KP065658	KP065598
***E. revolutum* (*s. str.*)**	ERHH4	C	*Stagnicola palustris*	Pond Hluboký u Hamru (Czech Republic)	–	–	KP065599
***Echinostoma* n. sp.**	ENG1	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	1	KP065659	
***Echinostoma* n. sp.**	ENG2	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	1	KP065660	
***Echinostoma* n. sp.**	ENG3	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	1	KP065661	
***Echinostoma* n. sp.**	ENB1	C	*Planorbarius corneus*	Pond Bohdaneč (Czech Republic)	1	KP065662	
***Echinostoma* n. sp.**	ENV1	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	1	KP065663	
***Echinostoma* n. sp.**	ENB2	C	*Planorbarius corneus*	Pond Bohdaneč (Czech Republic)	2	KP065664	
***Echinostoma* n. sp.**	ENB3	C	*Planorbarius corneus*	Pond Bohdaneč (Czech Republic)	2	KP065665	
***Echinostoma* n. sp.**	ENHH1	C	*Planorbarius corneus*	Pond Hluboký u Hamru (Czech Republic)	3	KP065666	
***Echinostoma* n. sp.**	ENV2	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	3	KP065667	KP065600
***Echinostoma* n. sp.**	ENHH2	C	*Planorbarius corneus*	Pond Hluboký u Hamru (Czech Republic)	4	KP065668	
***Echinostoma* n. sp.**	ENV3	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	4	KP065669	
***Echinostoma* n. sp.**	ENG4	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	5	KP065670	
***Echinostoma* n. sp.**	ENG5	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	6	KP065671	
***Echinostoma* n. sp.**	ENG6	C	*Planorbarius corneus*	Danube at Gabčíkovo (Slovakia)	7	KP065672	
***Echinostoma* n. sp.**	ENV4	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	8	KP065673	
***Echinostoma* n. sp.**	ENHH3	C	*Planorbarius corneus*	Pond Hluboký u Hamru (Czech Republic)	9	KP065674	KP065601
***Echinostoma* n. sp.**	ENV5	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	10	KP065675	
***Echinostoma* n. sp.**	ENBOH1	C	*Planorbarius corneus*	Pond Bohumilečský (Czech Republic)	11	KP065676	
***Echinostoma* n. sp.**	ENB4	C	*Planorbarius corneus*	Pond Bohdaneč (Czech Republic)	–	–	KP065602
***Echinostoma* n. sp.**	ENV6	C	*Planorbarius corneus*	Pond Vlkovský (Czech Republic)	–	–	KP065603
E. paraulum	EPP1	C	*Lymnaea stagnalis*	Pond near Poppenwind (Germany)	1	KP065677	
E. paraulum	EPP2	C	*Lymnaea stagnalis*	Pond near Poppenwind (Germany)	1	KP065678	
E. paraulum	EPM1	C	*Lymnaea stagnalis*	Nature Reserve Mohrhof (Germany)	2	KP065679	KP065604
E. paraulum	EPT1	A	*Aythya fuligula*	Vicinities of Tovačov (Czech Republic)	3	KP065680	KP065605
E. paraulum	EPM2	C	*Lymnaea stagnalis*	Nature Reserve Mohrhof (Germany)	4	KP065681	
***Echinostoma* sp. IG**	EIGH	C	*Radix auricularia*	Lake Hengsteysee (Germany)	2	KC618449*	KP065606
Hypoderaeum conoideum	AK44	C	*Lymnaea stagnalis*	Pond Bartoňovský (Czech Republic)	–	–	KP065607

*Published by Georgieva *et al.* [19].

at 94°C, 20 s primer annealing at 48°C, and 45 s at 72°C for primer extension), with a final extension step of 4 min at 72°C. Partial (domains D1–D3; c. 1,400 nt) 28S rDNA sequences were amplified using primer combinations U178F (5'-GCA CCC GCT GAA YTT AAG-3') and L1642R (5'-CCA GCG CCA TCC ATT TTC A-3') [33] or ZX-1 (5'-ACC CGC TGA ATT TAA GCA TAT-3') [34] and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') [35] with the following PCR profile: initial denaturation at 95°C for 5 min, followed by 40 cycles (30 s denaturation at 95°C, 30 s primer annealing at 55°C, and 45 s at 72°C for primer extension), and a final extension step of 7 min at 72°C.

PCR amplicons were purified using either a QIAquick™ Gel Extraction Kit or a Qiagen QIAquick™ PCR Purification

Kit (Qiagen Ltd., UK) and sequenced directly for both strands using the PCR primers [plus LSU1200R (5'-CAT AGT TCA CCA TCT TTC GG-3' [33]) for 28S rDNA]. Sequencing was performed on an ABI Prism 3130xl automated sequencer using ABI Big Dye chemistry (ABI Perkin-Elmer, UK) according to the manufacturer's protocol. Contiguous sequences were assembled and edited using MEGA v6 [36] and submitted to GenBank (accession numbers shown in Table 1).

Alignments and data analysis

Newly-generated and published *nad*1 and 28S rDNA sequences for *Echinostoma* spp. (Table 1; Additional file 1: Table S1) were aligned using Muscle implemented in MEGA v6; *nad*1 dataset was aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code [37], but analysed solely as nucleotides (first, second and third positions within the included codons were included in the analyses). Species boundaries were inferred with the application of the Neighbour-Joining (NJ) method using the Kimura's 2 parameter model (K2P) of substitution for pairwise distance calculations with MEGA v6 (1,000 bootstrap replicates) and Bayesian inference (BI) analyses using MrBayes v3.2 [38]. The best-fitting models of nucleotide substitution were estimated prior to BI analyses with jModelTest 2.1.4 [39,40]. These were the general time reversible model, with estimates of invariant sites and gamma distributed among-site rate variation (GTR + I + G) (*nad*1 dataset) and Hasegawa-Kishino-Yano model including estimates of invariant sites (HKY + I) (28S rDNA dataset). Log-likelihoods were estimated over 10^6 generations *via* 4 simultaneous Markov Chain Monte Carlo chains (nchains = 4) with a sampling frequency of 100. The first 25% of the samples were discarded (sump burnin = 2,500) as determined by the stationarity of lnL assessed with Tracer v.1.4 [41]; the remaining trees were used to construct the 50% majority-rule consensus tree and to estimate the nodal support as posterior probability values [42]. Genetic distances (uncorrected p-distance) were calculated with MEGA v6. Non-metric multidimensional scaling (NMDS) ordination performed with Primer v6 software [43] was used to visualise the raw pairwise distances. The significance of the relationship between the mean intra-specific divergence and the number of isolates sequenced was assessed with Spearman's correlation.

In addition to tree-based approaches to species delineation we used the distance-based identification method implemented in the function Species Identifier v1 within the program TAXONDNA [44]. The algorithm performs assignment to the correct species using K2P pairwise distances in comparisons of each sequence against the dataset using the "best close match" criterion. Assignment outcome is considered successful if the sequences exhibiting the lowest genetic distance (closest matches) are conspecific with the query sequence and the distance between the query and closest matches falls below a specified threshold. We used a distance threshold of 3%, which is a more conservative estimate than the two threshold values calculated after Meier *et al.* [44], i.e. 0.84% (distance below which 95% of all pairwise comparisons are found; n = 825) and 2.74% (distance below which 99% of all pairwise comparisons are found; n = 1,631). Relationships between haplotypes of *E. revolutum sensu lato* (*s.l.*) from Europe and North America were visualised with haplotype networks constructed with statistical parsimony analysis using TCS version 1.21 [45].

Species delineation

Delineation of the European species of *Echinostoma* was based on the integration of molecular, morphological and ecological data: (i) support for reciprocal monophyly in the *nad*1 phylogeny (a conservative approach to species delimitation); (ii) pairwise divergence at *nad*1 (including distance-based assignment) and 28S rRNA genes; (iii) matching of sequences for larval and adult stages (three of the species); (iv) comparisons with already published sequences; (v) morphological characterisation and identification of the cercarial and adult isolates; (vi) inference from the experimental completion of life-cycles (all five species); (vi) the use of different first intermediate hosts.

Results

Infections in natural host populations

The large-scale screening of natural snail populations in Europe revealed infections with five *Echinostoma* spp., including one species new to science: *E. revolutum* (type-species), *E. miyagawai*, *E. paraulum*, *E. bolschewense* and *Echinostoma* n. sp. Considering the recent results of Georgieva *et al*. [19] who delineated another putative new species (*Echinostoma* sp. IG), eight snail species are found to be infected with *Echinostoma* spp. in Europe, namely the lymnaeids *Lymnaea stagnalis*, *Radix auricularia*, *R. peregra* and *Stagnicola palustris*; the planorbids *Planorbis planorbis, Anisus vortex* and *Planorbarius corneus*; and the viviparid *Viviparus acerosus*. Five species acted as hosts of a single species of *Echinostoma*: *A. vortex* (*E. miyagawai*), *S. palustris* (*E. revolutum*), *P. planorbis* (*E. miyagawai*), *P. corneus* (*Echinostoma* n. sp.) and *V. acerosus* (*E. bolschewense*) and three lymnaeids hosted two *Echinostoma* spp. each: *L. stagnalis* (*E. revolutum* and *E. paraulum*), *R. auricularia* and *R. peregra* (*E. revolutum* and *Echinostoma* sp. IG) (see also [19]). *Echinostoma revolutum* exhibited the widest host range being recovered in the four lymnaeids studied (*L. stagnalis*, *R. auricularia*, *R. peregra* and *S. palustris*).

All cercariae exhibited characteristic features of the species belonging to the '*revolutum*' species complex of *Echinostoma*: (i) 37 collar spines with an arrangement 5-6-15-6-5 (5 angle and 6 lateral spines on each side and 15 dorsal spines in a double row); (ii) tail with a tip forming a highly contractile attenuated process and seven prominent tegumental fin-folds (2 dorsal, 3 ventral and 2 ventrolateral); and (iii) a flame-cell formula 2[(3 + 3 + 3) + (3 + 3 + 3)] = 36 [19]. However, detailed examination of cercarial morphology revealed specific differences with respect to a combination of characters, i.e. the number and distribution of the penetration and para-oesophageal gland-cells and the structure of the tail fin-folds (see Faltýnková *et al*. [46]).

Adult isolates representing four species were identified, three (*E. revolutum*, *E. miyagawai* and *E. paraulum*) recovered from naturally infected *Aythya fuligula* and *Anas platyrhynchos* and experimentally-raised specimens of *E. revolutum* and *E. paraulum*. In both life-cycle experiments the *nad*1 sequences of the adults were identical with the sequences of the cercariae used as starting material for infection (see also [28]). Morphological descriptions and sequences for *Echinostoma* sp. IG based on cercarial isolates sampled in Germany and Iceland have been published recently (Georgieva *et al*. [19]; see also Additional file 1: Table S1 for details). Formal description of this putative new species awaits the discovery of the adult stage. Detailed descriptions of the life-cycle stages of *Echinostoma* spp. from Europe and formal naming of the new species reported here will be published elsewhere [46], in order to avoid nomenclatural problems due to uncertainty concerning the first publication of the name.

Novel molecular data from Europe

Our study generated 74 novel partial *nad*1 sequences for five of the six European species of *Echinostoma* included in the analyses; these were collapsed into 39 unique haplotypes. Considering the sequences generated by Kostadinova *et al*. [28] and Georgieva *et al*. [19], the European *nad*1 dataset for *Echinostoma* spp. represented a total of 88 sequences and 50 unique haplotypes. Twenty haplotypes were identified in isolates of *E. revolutum* from four snail host species [*L. stagnalis* (ten haplotypes), *R. auricularia* (four haplotypes), *R. peregra* (seven haplotypes) and *S. palustris* (one haplotype)] with wide distribution in Germany (five localities), Czech Republic (four localities), Poland, Iceland, Finland and Bulgaria (one locality each) (Table 1; Additional file 1: Table S1). There was no differentiation within Europe (Table 2) with identical haplotypes shared across localities separated by as much as 2,500 km (haplotype 1, the most abundant haplotype found in *L. stagnalis* and *Radix* spp; see Table 1 and Additional file 1: Table S1).

Although most of the isolates of *E. miyagawai* originated from a single locality in the Czech Republic, we found high haplotype diversity (14 haplotypes). Notably, one haplotype was shared between adult isolates ex *An. platyrhynchos* from Poland and *Ay. fuligula* from the Czech Republic, "*E. revolutum* Germany, Europe" (AF025832) of Morgan & Blair [14,26] and *E. friedi* (Valencia, Spain; AJ564379), i.e. across localities separated by as much as 2,200 km. In contrast, *E. bolschewense*, a species that was also sampled at a single locality, was represented by two haplotypes; the most common haplotype (n = 13) was found at three closely-located sites within two different years.

Eleven haplotypes were identified from isolates of *Echinostoma* n. sp.; the most common haplotype was shared between locations in Slovakia (Gabčíkovo) and both northern (Pond Bohdaneč) and southern (Pond Vlkovský) locations in the Czech Republic. The two under-sampled (presumably rare) species, *Echinostoma* sp. IG and *E. paraulum*, were represented by three and four haplotypes, respectively. One haplotype of *Echinostoma* sp. IG was shared between cercarial isolates from *R. peregra* in Iceland and Wales, UK (AY168937), the latter provisionally identified on the basis of cercarial morphology as *E.* cf. *friedi* by Kostadinova *et al*. [28].

Phylogeny-based species delimitation

Both NJ and BI analyses resulted in consensus trees with similar topologies. Figures 1 and 2 represent the hypothesis for the relationships within the '*revolutum*' complex inferred from genetic distances (with indication of the nodal support from the BI analysis) of the *nad*1 dataset (159 sequences, 475 nt) that incorporated the sequences published by Morgan & Blair [14,26] (n = 11), Detwiler *et al*. [16,17] (n = 43), Georgieva *et al*. [19] (n = 14) and Kostadinova *et al*. [28] (n = 2); two otherwise unpublished sequences [AJ564379 (*E. friedi*) and AJ564378 (*E. caproni*)] of Marcilla *et al*. available on GenBank were also included in the analyses. NJ and BI analyses produced congruent results with minor topological differences. Six of the previously recognised species/cryptic lineages were represented by singletons thus preventing calculation of bootstrap support; however, most of these formed independent branches on the NJ and BI trees (Figures 1 and 2).

The newly-generated sequences from Europe fall into six distinct well-supported reciprocally monophyletic lineages corresponding to the species identifications based on morphology: *E. revolutum* ex *L. stagnalis*, *R. auricularia*, *R. peregra*, *S. palustris* and *Ay. fuligula*; *E. miyagawai* ex *P. planorbis*, *An. platyrhynchos* and *Ay. fuligula*; *E. paraulum* ex *L. stagnalis* and *Ay. fuligula*; *E. bolschewense* ex *V. acerosus*; *Echinostoma* sp. IG ex *R. auricularia* and *R. peregra*; and *Echinostoma* n. sp. ex *P. corneus*. Three species, *Echinostoma* sp. IG, *E. bolschewense* and *E. deserticum*

Table 2 Mean percent intraspecific (along the diagonal) and interspecific divergence (below the diagonal) for *Echinostoma* spp. in the *nad*1 dataset and number of pairwise nucleotide differences for 28S rDNA sequences (above the diagonal)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	***E. bolschewense***	**0.07**	12–13	11–12	18	11–12	13–16	12	8	–	–	–	13	–	–	–
2	***Echinostoma* n. sp.**	16.5	**0.53**	3–4	10	3–4	13	4	7	–	–	–	5	–	–	–
3	***E. miyagawai***	16.5	14.0	**0.83**	9	3–4	10	13	11	–	–	–	7	–	–	–
4	***E. revolutum* (*s. str.*) (Europe)**	14.3	13.0	11.4	**0.83**	9–10	15	12	13	–	–	–	13	–	–	–
5	***E. paraulum***	15.8	15.1	10.8	12.6	**0.55**	13–16	5–6	6–7	–	–	–	6–7	–	–	–
6	***Echinostoma* sp. IG**	19.3	18.9	19.0	18.2	19.4	**0.32**	13	11	–	–	–	16	–	–	–
7	***E. paraensei***	17.0	12.6	15.9	14.9	15.3	19.3	**0.21**	8	–	–	–	5	–	–	–
8	***E. caproni***	18.0	15.3	14.4	15.0	14.8	19.3	14.6	**1.82**	–	–	–	9	–	–	–
9	**"*E. robustum/friedi*" Lineage A**	16.9	13.9	4.9	11.3	10.8	17.3	15.3	14.0	**–**	–	–	–	–	–	–
10	**"*E. robustum/friedi*" Lineage C**	15.7	13.3	9.2	10.9	10.2	18.9	13.6	14.2	8.4	**–**	–	–	–	–	–
11	**"*E. robustum/friedi*" Lineage D**	16.9	13.1	8.4	12.2	10.6	19.1	14.7	15.4	8.6	5.3	**–**	–	–	–	–
12	***E. trivolvis* Lineage A***	16.3	11.8	14.6	13.0	14.7	18.0	13.6	14.3	14.1	12.7	12.9	**0.80**	–	–	–
13	***E. trivolvis* Lineage B**	15.6	12.7	15.2	14.0	15.8	19.6	14.3	16.6	14.9	14.0	13.0	8.1	**0.91**	–	–
14	***E. trivolvis* Lineage C**	14.4	11.2	15.5	13.5	15.8	19.0	14.1	16.6	15.6	13.2	13.0	7.9	2.7	**0.46**	–
15	**"*E. revolutum*" (USA)**	15.2	13.2	12.0	5.9	13.3	18.8	15.6	14.4	11.8	11.7	13.5	13.9	14.6	13.6	**0.88**

*28S rDNA sequence (AY222246) published as *E. revolutum* by Olson *et al*. [47].

(a laboratory strain from Niger maintained by Dr J. Jordane (France) with sequences previously reported as *Echinostoma* sp. I by Morgan & Blair [14,25,26]), appeared with maximum support as the earliest species to diverge among the '*revolutum*' group. The remaining species/lineages formed two main clades (A and B), shown in Figures 1 and 2, respectively.

The first clade (A) comprised the isolates of *E. revolutum sensu lato* (*s.l.*), *Echinostoma* sp. NZ-Ad, *E. paraulum*, *E. miyagawai* and the three lineages (labelled A–C) of "*E. robustum/friedi*" *sensu* Detwiler *et al*. [16,17] (Figure 1). Within this clade, the isolates ex *Stagnicola elodes* from the USA labelled as "*E. revolutum*" by Detwiler *et al*. [16,17] and the European isolates from four species of lymnaeids and wild and experimentally raised adults identified by us as *E. revolutum sensu stricto* (*s. str.*) based on morphology (see also [28]), formed sister reciprocally monophyletic lineages (Figure 1) with high support (as in Georgieva *et al*. [19]). The average sequence divergence between the two lineages was 5.9% and there were no shared haplotypes; the average intra-lineage divergence was low (0.88 and 0.83%, respectively; Table 2). Maximum parsimony haplotype network analysis depicted two unconnected networks at 95% connection limit for the isolates of *E. revolutum* (*s.l.*) from Europe and the USA (Figure 3). These results strongly support the suggestion of Georgieva *et al*. [19] that the North American isolates of "*E. revolutum*" of Detwiler *et al*. [16,17] represent a distinct cryptic species of the '*revolutum*' group.

The European cercarial and adult isolates of *E. miyagawai* clustered together with: (i) one North American isolate (GQ463053), Lineage A of "*E. robustum/friedi*" *sensu* Detwiler *et al*. [16,17]; (ii) the isolate "*E. revolutum* Germany, Europe" (AF025832) of Morgan & Blair [14,25,26]; (iii) three Australian isolates (AF026286–AF026288) identified as *E. revolutum* by Morgan & Blair [14] and representing Lineage B of "*E. robustum/friedi*" *sensu* Detwiler *et al*. [16,17]; and (iv) the isolate of *E. friedi* of Marcilla *et al*. (AJ564379; sequence otherwise unpublished). The isolates (ii) and (iv) shared the most common haplotype of *E. miyagawai* from Europe thus confirming their conspecificity. When the North American isolate (i) was considered separately, the average intraspecific divergence for *E. miyagawai* was 0.83% and the average divergence between this isolate and *E. miyagawai* was 4.9% (range 4.2–5.3%) (Table 2). Surprisingly, the North American "*E. robustum/friedi*" of Detwiler *et al*. [16] was recovered as paraphyletic with lineages C and D divergent from Lineages A and B (i and iii above) (Figure 1) and comprising a pair of sister taxa that exhibited a strongly supported sister-group relationship with the European *E. paraulum* in the BI analysis.

The second clade (B) was characterised by maximum support at almost all nodes and comprised isolates of *Echinostoma* n. sp., *E. paraensei* and the isolates of the three lineages (A–C) of *E. trivolvis* identified by Detwiler *et al*. [16,17], joined by three isolates of *E. caproni* (NJ analysis only; Figure 2). There was poor support for Lineage C of *E. trivolvis* in the BI tree.

Overall, the analyses of the *nad*1 dataset provided evidence for 12 monophyletic groups and five singletons, which represent seven described/named species

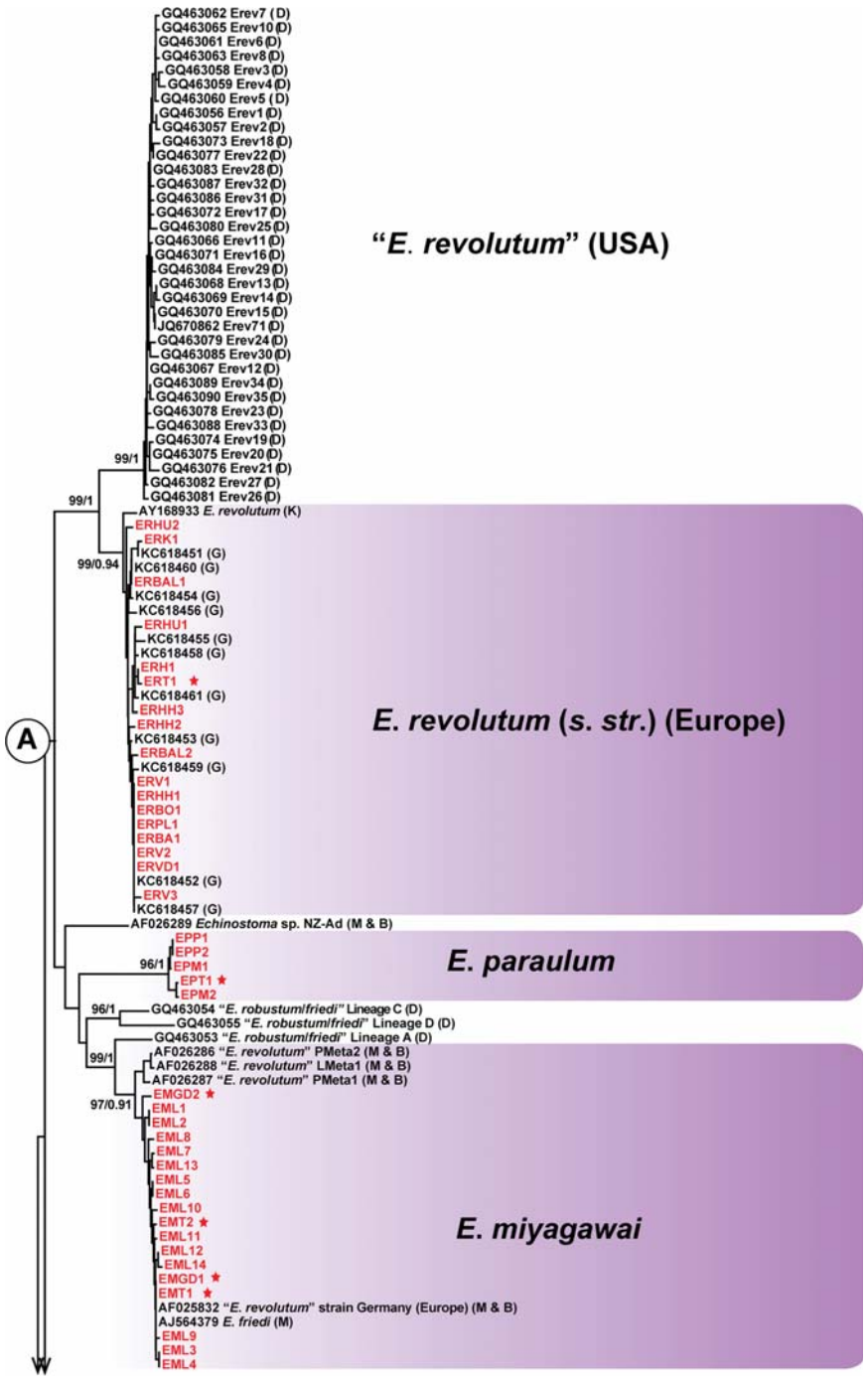

Figure 1 Neighbour-Joining (NJ) tree for 16 species-level lineages within the '*revolutum*' group of *Echinostoma* based on the mitochondrial gene *nad*1: Clade A. Based on a 475-nt fragment of *nad*1. Outgroups: *Echinoparyphium aconiatum* and *Hypoderaeum conoideum*. Numbers represent node supports from NJ and Bayesian inference (50% majority rule consensus tree) analyses (only values greater than 70 and 0.95, respectively, are shown). The newly-sequenced European isolates are shown in red; stars indicate adult isolates from natural infections. Sequence identification is as in GenBank, followed by a letter: D, Detwiler *et al.* [16,17]; G, Georgieva *et al.* [19]; K, Kostadinova *et al.* [28]; M, Marcilla *et al.* (unpublished); M & B, Morgan & Blair [14,26]. The scale-bar indicates the expected number of substitutions per site.

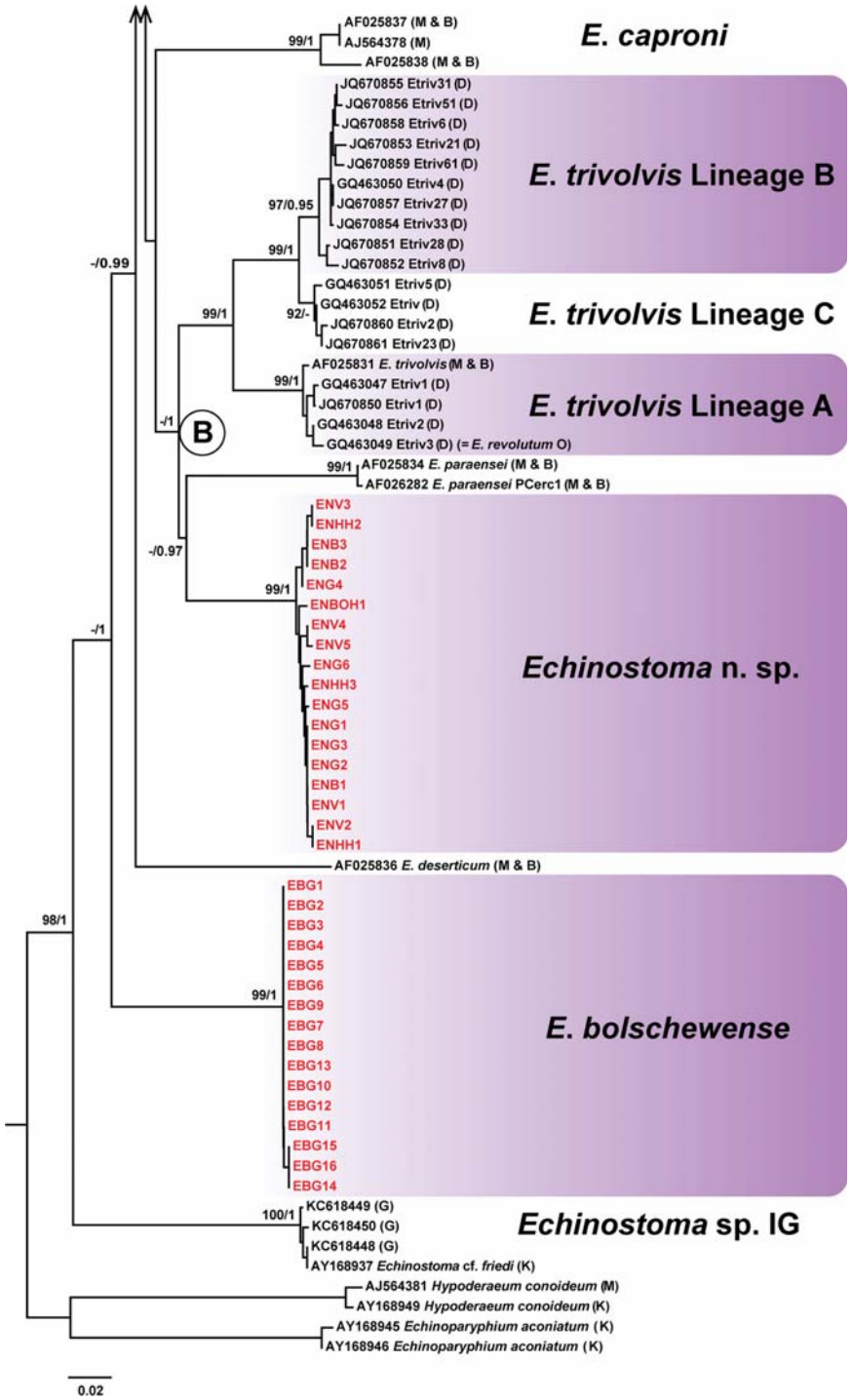

Figure 2 Neighbour-Joining (NJ) tree for 16 species-level lineages within the '*revolutum*' group of *Echinostoma* based on the mitochondrial gene *nad*1: Clade B and the remaining species, continuation of Figure 1. The newly-sequenced European isolates are shown in red; stars indicate adult isolates from natural infections. Sequence identification is as in GenBank, followed by a letter: D, Detwiler *et al.* [16,17]; G, Georgieva *et al.* [19]; K, Kostadinova *et al.* [28]; M, Marcilla *et al.* (unpublished); M & B, Morgan & Blair [14,26]; O, Olson *et al.* [47]. The scale-bar indicates the expected number of substitutions per site.

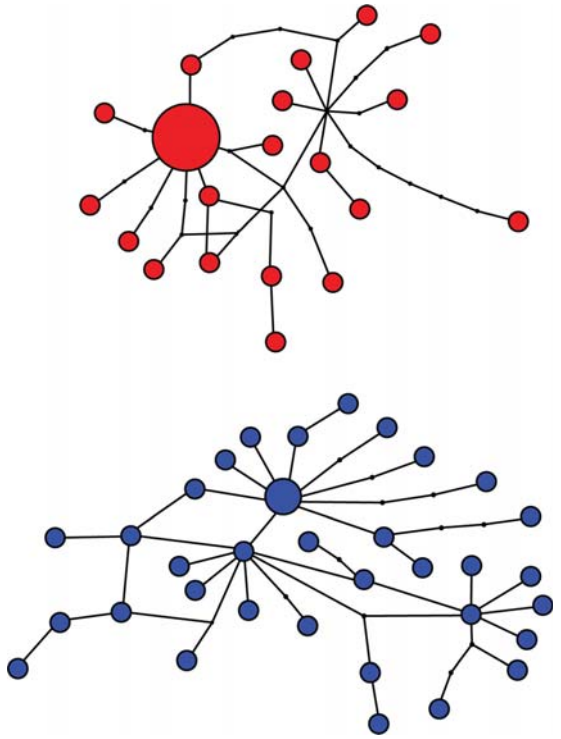

Figure 3 Haplotype network for isolates of *Echinostoma revolutum* (*sensu lato*). Isolates of *E. revolutum* (*sensu stricto*) sampled in Europe (present study; Kostadinova *et al.* [28]; Georgieva *et al.* [19]) are shown in red and isolates of "*E. revolutum*" sampled in the USA by Detwiler *et al.* [16,17] are shown in blue.

of *Echinostoma*, i.e. *E. revolutum* (*s. str.*), *E. bolschewense*, *E. caproni*, *E. deserticum*, *E. miyagawai*, *E. paraensei* and *E. paraulum*), and ten cryptic species-level lineages: *Echinostoma* n. sp. and *Echinostoma* sp. IG from Europe; "*E. revolutum*", three lineages (A–C) of *E. trivolvis* (*s.l.*) and three lineages (A, C and D) of "*E. robustum*/*friedi*" *sensu* Detwiler *et al.* [16,17] from the USA; and *Echinostoma* sp. from New Zealand. Notably, the identification of the newly-sequenced adult isolates based on morphology alone, using the concept of Kostadinova *et al.* [1,2,28] for *E. revolutum* (*s. str.*), *E. miyagawai* and *E. paraulum*, matched the identification using molecular data.

The 16 newly-generated 28S rDNA sequences corroborated with strong support the distinct species status of the six *nad*1 lineages of *Echinostoma* spp. studied in Europe (Figure 4). The only supported sister-group relationship was between *E. revolutum* and *Echinostoma* sp. IG but this is likely due to the incomplete taxon sampling for the 28S rRNA gene. No intraspecific variation was detected for species with multiple sequences, i.e. *E. revolutum*, *Echinostoma* n. sp. and *E. bolschewense*, and the two sequences (from one cercarial and one adult isolate) for *E. paraulum* differed at a single nucleotide position. The lower divergence range was 3–5 nucleotide positions (0.25–0.41%) between *Echinostoma* n. sp. and *E. paraulum*, *E. trivolvis*, *E. miyagawai* and *E. paraensei*; *E. paraulum* and *E. miyagawai*; and *E. paraensei* and *E. trivolvis* and *E. paraulum* (see Table 2 for details).

Distance-based species delimitation

The NMDS two-dimensional plot based on raw pairwise divergence data for all isolates with indication of the content of the two main clades discussed above is presented in Figure 5. The mean intraspecific divergence within the *nad*1 dataset was 0.81% (S.D. = 0.57%; range for mean divergence values of 0.21–1.82%; range for raw values of 0–3.59%, with just four comparisons exceeding 3%; see Table 2). These values were much lower than the mean divergence of 13.3% (S.D. = 3.1%) in the interspecific comparisons (range for mean divergence values of 2.7–19.6%; range for raw divergence values of 4.2–21.5%). There was no significant correlation between the number of isolates per species/lineage and mean intraspecific variation (Spearman's rho = 0.248; $P > 0.05$). The mean interspecific divergence was 16-fold higher than mean intraspecific divergence but three sister-species groups [*E. trivolvis* Lineages A–C; *E. miyagawai* – "*E. robustum*/*friedi*" Lineage A; *E. revolutum* (*s. str.*) (Europe) – "*E. revolutum*" (USA)] exhibited ratios at the margin or below the '10× rule' proposed by Hebert *et al.* [48], thus indicating a possible problem of overlapping variability at *nad*1 in the '*revolutum*' species complex (see also Figure 5). However, there was no overlap in the distributions of intraspecific and interspecific (sister-taxa only) divergences (Figure 6). Furthermore, all sister-species groups could be resolved using diagnostic nucleotide sites: 65 for *Echinostoma* n. sp. – *E. paraensei*; 44 and 47 for *E. paraulum* – "*E. robustum*/*friedi*" Lineages C and D of Detwiler *et al.* [16], respectively; 28 for *E. trivolvis* Lineage A – *E. trivolvis* Lineages B and C; 24 for "*E. robustum*/*friedi*" Lineage C – "*E. robustum*/*friedi*" Lineage D of Detwiler *et al.* [16]; 19 for *E. revolutum* (*s. str.*) – "*E. revolutum*" (USA); and 16 for *E. miyagawai* – "*E. robustum*/*friedi*" Lineage A of Detwiler *et al.* [16]. Finally, excluding singletons, successful identification of all isolates was achieved for all 12 species/lineages at 3% divergence threshold in Species Identifier v.1.

Discussion

The phylogenetic analyses depicted 17 genetically distinct lineages within the data set studied and, excluding singletons, successful identification of all isolates was achieved by the distance-based identification method implemented in Species Identifier v.1 for all 12 species/lineages. Our results are congruent with the phylogenies obtained by Detwiler *et al.* [16,17] on datasets dominated by isolates from the USA. The increase in the estimated

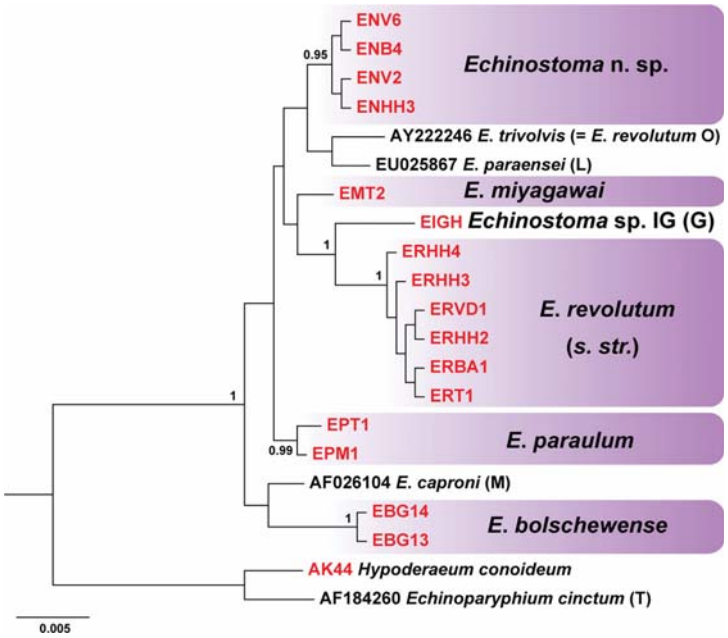

Figure 4 Bayesian inference phylogram reconstructed using partial 28S rDNA sequences for nine *Echinostoma* spp. The alignment comprised 1,219 nucleotide positions. Outgroups: *Hypoderaeum conoideum* and *Echinoparyphium cinctum*. The newly-sequenced European isolates are shown in red. Sequence identification is as in GenBank, followed by a letter: G, Georgieva *et al.* [19]; O, Olson *et al.* [47]; L, Lotfy *et al.* [49]; M, Mollaret *et al.* [50]. The scale-bar indicates the expected number of substitutions per site.

number of species in the '*revolutum*' group is largely due to the increased sampling within Europe. The novel sequence data generated here in association with the morphological characterisation of the life-cycle stages of *Echinostoma* spp. provides an integrative framework for future studies on species diversity within this difficult group.

European species within the '*revolutum*' group

This first large-scale sequencing study of species of *Echinostoma* across Europe provided evidence for six molecularly distinct species of the '*revolutum*' group. Their independent status was supported by the concordant signal of the mitochondrial *nad*1 and nuclear 28S rRNA

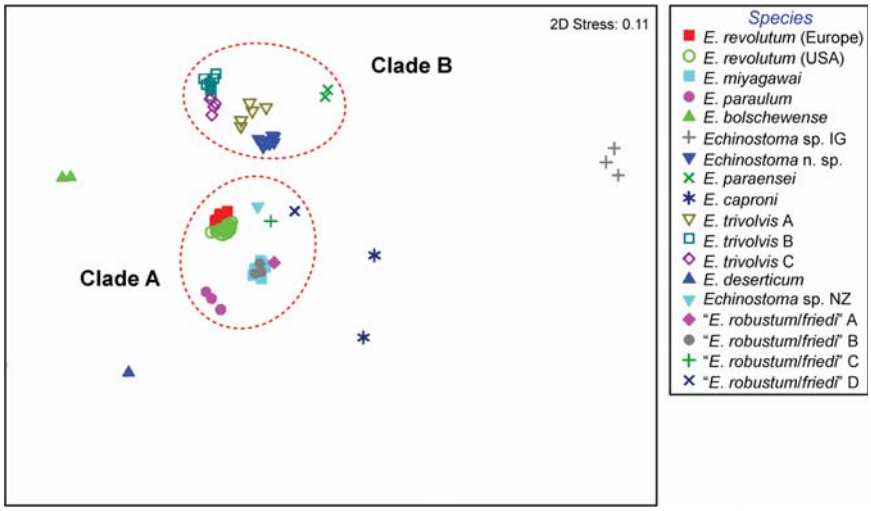

Figure 5 Non-metric multidimensional scaling ordination plot derived from the raw pairwise distances calculated for the *nad*1 dataset. Labels for the lineages of *E. trivolvis* and "*E. robustum/friedi*" are after Detwiler *et al.* [16]. Ellipses indicate the two main clades.

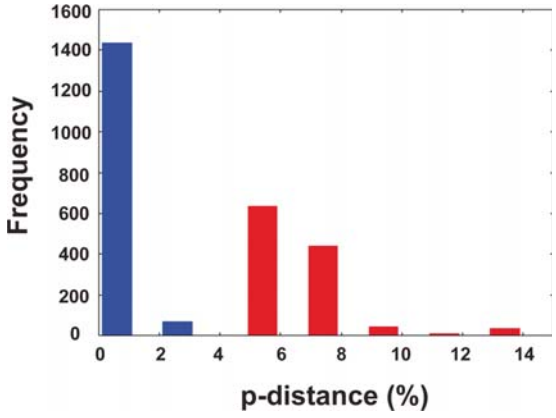

Figure 6 Patterns of intra- and interspecific divergence in *Echinostoma* spp. using the *nad*1 dataset. Red bars indicate intraspecific divergence; blue bars indicate iterspecific divergence (raw p-distances in %, pairwise comparisons between sister-species only).

genes, distance-based identification and morphological evidence. The integration of molecular and morphological data for two of the species-level lineages strongly indicates that these represent species new to science (see Georgieva *et al*. [19] for a description of the cercaria of *Echinostoma* sp. IG and Faltýnková *et al*. [46] for a description of the life-cycle stages of *Echinostoma* n. sp.).

Our extensive sampling resulted in a successful match of sequences based on life-cycle stages from naturally infected intermediate and definitive hosts for three of the European species whose life-cycles have been completed experimentally, *E. revolutum*, *E. miyagawai* and *E. paraulum* (see [1,2,46]). Notably, the identification of the adult isolates from natural infections based on morphology alone using the concept of Kostadinova *et al*. [1,2,28] and the morphological data from adult experimental isolates, matched the identification using molecular data. Sequencing of isolates from wild mammalian hosts within Europe may contribute to resolving the natural definitive hosts in the life-cycles of *E. bolschewense* and *Echinostoma* n. sp. The large-scale sampling of natural snail populations also shed light on the intermediate host range of *Echinostoma* spp. Whereas *E. bolschewense*, *E. miyagawai*, *E. paraulum* and *Echinostoma* n. sp. were found to infect single first intermediate snail species (*Viviparus acerosus*, *Planorbis planorbis*, *Lymnaea stagnalis* and *Planorbarius corneus*, respectively), *Echinostoma* sp. IG was detected in two snail hosts (*Radix auricularia* and *R. peregra*) and *E. revolutum* (*s. str*.) exhibited the widest intermediate host range (*L. stagnalis*, *R. auricularia*, *R. peregra* and *Stagnicola palustris*). These results further stress the importance of precise identification of cercarial isolates of *Echinostoma* spp. in hosts found to harbour more than one species: *L. stagnalis* (parasitised by two species, *E. revolutum* (*s. str*.) and *E. paraulum*), *R. auricularia* (*E. revolutum* (*s. str*.) and *Echinostoma* sp. IG) and *R. peregra* (*E. revolutum* (*s. str*.) and *Echinostoma* sp. IG). As shown by Georgieva *et al*. [19] and Faltýnková *et al*. [46], these species combinations can be distinguished based on cercarial morphology.

Perhaps the most important result of our study is that the integration of morphological and molecular data from both experimental and wildlife infections clarified the status of *E. revolutum* (*s. str*.) and *E. paraulum*. Both species use *L. stagnalis* as the first intermediate host but the cercariae differ in the number and location of the paraoesophageal gland-cells. The cercarial isolates from *L. stagnalis*, with a pattern of paraoesophageal gland-cells dissimilar to *E. revolutum* and experimentally obtained and wild adult isolates, formed a distinct strongly-supported clade with "*E. robustum/friedi*" Lineages C and D of Detwiler *et al*. [16,17] as nearest neighbours (Figure 1). A detailed examination of adult morphology (experimental set and the voucher specimen from natural infection used for sequencing; see [46]) confirmed their identification as *E. paraulum*, a species long considered a synonym of *E. revolutum* (see e.g. [4,5]). Combining morphological and molecular evidence from different life-cycle stages, we can confidently restore the validity of this species. All life-cycle stages of *E. revolutum* (*s. str*.) and *E. paraulum* linked to the sequences from Europe reported here are described in detail by Faltýnková *et al*. [46].

Our study provided the first datasets of sequences for *E. miyagawai* and *E. bolschewense*. *Echinostoma miyagawai* was re-validated after experimental completion of its life-cycle and detailed re-description of the morphology of all stages based on European material [1,2]; however, no sequences for this species were available. The incorporation of a large set of sequences for larval and adult *E. miyagawai* in our analyses solved the taxonomy of the German and Australian isolates identified as *E. revolutum* by Morgan & Blair [14,26]. Kostadinova *et al*. [28] examined a single voucher specimen (Australian isolate PMeta-2) of Morgan & Blair [14] and concluded that the morphology of this adult worm suggests an affiliation to *E. robustum*. However, they stated "… at present we prefer not to favour this specific identification for the 'Australian-German' clade of *Echinostoma* sp., pending a redescription of both larval and adult stages". The inclusion of the sequences for four of the "*E. revolutum*" isolates of Morgan & Blair [14,26] within the well-supported clade of *E. miyagawai* (containing both cercarial and adult isolates identified using the concept of Kostadinova *et al*. [1,2]) suggests that these, in fact, belong to the latter species. The "German" isolate of "*E. revolutum*" (a laboratory strain identified by I. Kanev and sequenced by Morgan & Blair [14,25,26]) clearly represents a misidentification. As shown by Kostadinova *et al*. [1] based on re-examination of the voucher material, the re-description of *E. revolutum* by Kanev [5] was

based on a mixture of material and likely represents a composite of at least two species of the '*revolutum*' group. The position of *E. friedi* of Marcilla *et al*. (Valencia, Spain; AJ564379; published in GenBank only) within the *E. miyagawai* clade supports the inclusion of this species among the synonyms of *E. miyagawai*. Moreover, "*E. revolutum* Germany, Europe" of Morgan & Blair [14,26] (AF025832) and *E. friedi* (Valencia, Spain; AJ564379) represented a haplotype shared with adult isolates of *E. miyagawai* ex *An. platyrhynchos* from Poland and *Ay. fuligula* from the Czech Republic. The close association of *E. friedi* with the Australian isolates of Morgan & Blair [14,26] listed above was also confirmed in the recent study of Detwiler *et al*. [16] on a different set of taxa. However, a mislabelling of the sequence for *E. friedi* of Marcilla *et al.* (AJ564379) as the sequence for an isolate of Kostadinova *et al*. [28] provisionally identified as *E.* cf. *friedi* (AY168937) leaves a wrong impression that the latter isolate also represents *E. friedi* (see Georgieva *et al*. [19] for detailed discussion). As shown by Georgieva *et al*. [19] and the present study, the isolate of Kostadinova *et al*. [28] belongs to an as yet undescribed species of *Echinostoma* (*Echinostoma* sp. IG); this is strongly supported in the present analyses.

The life-cycle of *Echinostoma bolschewense* (possible synonym *E. jurini* (Skvortsov, 1924) of Kanev *et al*. [7]; for detailed comment on taxonomy see Faltýnková *et al*. [46]) has been elucidated by Našincová [21] who described in detail the life-cycle stages (rediae and cercariae from naturally infected prosobranch snails, *Viviparus contectus*, metacercariae from a range of prosobranch and pulmonate snails and adults from hamsters) of this species. To the best of our knowledge, this is the only species of *Echinostoma* developing in prosobranch snails; our study elucidated another first intermediate host, *Viviparus acerosus*.

In addition to the large *nad*1 dataset, we also generated 28S rDNA sequences for the six European species of the '*revolutum*' group; these can be used in future phylogenetic studies at the supraspecific level. The minima for sequence divergence (0.25–0.41%) between *Echinostoma* spp. for which 28S rDNA data were available are comparable with the minima observed between closely related but distinct digenean species (e.g. 0.2–0.4% in the Cryptogonimidae, see Miller & Cribb [51,52].

American species within the '*revolutum*' group

The taxonomy of the American species of *Echinostoma* belonging to the '*revolutum*' group is in urgent need of revision. First, consistent with the recent study of Georgieva *et al*. [19], we found strong evidence for genetic differentiation between the North American and European populations within *E. revolutum* (*s.l.*) as evidenced by the phylogenetic reconstructions and distance-based identification. Therefore, the increased sampling within Europe reinforces the results of the network analysis of *E. revolutum* (*s.l.*) indicating lack of gene flow between Europe and North America [16].

Secondly, although the *nad*1 dataset was substantially expanded, the same lineages of *E. trivolvis* and "*E. robustum*/*friedi*" were recovered as identified by Detwiler *et al*. [16,17] suggesting that the lineages within *E. trivolvis* (A–C) and "*E. robustum/friedi*" (A, C and D) *sensu* Detwiler *et al*. [16] may represent distinct, closely-related cryptic species. However, this finding calls for further molecular and taxonomic scrutiny. In particular, comprehensive sampling in both North and South America is required to enlarge the sample size for the three lineages of "*E. robustum*/*friedi*" (note that this label is no more appropriate in view of the synonymy indicated above; we use it just for consistency in referring to the isolates of Detwiler *et al*. [16,17] currently represented by singletons). This would provide data for testing the monophyly of the lineages and alternative hypotheses for patterns of diversification associated with e.g. specificity to the snail host or geography. The strong support for different sister-group relationships of the three isolates of "*E. robustum*/*friedi*" further reinforce our suggestion; it is also worth noting that one of the isolates (Lineage D) originates from naturally infected *Biomphalaria glabrata* in South America (Brazil; see Detwiler *et al*. [16], whereas the other two (Lineages A and C) represent cercarial isolates ex *Lymnaea elodes* in the USA. It is also necessary to test if the structuring inferred from the *nad*1 sequences (Detwiler *et al*. [16,17]; this study) is reflected in divergences in the nuclear genes and consistent differences in morphology.

Although species boundaries are delimited, naming the American species would appear the most complicated task. Five nominal species assigned by different authors to the '*revolutum*' group have been described in North America (USA), i.e. *Echinostoma armigerum*; *E. callawayense* Barker & Noll in Barker, 1915; *E. coalitum*; *E. trivolvis* and *Echinoparyphium contiguum* Barker & Barston in Barker, 1915 [6,53,54], and further eight species have been described in South America (Brazil), i.e. *E. barbosai*; *E. erraticum* Lutz, 1924; *E. luisreyi* Maldonado, Vieira & Lanfredi, 2003; *E. microrchis* Lutz, 1924; *E. neglectum* Lutz, 1924; *E. nephrocystis* Lutz, 1924; *E. rodriguesi* Hsu, Lie & Basch, 1968; *E. paraensei* Lie & Basch, 1967 [24,55-59]. In contrast to the opinions of Beaver [4] and Kanev *et al*. [6] regarding the synonymy of all North American species listed above with *E. trivolvis*, detailed studies on the morphology of some of the South American species have revealed that these exhibit distinguishing differences [18,24,57,59]. Comparative approaches to the morphology of North American strains of "*E. revolutum*" and *E. trivolvis* during the 'pre-molecular era' have shown that morphometric features of the experimentally raised adult worms can be used to distinguish closely related species [60,61].

Therefore, although the sequence information and analyses of Detwiler *et al.* [16,17] and the present study provide a sound framework for alpha taxonomy, revealing the species diversity of the '*revolutum*' group of *Echinostoma* in the Americas requires an integrative approach linking the molecular data with detailed phenotypical characterisation of the isolates studied. Although the species within this group qualify as cryptic, the comprehensive morphological analysis in the course of our study revealed useful features for distinguishing two life-cycle stages, cercariae and adults, of the European *Echinostoma* spp. (Faltýnková *et al.* [46]; see also [19]). This stresses the importance of detailed morphological examination of live cercarial isolates prior to sequencing and the availability of voucher specimens identified by experts for the adult isolates sequenced (e.g. present study – see Faltýnková *et al.* [46]; Maldonado *et al.* [18]). The latter, even if unidentified at the time of DNA sequence publication, are of primary importance for accelerating further integrative taxonomy studies. Unfortunately, although a large number (32) of adult specimens of "*E. revolutum*", *E. trivolvis* (Lineages A–C) and "*E. robustum/friedi*" (Lineage D) (see Additional file 1: Table S1) from natural infections or raised experimentally were sequenced by Detwiler *et al.* [16,17], these have not been submitted to a museum collection.

Asian species within the '*revolutum*' group

Several notes of caution are required before considering the recent papers on "*Echinostoma*" spp. reported recently from Asian locations (Saijuntha *et al.* [62-64]; Noikong *et al.* [65]). First, the authors should be aware that annotations in GenBank solely reflect the identification (in most cases not supported by voucher material and/or morphological data) of the authors submitting the sequences. Whereas the identifications based on comparisons with original species descriptions may be correct, failure to follow the subsequent taxonomic/systematic changes may results in 'discoveries' such as "Interestingly, this study revealed that *E. revolutum* was more closely aligned with *E. recurvatum* than the other species of genus *Echinostoma* (e.g., *E. malayanum*), contradicting traditional morphological taxonomy." (Saijuntha *et al.* [63]) and "Interestingly, this study revealed that two species of genus *Echinostoma*, i.e. *E. revolutum* and *E. malayanum* do not cluster as a monophyletic clade and/or sister taxa." (Saijuntha *et al.* [62]). Just reading the subtitle for this species in the taxonomic revision of Kostadinova & Gibson [66], i.e. "*Artyfechinostomum malayanum* (Leiper, 1911) Railliet, 1925 [Syns *Echinostoma malayanum* Leiper, 1911; *Euparyphium malayanum* (Leiper, 1911) Leiper, 1915; *Echinoparyphium malayanum* (Leiper, 1911) Skrjabin & Shul'ts, 1929]" makes it clear that *E. malayanum* has been transferred to the genus *Artyfechinostomum* Lane, 1915 by Railliet nearly a century ago and that the only different generic placements of this species are those of Leiper (in *Euparyphium*) and Skrjabin & Shul'ts (in *Echinoparyphium*). Therefore, there is nothing "contradicting traditional morphological taxonomy" since the clustering pattern in Saijuntha *et al.* [62] simply reflects a distinction at the generic level which the authors failed to recognise because of lack of knowledge on the taxonomy of the group. Along this line, *Echinostoma hortense* Asada, 1926 has been transferred to the genus *Isthmiophora* as *I. hortensis* (Asada, 1926) in the revision of Kostadinova & Gibson [66]. The examination of the experimental material of *E. hortense* used for obtaining the sequence data of Morgan & Blair [14,25,26] confirmed its affiliation to *Isthmiophora* (see Kostadinova *et al.* [28]). However, this species is still referred to as *E. hortense* by Saijuntha *et al.* [62] and Noikong *et al.* [65].

A second problem in recent studies on Asian echinostomatids is the failure to understand/integrate existing knowledge (e.g. re-identifications of sequenced isolates based on morphological evidence, e.g. *Echinoparyphium ellisi* (AF026791, isolate PMeta3 of Morgan & Blair [14,26]) and *Echinoparyphium hydromyos* (AF026290, isolate Rat-Ad of Morgan & Blair [14]) re-identified by Kostadinova *et al.* [28] based on examination of the available voucher material, are still being referred to as "*Echinostoma* sp." (see Noikong *et al.* [65]).

Thirdly, there are wrong interpretations of published work, e.g. "These results were relatively concordant to a previous report by Kostadinova *et al.*, 2003, which confirmed that not all species within the genus *Echinostoma* represent a monophyletic group." (Saijuntha *et al.* [62]). In fact, the opening sentence of the section "Molecular identification and relationships between *Echinostoma*, *Echinoparyphium*, *Hypoderaeum* and *Isthmiophora*" in Kostadinova *et al.* [28] states: "Considering **the initial identification (as given by Morgan & Blair, 1998a, b) and the names of the taxa as existing at present in the GenBank database** (our emphasis), *Echinostoma* is represented as a paraphyletic taxon with *Echinoparyphium recurvatum* (ITS and ND1 trees) and *Isthmiophora melis* (ND1 trees), *Echinoparyphium aconiatum* (ND1 trees) and *Hypoderaeum conoideum* (ND1 trees) nested within it." [28]. Unfortunately, the findings of the study of Kostadinova *et al.* [28] were not understood by Saijuntha *et al.* [62].

Fourthly, the original papers should be consulted in order that the correct origin of the material sequenced is identified. For example, Saijuntha *et al.* [63] assumed that the sequence U58102 of Morgan & Blair [25] was of an "Australian isolate". The provenance of this isolate is not annotated in GenBank but is clearly identified (i.e. Germany, Europe) in the original papers (see Table 1 in Morgan & Blair [25,26], respectively). The status of this isolate was discussed by Kostadinova *et al.* [28] who

suggested a provisional identification as *Echinostoma* cf. *robustum* based on the additional molecular data. Failure to detect the origin of this isolate has resulted in a wrong conclusion, i.e. "Moreover, the phylogenetic relationships of *E. revolutum* presented in the present study suggested that genetic clustering is related to the geographical origin of the isolates, i.e., the American isolates closely aligned to the European isolate, whereas the Australian isolate closely aligned to Southeast Asian isolates." (Saijuntha *et al*. [63]). In fact, the isolate of "*E. revolutum*" from Thailand exhibits close affinity to the European isolate studied by Morgan & Blair [14,26], which we have shown to represent *E. miyagawai* (see above). Finally, to our astonishment we found out that not a single sequence has been deposited in GenBank from the sequencing study in Thailand by Noikong *et al*. [65]. The lack of evidence for further comparative evaluation renders the findings reported by these authors useless.

Overall, these problems with the recent molecular studies based on Asian echinostomatids result in a rather bleak picture with regard to the identity of the isolates sequenced. It is likely that the papers by Saijuntha and colleagues deal with two species of the '*revolutum*' group, one misidentified as *E. revolutum* and one misidentified as "*E. recurvatum* 43–50 collar spines" (*E. recurvatum* is a species with 45 collar spines), both exhibiting affinities with *E. miyagawai*. Whereas the identification of *Artyfechinostomum malayanum* (as *Echinostoma malayanum* in their papers) may be correct, that of "*Hypoderaeum conoideum* 41–45 collar spines" is likely wrong. Species of *Hypoderaeum* possess 43–82 collar spines [67] so that the minimum number of spines provided for the isolate (i.e. 41–45) is probably a miscount. Further, *H. conoideum* is characterised by the possession of 47–53 spines [68], i.e. above the range given by Saijuntha *et al*. [62]. Unfortunately, no data other than a short *cox*1 (250 nt) sequence are available to check their identification of "*H. conoideum*". All these considerations indicate that further molecular work based on precise identification of the Asian isolates associated with the description and deposition of vouchers is required in order to make progress in elucidating the species diversity of the '*revolutum*' group in Asia.

*Nad*1 for a barcode?

The first assessment of the usefulness of the partial mitochondrial *nad*1 gene sequences for species identification and inferring the relationships within the '*revolutum*' group was carried out in a comparative framework by Morgan & Blair [26]. Their findings suggested that *nad*1 is diverging significantly faster than the *cox*1 and ITS gene regions and thus appears to be the most informative region. These authors reported interspecific sequence divergence for *nad*1 within the '*revolutum*' group of 12.3–30.8% [26] and 9.6–30.8% [14]. However, the very high upper limits of these ranges were due to inclusion in their comparisons of "*Echinostoma*" *hortense*, which was shown to belong to a different echinostomatid genus, *Isthmiophora* [66]. Detwiler *et al*. [16] reported a range of 1.2–5.4% and 8.1–12.4% for *nad*1 mean intra- and interspecific genetic divergence, respectively, for three sibling species groups of the '*revolutum*' complex designated as "*E. revolutum*", *E. trivolvis* (Lineages A–C) and "*E. robustum/friedi*" (Lineages A–D).

These values are generally comparable to the ranges obtained in our study (i.e. means of 0.2–1.8% and 2.7–19.4%, respectively), the mean pairwise divergence within the named and putative species in the present expanded dataset being much lower than the data reported by Detwiler *et al*. [16]. Although *nad*1 differentiation within species-level lineages was generally low compared with divergences between species with cases where the same haplotype was detected in remote geographical locations [*E. revolutum* (*s. str*.) and *E. miyagawai*], the overall mean interspecific divergence was 16-fold higher than the mean intraspecific divergence. The molecular divergences among three sister-species groups (i.e. *E. trivolvis* Lineages A–C; *E. miyagawai* – "*E. robustum*/*friedi*" Lineage A; *E. revolutum* (*s. str*.) (Europe) – "*E. revolutum*" (USA)) were relatively low (range for means 2.7–8.6%). However, a barcode gap (i.e. a discontinuity in levels of intraspecific compared with interspecific genetic divergence) was detected and all sister-species groups could be resolved using diagnostic nucleotide sites.

Conclusion

Taking into account that a large comparative database of sequences exists, we conclude that *nad*1 should be the first choice for large-scale barcode-based identification of the species of the '*revolutum*' group of *Echinostoma*. Our study provides a comprehensive reference library for precisely identified isolates of the European species and highlights the importance of an integrative approach for species identification linking molecular, morphological and biological data.

Additional file

Additional file 1: Summary data for *nad*1 sequences of *Echinostoma* spp. retrieved from the GenBank.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AK and AF: conceived and designed the study, discussed the results and helped draft the MS. AF, MS, SG and JS: obtained samples, discussed the results and took part in the preparation of the MS. AF, MS, SG and RB: undertook the identification and morphological characterisation of the isolates. RB and IB-C contributed to sequencing and drafting the results. SG

carried out the major part of the sequencing, performed the phylogenetic analyses and prepared the first draft of the MS. TS coordinated the project, discussed the results and helped draft the MS. All authors read and approved the final manuscript.

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Supplementary Table S1. Summary data for *nad*1 sequences of *Echinostoma* spp. retrieved from the GenBank.

Species	Isolate	Life-cycle stage	Host	Locality
Echinostoma revolutum (*s. str.*)	J5	Adult	*Radix peregra*/*Columba livia* (exp.)	Grigorevo (Bulgaria)
Echinostoma revolutum (*s. str.*)	RPI2	Cercaria	*Radix peregra*	Lake Mývatn (Iceland)
Echinostoma revolutum (*s. str.*)	RPI3	Cercaria	*Radix peregra*	Lake Mývatn (Iceland)
Echinostoma revolutum (*s. str.*)	RPI4	Cercaria	*Radix peregra*	Lake Mývatn (Iceland)
Echinostoma revolutum (*s. str.*)	SPG1	Cercaria	*Stagnicola palustris*	Lake Hengsteysee (Germany)
Echinostoma revolutum (*s. str.*)	RAG3	Cercaria	*Radix auricularia*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RPG1	Cercaria	*Radix peregra*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RPG2	Cercaria	*Radix peregra*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RPG3	Cercaria	*Radix peregra*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RPG5	Cercaria	*Radix auricularia*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RPG4	Cercaria	*Radix peregra*	Lake Hennetalsperre (Germany)
Echinostoma revolutum (*s. str.*)	RAG4	Cercaria	*Radix auricularia*	Lake Hennetalsperre (Germany)
Echinostoma revolutum	Erev1	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev2	Adult	Lymnaea elodes/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev3	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev4	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev5	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev6	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev7	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev8	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev10	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev11	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev12	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev13	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev14	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev15	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Worm 71	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma revolutum	Erev16	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev17	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev18	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev19	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev20	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)

Supplementary Table S1. Continued.

Species	GenBank (*nad*1)	Note	Reference
Echinostoma revolutum (*s. str.*)	AY168933		Kostadinova et al. (2003)
Echinostoma revolutum (*s. str.*)	KC618451	Haplotype 12	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618452	Haplotype 1	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618453	Haplotype 13	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618454	Haplotype 14	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618455	Haplotype 15	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618456	Haplotype 16	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618457	Haplotype 1	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618458	Haplotype 17	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618459	Haplotype 18	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618460	Haplotype 2	Georgieva et al. (2013)
Echinostoma revolutum (*s. str.*)	KC618461	Haplotype 19	Georgieva et al. (2013)
Echinostoma revolutum	GQ463056		Detwiler et al. (2010)
Echinostoma revolutum	GQ463057		Detwiler et al. (2010)
Echinostoma revolutum	GQ463058		Detwiler et al. (2010)
Echinostoma revolutum	GQ463059		Detwiler et al. (2010)
Echinostoma revolutum	GQ463060		Detwiler et al. (2010)
Echinostoma revolutum	GQ463061		Detwiler et al. (2010)
Echinostoma revolutum	GQ463062		Detwiler et al. (2010)
Echinostoma revolutum	GQ463063		Detwiler et al. (2010)
Echinostoma revolutum	GQ463065		Detwiler et al. (2010)
Echinostoma revolutum	GQ463066		Detwiler et al. (2010)
Echinostoma revolutum	GQ463067		Detwiler et al. (2010)
Echinostoma revolutum	GQ463068		Detwiler et al. (2010)
Echinostoma revolutum	GQ463069		Detwiler et al. (2010)
Echinostoma revolutum	GQ463070		Detwiler et al. (2010)
Echinostoma revolutum	JQ670862		Detwiler et al. (2010)
Echinostoma revolutum	GQ463071		Detwiler et al. (2010)
Echinostoma revolutum	GQ463072		Detwiler et al. (2010)
Echinostoma revolutum	GQ463073		Detwiler et al. (2010)
Echinostoma revolutum	GQ463074		Detwiler et al. (2010)
Echinostoma revolutum	GQ463075		Detwiler et al. (2010)

Supplementary Table S1. Continued.

Species	Isolate	Life-cycle stage	Host	Locality
Echinostoma revolutum	Erev21	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev22	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev23	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev24	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev25	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev26	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev27	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev28	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev29	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev30	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev31	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev32	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev33	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	Erev34	Adult	*Lymnaea elodes*/*Gallus gallus* f. dom. (exp.)	Indiana (USA)
Echinostoma revolutum	Erev35	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma revolutum	PMeta 2	Metacercaria	*Glyptophysa* sp	Townsville (Australia)
Echinostoma revolutum	PMeta 1	Metacercaria	*Glyptophysa* sp.	Townsville (Australia)
Echinostoma revolutum	LMeta 1	Metacercaria	*Austropeplea lessoni*	Townsville (Australia)
Echinostoma caproni	Madagascar (c);	Adult?	*Laboratory isolate*	Madagascar
Echinostoma caproni	Egypt (l)	Adult	*Rattus norvegicus*	Cairo (Egypt)
Echinostoma caproni	Cameroon (k) (=E. sp. II of Morgan & Blair (1995)	Adult?	Laboratory isolate	Cameroon
Echinostoma deserticum	*Echinostoma* sp. I Niger	Adult?	Laboratory isolate	Niger
Echinostoma paraensei		Adult?	Laboratory isolate	Brazil
Echinostoma paraensei	PCerc1	Cercaria	*Glyptophysa* sp.	Townsville (Australia)
Echinostoma robustum/*friedi*	Erob/fri1	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma robustum/*friedi*	Erob/fri2	Cercaria	*Lymnaea elodes*	Minnesota (USA)
Echinostoma robustum/*friedi*	Erob/fri3	Adult	*Biomphalaria glabrata*/*Gallus gallus* f. dom. (exp.)	Brazil
Echinostoma trivolvis		Adult?	Laboratory isolate	North America

Supplementary Table S1. Continued.

Species	GenBank (*nad*1)	Note	Reference
Echinostoma revolutum	GQ463076		Detwiler et al. (2010)
Echinostoma revolutum	GQ463077		Detwiler et al. (2010)
Echinostoma revolutum	GQ463078		Detwiler et al. (2010)
Echinostoma revolutum	GQ463079		Detwiler et al. (2010)
Echinostoma revolutum	GQ463080		Detwiler et al. (2010)
Echinostoma revolutum	GQ463081		Detwiler et al. (2010)
Echinostoma revolutum	GQ463082		Detwiler et al. (2010)
Echinostoma revolutum	GQ463083		Detwiler et al. (2010)
Echinostoma revolutum	GQ463084		Detwiler et al. (2010)
Echinostoma revolutum	GQ463085		Detwiler et al. (2010)
Echinostoma revolutum	GQ463086		Detwiler et al. (2010)
Echinostoma revolutum	GQ463087		Detwiler et al. (2010)
Echinostoma revolutum	GQ463088		Detwiler et al. (2010)
Echinostoma revolutum	GQ463089		Detwiler et al. (2010)
Echinostoma revolutum	GQ463090		Detwiler et al. (2010)
Echinostoma revolutum	AF026286	Lineage B of Detwiler et al. (2010)	Morgan & Blair (1998b)
Echinostoma revolutum	AF026287	Lineage B of Detwiler et al. (2010)	Morgan & Blair (1998b)
Echinostoma revolutum	AF026288	Lineage B of Detwiler et al. (2010)	Morgan & Blair (1998b)
Echinostoma caproni	AF025837		Morgan & Blair (1998a)
Echinostoma caproni	AJ564378		Marcilla et al. (GenBank only)
Echinostoma caproni	AF025838		Morgan & Blair (1998a)
Echinostoma deserticum	AF025836		Morgan & Blair (1998a)
Echinostoma paraensei	AF025834		Morgan & Blair (1998a)
Echinostoma paraensei	AF026282		Morgan & Blair (1998b)
Echinostoma robustum/*friedi*	GQ463053	Lineage A of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma robustum/*friedi*	GQ463054	Lineage C of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma robustum/*friedi*	GQ463055	Lineage D of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma trivolvis	AF025831		Morgan & Blair (1998a)

Supplementary Table S1. Continued.

Species	Isolate	Life-cycle stage	Host	Locality
Echinostoma trivolvis	Etriv1	Cercaria	*Helisoma trivolvis*	Indiana (USA)
Echinostoma trivolvis	Etriv2	Cercaria	*Helisoma trivolvis*	Indiana (USA)
Echinostoma trivolvis	Etriv3	Adult	Adult tissue of *E. revolutum* of Olson et al. (2003)	
Echinostoma trivolvis	Worm1	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Etriv4	Cercaria	*Lymnaea elodes*	Indiana (USA)
Echinostoma trivolvis	Worm27	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm28	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm8	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm21	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm33	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm31	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm51	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm6	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm61	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Etriv5	Adult	*Ondatra zibethicus*	Wisconsin (USA)
Echinostoma trivolvis	Etriv6	Adult	*Ondatra zibethicus*	Wisconsin (USA)
Echinostoma trivolvis	Worm2	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma trivolvis	Worm23	Adult	*Ondatra zibethicus*	Virginia (USA)
Echinostoma sp. IG	RAG1	Cercaria	*Radix auricularia*	Lake Hengsteysee (Germany)
Echinostoma sp. IG	RAG2	Redia	*Radix auricularia*	Lake Hengsteysee (Germany)
Echinostoma sp. IG	RPI1	Cercaria	*Radix peregra*	Lake Nordic House (Iceland)
Echinostoma sp. IG	J6	Cercaria	*Radix peregra**	Lake Pwll Penarth (Wales, UK)
Echinostoma sp. NZ-Ad	NZ-Ad	Adult	*Branta candensis*	New Zealand
Hypoderaeum conoideum		Cercaria	*Radix peregra*	Grigorevo (Bulgaria)
Echinoparyphium aconiatum	FA1	Cercaria	*Lymnaea stagnalis*	Pond Vehkalampi (Finland)
Echinoparyphium aconiatum	FA3	Cercaria	*Lymnaea stagnalis*	Lake Pyykosjärvi (Finland)

Supplementary Table S1. Continued.

Species	GenBank (*nad*1)	Note	Reference
Echinostoma trivolvis	GQ463047	Lineage A of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma trivolvis	GQ463048	Lineage A of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma trivolvis	GQ463049	Lineage A of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma trivolvis	JQ670850	Lineage A of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	GQ463050	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2010)
Echinostoma trivolvis	JQ670857	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670851	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670852	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670853	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670854	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670855	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670856	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670858	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670859	Lineage B of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	GQ463051	Lineage C of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	GQ463052	Lineage C of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670860	Lineage C of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma trivolvis	JQ670861	Lineage C of Detwiler et al. (2010)	Detwiler et al. (2012)
Echinostoma sp. IG	KC618449	Haplotype 2	Georgieva et al. (2013)
Echinostoma sp. IG	KC618450	Haplotype 3	Georgieva et al. (2013)
Echinostoma sp. IG	KC618448	Haplotype 1	Georgieva et al. (2013)
Echinostoma sp. IG	AY168937	Haplotype 1 (= *Echinostoma* cf. *friedi*)	Kostadinova et al. (2003)
Echinostoma sp. NZ-Ad	AF026289		Morgan & Blair (1998b)
Hypoderaeum conoideum	AY168949		Kostadinova et al. (2003)
Echinoparyphium aconiatum	AY168945		Kostadinova et al. (2003)
Echinoparyphium aconiatum	AY168946		Kostadinova et al. (2003)

* Erroneously annotated in GenBank as *Planorbis* sp.

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3.2.3. Paper VIII

A re-assessment of species diversity within the ‘revolutum’ *group of* Echinostoma *Rudolphi, 1809 (Digenea: Echinostomatidae) in Europe*

Faltýnková, A., Georgieva, S., Soldánová, M. & Kostadinova, A.

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DOI 10.1007/s11230-014-9530-3

A re-assessment of species diversity within the '*revolutum*' group of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) in Europe

Anna Faltýnková · Simona Georgieva · Miroslava Soldánová · Aneta Kostadinova

Received: 3 October 2014 / Accepted: 14 October 2014

Abstract Species of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) belonging to the '*revolutum*' species complex were re-examined based on material gathered in an extensive sampling programme in eight countries in Europe. The morphology of the life-cycle stages was studied in naturally and experimentally infected snail and bird hosts. A review, with an updated synonymy, is presented for six European species, including one new to science, i.e. *Echinostoma revolutum* (Frölich, 1802) (*sensu stricto*) (type-species), *E. bolschewense* (Kotova, 1939), *E. miyagawai* Ishii, 1932, *E. nasincovae* n. sp., *E. paraulum* Dietz, 1909 and *Echinostoma* sp. IG), and keys to the identification of their cercariae and adults are provided.

Introduction

The species of the '*revolutum*' group of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) are characterised by a substantial interspecific homogeneity of the morphological characters of the life-cycle stages which have been used for species discrimination (see Kostadinova & Gibson, 2000, for a detailed review). This emphasises the need of a more precise assessment of the degree of the morphological intra- and inter-specific variability of the species within the '*revolutum*' group (e.g. Kostadinova et al., 2000a, b) and the application of an integrated approach to the problem of species diversity within this group (Kostadinova et al., 2003; Georgieva et al., 2013).

Studies on the genetic and morphological diversity of natural echinostome populations are vital for our understanding of the phylogeny and the likely mode of speciation within the '*revolutum*' group (Kostadinova & Gibson, 2000). However, to date these two aspects have been treated separately (e.g. Našincová, 1986, 1991; Kanev, 1994, Kanev et al., 1995; Morgan & Blair, 1995, 1998a, b; Kostadinova et al., 2000a, b; Toledo et al., 2000; Detwiler et al., 2010, 2012). Wider taxon sampling from natural host populations in Europe is especially important in view of the higher genetic diversity revealed in North America (Detwiler et al., 2010, 2012). However, although morphological evidence indicates higher than previously thought species diversity within the '*revolutum*' group, molecular data from Europe are virtually lacking.

This paper is part of a complex study addressing re-assessment of the species within the '*revolutum*' group of *Echinostoma* in Europe through an integrated taxonomic approach, linking morphological and

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3.2.4. Paper IX

The life-cycle of Petasiger islandicus *Kostadinova & Skirnisson, 2007 (Digenea: Echinostomatidae) elucidated with the aid of molecular data*

Georgieva, S., Kostadinova, A. & Skírnisson, K.

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The life-cycle of *Petasiger islandicus* Kostadinova & Skirnisson, 2007 (Digenea: Echinostomatidae) elucidated with the aid of molecular data

Simona Georgieva · Aneta Kostadinova · Karl Skirnisson

Received: 2 January 2012 / Accepted: 21 January 2012

Abstract The small planorbid snail *Gyraulus* cf. *laevis* (Alder) from Lake Mývatn in Iceland was found to emit large-tailed cercariae with 19 collar spines, and three-spined sticklebacks *Gasterosteus aculeatus* L. were infected with metacercariae of a species of *Petasiger* Dietz, 1909. Comparative sequence analysis using ND1 mitochondrial DNA sequences revealed that the rediae and cercariae are conspecific with *P. islandicus* Kostadinova & Skirnisson, 2007, recently described from an isolated population of the horned grebe *Podiceps auritus* (L.) at the lake. The redia, cercaria and metacercaria are described and compared with related forms.

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Introduction

As part of a study of a demographically isolated population of the horned grebe *Podiceps auritus* (L.) at Lake Mývatn, Iceland, we recently described *Petasiger islandicus* Kostadinova & Skirnisson, 2007 based on abundant material of adult worms (Kostadinova & Skirnisson, 2007). Subsequent investigation of the digenean fauna at the lake revealed that the small planorbid snail *Gyraulus* cf. *laevis* (Alder) emitted cercariae of the 'Magnacauda' group; we also found that three-spined sticklebacks *Gasterosteus aculeatus* L. were infected with metacercariae of a species of *Petasiger* Dietz, 1909.

Comparative analysis revealed that sequences obtained from the intramolluscan larval stages are almost identical with those of the adult worms upon which *P. islandicus* was described. This paper reports on the life-cycle of *P. islandicus* elucidated with the aid of molecular evidence and describes the life-history stages in intermediate hosts of this species.

Materials and methods

Samples of suspected intermediate hosts of *Petasiger islandicus*, the freshwater snail *Gyraulus* cf. *laevis* and the three-spined stickleback *Gasterosteus aculeatus*, were collected from Lake Mývatn in Iceland (65°37′N, 16°59′W) and examined for infections during October, 2009 and October, 2011. Rediae and

3.2.5. Paper X

Morphological and molecular data for larval stages of four species of Petasiger *Dietz, 1909 (Digenea: Echinostomatidae) with an updated key to the known cercariae from the Palaearctic*

Selbach, C., Soldánová, M., Georgieva, S., Kostadinova, A., Kalbe, M. & Sures, B.

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Morphological and molecular data for larval stages of four species of *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) with an updated key to the known cercariae from the Palaearctic

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Received: 26 June 2014 / Accepted: 17 July 2014

Abstract Large-tailed echinostomatid cercariae of the genus *Petasiger* Dietz, 1909 (Digenea: Echinostomatidae) from the planorbid snails *Gyraulus albus* (Müller) and *Planorbis planorbis* (L.) collected in Germany and the Czech Republic and metacercariae from *Gasterosteus aculeatus* L. (Gasterosteiformes: Gasterosteidae) collected in Canada are characterised morphologically and molecularly. The rediae, cercariae and metacercariae are described in detail and compared with the existing data on the larval stages of *Petasiger* spp. Comparative molecular analyses using 28S rDNA and *nad*1 mitochondrial sequences supported the distinct status of four species of *Petasiger*. Molecular and morphological evidence for their distinction and an updated key to the known large-tailed cercariae of *Petasiger* from the Palaearctic are provided.

Christian Selbach and Miroslava Soldánová contributed equally to this work.

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Introduction

Echinostomatids of the genus *Petasiger* Dietz, 1909 constitute a relatively large group of digenean trematodes (33 nominal species, of these 23 species described from the Palaearctic; see Faltýnková et al., 2008). The most recent revision of the genus recognised a total of 18 valid species (Faltýnková et al., 2008). Of these, seven have been described or recorded in Europe: two species possessing 27 collar spines [*P. exaeretus* Dietz, 1909 and *P. phalacrocoracis* (Yamaguti, 1939)] and five species with 19 collar spines [*P. grandivesicularis* Ishii, 1935, *P. islandicus* Kostadinova & Skírnisson, 2007, *P. megacanthus* (Kotlán, 1922), *P. neocomense* Fuhrmann, 1927 and *P. pungens* (Linstow, 1893)] (Faltýnková et al., 2008).

Species of *Petasiger* utilise snails of the family Planorbidae Rafinesque as first intermediate hosts, fish as second intermediate hosts and fish-eating birds as definitive hosts. However, in spite of the numerous records of *Petasiger* spp. in the bird hosts, most noticeably grebes (Faltýnková et al., 2008), data on the

3.3.1. Paper XI

Species diversity of Plagiorchis *Lühe, 1899 (Digenea: Plagiorchiidae) in lymnaeid snails from freshwater ecosystems in central Europe revealed by molecules and morphology*

Zikmundová, J., Georgieva, S., Faltýnková, A., Soldánová, M. & Kostadinova, A.

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Species diversity of *Plagiorchis* Lühe, 1899 (Digenea: Plagiorchiidae) in lymnaeid snails from freshwater ecosystems in central Europe revealed by molecules and morphology

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Received: 18 February 2014 / Accepted: 6 March 2014

Abstract Larval stages of *Plagiorchis* spp. are both ubiquitous and ecologically important parasites in snail populations of freshwater ecosystems in Europe. However, difficulties in distinguishing the morphologically similar cercariae used for species identification, may lead to underestimation of species diversity. In this study, 38 isolates of *Plagiorchis* spp. infecting two lymnaeid snails, *Lymnaea stagnalis* (L.) and *Radix auricularia* (L.), in five central European freshwater ecosystems were subjected to morphological and molecular assessment. Five morphologically homogeneous and genetically distinct lineages of *Plagiorchis* spp. were identified *via* matching molecular data for the mitochondrial cytochrome *c* oxidase subunit I (*cox*1) gene with detailed morphological and morphometric data of the cercariae. Comparative sequence analysis using partial 28S rDNA and ITS1-5.8S-ITS2 sequences revealed that three distinct *cox*1 lineages are conspecific with *Plagiorchis elegans* (Rudolphi, 1802), *P. maculosus* (Rudolphi, 1802) and *P. koreanus* Ogata, 1938, respectively, whereas the lineage identified based on cercarial morphology as *P. neomidis* Brendow, 1970 plus a single isolate that could not be assigned to a described species, did not match any of the available sequences for *Plagiorchis* spp. A key to the cercariae of *Plagiorchis* spp. parasitising lymnaeid populations in central Europe is provided to facilitate identification.

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Introduction

Plagiorchis Lühe, 1899 is the type- and perhaps the most speciose genus of the family Plagiorchiidae Lühe, 1901. Species of *Plagiorchis* utilise a three-host life-cycle using lymnaeid snails as first intermediate hosts, aquatic insects and freshwater crustaceans as second intermediate hosts, and birds and mammals, accidentally amphibians and reptiles, as definitive hosts.

Larval stages of *Plagiorchis* spp. are both ubiquitous and ecologically important parasites in snail populations of freshwater ecosystems in Europe (e.g. Faltýnková et al., 2007; Soldánová et al., 2011). Notably, a single species, *Plagiorchis elegans* (Rudolphi, 1802), is among the most frequently recorded in the inventories of larval trematodes of *Lymnaea stagnalis* (L.) in Europe (Väyrynen et al., 2000; Faltýnková, 2005; Faltýnková & Haas, 2006; Żbikowska et al., 2006; Żbikowska, 2007; Faltýnková et al., 2007). This species has been recognised in these

4. CONCLUDING REMARKS

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4. CONCLUDING REMARKS

This study is the first attempt to alleviate confusion associated with the taxonomy of two complex and widely distributed in the freshwater ecosystems digenean groups, the genus *Diplostomum* (Diplostomidae) and the '*revolutum*' species complex of *Echinostoma* (Echinostomatidae), in future molecular, morphological and ecological studies. Profiting from a large-scale sampling and fruitful collaborations, we have generated large sequence databases for the European species of these groups, linking mitochondrial (*cox*1 or *nad*1) and nuclear (ITS or 28S rDNA) sequences for isolates from intermediate and definitive hosts that were identified based on parasite morphology and by assessing their usefulness for species discrimination. This study is also the first to use morphological and molecular data in conjunction to distinguish between morphologically similar larval stages of *Plagiorchis* spp. (Plagiorchiidae), *Tylodelphys* spp. (Diplostomidae) and *Petasiger* spp. (Echinostomatidae) and the first to apply *cox*1/*nad*1 'barcoding' to species prospecting within these groups in natural host populations.

The application of an integrative approach to the species diversity in the digenean groups studied consisted in combining different lines of evidence for species delineation:

- morphological examination and identification (where possible) of life-cycle stages;
- linking larval and adult stages experimentally or *via* matching sequence data and assessment of the degree of morphological/morphometric differentiation;
- support for reciprocal monophyly and/or sister group relationships in the distance- and model-based phylogenies;
- concordance of phylogenies inferred for mitochondrial and nuclear loci;
- pairwise divergence at mitochondrial loci above the known range of intraspecific variation;
- comparisons with published sequences;
- host use and (where possible) microhabitat specialisation.

4.1. SYNOPSIS OF THE MAIN FINDINGS

FAMILY DIPLOSTOMIDAE

4.1.1. Intensive sampling of life-cycle stages of *Diplostomum* spp. carried out in the northern, central and southern regions of Europe expanded the existing molecular datasets with 144

*cox*1 and 64 ITS1-5.8S-ITS2 novel sequences for isolates from snails, fish and birds. Taken together, our analyses depicted 26 molecularly characterised species and lineages of *Diplostomum* globally. These include three complexes of genetically distinct lineages, i.e. '*D. mergi*', '*D. baeri*' and '*D. huronense*', that would require further appraisal with the application of molecular and morphological approaches focusing on the adult life-cycle stages. Two of the named species and 11 of the lineages (arguably species) delineated in the datasets studied originate from Europe thus indicating a substantial unrecognised genetic diversity inferred from molecular evidence (Papers I–IV; see Appendix 1 for a summary of the European taxa).

4.1.2. This study provided the first estimates of the diversity of *Diplostomum* in sub-Arctic freshwater ecosystems. Our analyses integrating different analytical approaches, phylogenetic analyses, estimates of genetic divergence, character-based barcoding, morphological examination of live larval stages, precise detection of microhabitat specialisation and host use, revealed that at least six species of *Diplostomum* (one described and five putative new species) complete their life-cycles within a fairly narrow geographic area in southern Iceland. This finding increases the species richness of *Diplostomum* in Iceland by 200% and raises the number of molecularly characterised *Diplostomum* spp. from the Palaearctic to 15 species. The cercariae of three and the metacercariae of the six lineages are described in detail and compared with similar forms (Papers II, III).

4.1.3. The first application of a DNA-based approach to diplostomid diversity in the African continent provided evidence for the existence of three distinct brain-infecting species co-occurring in natural populations of the catfish *Clarias gariepinus* in four water bodies of the Lake Victoria, Rufiji and Vami Ruvu basins in Tanzania. The phylogenetic hypotheses estimated from Bayesian inference and maximum likelihood analyses of ITS1-5.8S-ITS2 data exhibited congruent strong support for the *cox*1-derived lineages. Mitochondrial and ribosomal sequences were also generated for a novel species of *Diplostomum* parasitising another African fish host, *Synodontis nigrita* (Paper V; Appendix 1).

FAMILY ECHINOSTOMATIDAE

4.1.4. Delineation of the European species of *Echinostoma* of the '*revolutum*' complex of cryptic species was carried out based on the integration of molecular, morphological and ecological data. The large-scale screening revealed infections with six *Echinostoma* spp.,

including two new species: *E. revolutum* (*sensu stricto*), *E. miyagawai*, *E. paraulum*, *E. bolschewense*, *E. nasincovae* n. sp. and *Echinostoma* sp. IG. The 88 newly-generated *nad*1 sequences from European isolates fall into six distinct well-supported reciprocally monophyletic lineages corresponding to the species identifications based on morphology. Sequences from larval and adult stages were matched for three species, *E. revolutum* (*s. str*.), *E. miyagawai* and *E. paraulum*. The 16 newly-generated 28S rDNA sequences corroborated the distinct species status of the six European *nad*1 lineages (Papers VI, VII).

4.1.5. Taken together, the phylogenetic analyses depicted 17 genetically distinct lineages (12 monophyletic groups and five singletons) within the total *nad*1 data set for *Echinostoma* spp. studied. These represent seven described/named species and ten cryptic species-level lineages of *Echinostoma*, including one cryptic lineage of *E. revolutum* (*sensu lato*) from North America depicted in the present study. This increase in the estimated number of species in the '*revolutum*' group is largely due to the denser sampling conducted within Europe. Excluding singletons, successful assignment of all isolates was achieved by the distance-based identification method implemented in Species Identifier v.1 for all 12 species/lineages. The novel sequence data, in association with the morphological characterisation of the larval and adult life-cycle stages of *Echinostoma* spp. provides an integrative framework for future diversity assessments within this difficult group. These data are discussed in relation to the validity, synonymy and distribution of the species of the '*revolutum*' group worldwide (Papers VI, VII; see Appendix 2 for a summary of the species and re-assigned isolates).

4.1.6. A review, with an updated synonymy, was elaborated for the six European species, including the description of the life-cycle stages for the type-species of *Echinostoma*, *E. revolutum* (*s. str*.), *E. paraulum* and *E. nasincovae* n. sp. Keys to the identification of the cercariae and adults of the European *Echinostoma* spp. are constructed (Paper VIII).

4.1.7. The life-cycle of *Petasiger islandicus*, described recently from an isolated population of the horned grebe *Podiceps auritus* at Lake Mývatn (Iceland), was elucidated with the aid of comparative sequence analysis using *nad*1 sequences for isolates of rediae, large-tailed cercariae and adults. This species utilises the small planorbid snail *Gyraulus* cf. *laevis* as the first intermediate host and the three-spined stickleback *Gasterosteus aculeatus* as the second intermediate host. The redia, cercaria and metacercaria of *P. islandicus* are described and compared with related forms (Paper IX).

4.1.8. Large-tailed cercariae of the genus *Petasiger* emerging from the planorbid snails *Gyraulus albus* and *Planorbis planorbis* collected in Europe and metacercariae from *Gasterosteus* aculeatus collected in Canada were characterised morphologically and molecularly. Comparative morphological and molecular analyses using *nad*1 and 28S rDNA sequences supported the distinct status of four species of *Petasiger*. The rediae, cercariae and metacercariae are described in detail and compared with the existing data on the larval stages of *Petasiger* spp. and an updated key to the known large-tailed cercariae of *Petasiger* from the Palaearctic is constructed (Paper X; Appendix 2).

FAMILY PLAGIORCHIIDAE

4.1.9. The first study to apply *cox*1 'barcoding' to prospect for *Plagiorchis* spp. in natural snail populations and to use morphological and molecular data in conjunction to distinguish between morphologically similar larval stages, revealed five morphologically homogeneous and genetically distinct lineages of *Plagiorchis* spp. infecting the lymnaeid snails *Lymnaea stagnalis* and *Radix auricularia* in five freshwater ecosystems in central Europe. The cercariae of all five species were clearly distinguishable with respect to the novel molecular (*cox*1 and 28S rDNA sequences) and morphometric data. Comparative analysis using partial 28S rDNA and ITS1-5.8S-ITS2 sequences revealed that three distinct *cox*1 lineages are conspecific with *Plagiorchis elegans*, *P. maculosus* and *P. koreanus*, respectively. A key to the cercariae of *Plagiorchis* spp. parasitising lymnaeid populations in central Europe is constructed to facilitate identification (Paper XI).

4.2. CONCLUSIONS

4.2.1. The establishment of *cox*1 reference libraries for the European species of *Diplostomum* and *Plagiorchis* and the African species of *Tylodelphys* and of *nad*1 libraries for the European species of *Echinostoma* and *Petasiger*, provides a foundation that will allow identification and/or assignment of life-cycle stages, elucidation of the life-cycles and further molecular and taxonomic research on these important freshwater parasite groups worldwide. The existence of large comparative databases and the detection of a barcoding gap for both mitochondrial markers indicate that *cox*1 and *nad*1 should be the first choice for large-scale barcode-based identification of the species of the diplostomids and plagiorchiids, and the echinostomatids, respectively.

4.2.2. The concordance of the results of at least three protocols/lines of evidence used for species delimitation in the digenean groups studied provides strong support to our morphology-based hypotheses for the distinct status of the species/lineages. The overall good agreement between the genetic and morphological distinctness of the species/lineages provides a framework for monitoring the diversity and transmission of the digenean groups studied in the freshwater ecosystems in Europe. The sets of morphological and/or genetic markers defined in the course of the study will allow confident identification of species/lineages in the focal freshwater digenean groups. Identification of the larval stages of lineages within the '*revolutum*' species complex of *Echinostoma* and of *Petasiger* spp. in Europe can be reliably based on morphological methods (light microscopy examination of live cercariae) in association with biological data (host use). This approach appears also plausible for most of the species of *Plagiorchis* and there is a possibility to develop predictive algorithms for morphometric discrimination of fixed cercariae. However, identification of the larval stages of the cryptic lineages within the '*D. mergi*' and '*D. baeri*' species complexes should be based on light and scanning electron microscopy examination (cercariae) and sequencing (cercariae, metacercariae) in association with microhabitat selection and host use data (metacercariae); predictive models for morphometric delineation of the metacercariae of *Diplostomum* spp. may represent a promising approach to larval identification.

4.2.3. The species diversity of the freshwater digenean groups studied is higher than previously thought, as evidenced by the description of one new species, *Echinostoma nasincovae*, and the detection of 26, 5, 17, 4 and 5 genetically and, in most cases, morphologically distinct lineages (named and putative new species) within the genera *Diplostomum*, *Tylodelphys*, *Echinostoma*, *Petasiger* and *Plagiorchis*.

4.2.4. Phylogenetic analyses indicated high rates of speciation within the '*D. baeri*' species complex, which comprises at least eight molecularly characterised species: five ["*D. baeri* NA", *Diplostomum* spp. 2, 5, 6 and 7 of Locke et al. (2010a, b)] recorded in North America, two ['*D. baeri* 1 (trout)' and '*D. baeri* 2 (perch)' of Georgieva et al. (2013)] in both Europe and Iceland and one [*Diplostomum* sp. 'Lin 5I' of Blasco-Costa et al. (2014)] in Iceland only. '*Diplostomum baeri*' thus appears to be the most diverse species group within the genus *Diplostomum*.

4.2.5. A preliminary exploration of the evolutionary history in the context of microhabitat selection and geography suggests that speciation in *Diplostomum* is a result of expansion-

contraction events of both the ecological niche and geographic ranges of the common ancestor, which is inferred as a lens-infecting species with a Holarctic distribution. The basal clustering in the phylogeny appears to be better explained by the microhabitat specialisation, the species inhabiting lens being clearly divergent from the species found in non-lens microhabitats.

4.2.6. The diversity of *Diplostomum* spp. appears underestimated globally in the high latitude ecosystems. The increased taxonomic resolution achieved in this study of the larval stages in snails and fish of the sub-Arctic lakes in Iceland resulted in the addition of four putative species each to the parasite lists for salmonids and gasterosteids and thus has important implications for parasite diversity baselines in salmonid and gasterosteid hosts in the Arctic. The high species richness of *Diplostomum* in the limited area of the study calls for a cautionary approach to pathogen identification in developing the much needed baselines of pathogen diversity that may help detect effects of climate change in the freshwater environment of the sub-Arctic.

4.2.7. The high abundance of *Diplostomum* spp. in the fishes from the lakes in and around Reykjavik observed recently may provide circumstantial evidence for possible association with increased parasite transmission rates due to the rise of the temperature in the area. Furthermore, the higher infection levels in *Salvelinus alpinus* in relation to these in *Salmo trutta fario* indicate that infections with pathogenic *Diplostomum* spp. may mediate the outcomes of the competition between the two species with consequences for patterns of potential extinctions of *Sa. alpinus* in the high latitude ecosystems under the climate change scenario.

4.2.8. Molecular elucidation of the life-cycles of nine species (the diplostomids *Diplostomum spathaceum* and *D. pseudospathaceum*; the echinostomatids *Echinostoma revolutum*, *E. miyagawai*, *E. paraulum* and *Petasiger islandicus*; and the plagiorchiids *Plagiorchis elegans*, *P. koreanus* and *P. maculosus*) was achieved *via* matching of multiple sequences for isolates from natural and/or experimental infections identified on the basis of the morphology of life-cycle stages from snails, fish and birds.

4.2.9. Critical assessment of the relationships between isolates in the analyses based on mitochondrial and/or nuclear gene sequences, associated with morphological data where possible, is essential for refined delineation of and formulation of further hypotheses for

species boundaries within the freshwater digenean groups studied. The application of this approach resulted in the re-assignment of 40 isolates of *Diplostomum* spp. (detailed in Appendix 1) and of 41 isolates of *Echinostoma* spp. (detailed in Appendix 2) and in taxonomic and nomenclatural changes as follows: new combination for *Diplostomum mashonense* (as *Tylodelphys mashonensis*), description of a new species of *Echinostoma*, re-validation of *E. paraulum* and *E. miyagawai* and synonymisation of *E. friedi* and the German and Australian isolates identified as *E. revolutum* by Morgan & Blair (1995; 1998a, b) with the latter species. Of particular importance for further taxonomic scrutiny within the major groups studied, is the fact that the type-species of the genera *Diplostomum* and *Echinostoma* are redefined in the strict sense, based on molecular and morphological evidence for adult and larval life-cycle stages.

4.3. FUTURE PROSPECTS

The answer of the long-standing question "How many species?" in relation to the focal digenean groups is currently "More than we were aware of" ... but how many more globally? And which ones? The findings of this study indicate that future efforts should be focused on at least four logically interconnected actions, which we name ELDR strategy: (i) **Expand** (sampling effort): sample more, sample widely (on a continental and host scale), sample adults; (ii) **Link**: molecular, morphological and ecological evidence, larvae and adults; newly-collected and museum material (iii) **Describe**: all life-cycle stages; and (iv) **Revise**: species, genera, synonymies.

Funding agencies are generally happy with (i) and (ii) (excluding examination of the 'dust-covered' museum material) but not with (iii) and (iv) (A. Kostadinova, personal communication) in spite of the critical importance of a good taxonomy for biodiversity assessments and the sporadic outcries about the taxonomic impediment. However, although significant progress towards uncovering the diversity of the focal groups in Europe has been achieved, the results of the present study and ongoing work strongly suggest that thorough global taxonomic revisions are required for *Diplostomum*, *Echinostoma* and *Plagiorchis*. Historically, the studies of freshwater parasites were initiated in Europe in the 18th Century and carried out actively by several prolific teams of faunists and taxonomists within the Palaeacrtic during the 19th and 20th Century. This is reflected in the number of taxa described from Europe and the Palaearctic and their 'age', i.e. date of original description, e.g. *E. revolutum* (Frölich, 1802), *Diplostomum spathaceum* (Rudolphi, 1819), *Plagiorchis elegans* (Rudolphi, 1802), but also in a convoluted synonymy. As our results have shown, the Nearctic

and Palaearctic species cannot be treated in isolation. For example, the clarification of the status of the type-species of *Echinostoma*, *E. revolutum* (*s. str*.), revealed the presence of a cryptic species in the USA and the clarification of the status of the type-species of *Diplostomum*, *D. spathaceum*, seems to have partially prevented flooding of genetic databases (e.g. BOLD) with sequences labelled as *D. paracaudum*, a species with no documented natural fish and bird hosts that otherwise would have appeared the most abundant Holarctic species of *Diplostomum*. Ongoing research also has revealed five and four additional novel lineages of *Plagiorchis* in snail and invertebrate hosts in Norwegian and Canadian lakes, respectively, and several additional novel lineages of *Diplostomum* spp. parasitising fishes in China.

The ELDR strategy, therefore, should be focused on clarification of the taxonomy of the Palaearctic species of the focal groups of freshwater digeneans before undertaking rationalisation of the Nearctic faunas. So far, this state has been reached for the species of the '*revolutum*' group of *Echinostoma* only, although the North American species require taxonomic scrutiny and the critical evaluation of the recent Asian studies on the group indicates a need of sampling and expert assessment farther east. However, the life-cycles and taxonomy of the cryptic lineages within the '*D. baeri*', '*D. mergi*' and '*D. huronense*' species complexes are yet to be fully elucidated.

Untying the complex knot of the '*D. baeri*' species complex is still pending ELDR. First, we should expand the sampling within Europe focusing on the adult stages and link the molecular and morphological evidence for the European 'larval' lineages with corresponding evidence for the adult forms. The diversity of the group in the sub-Arctic lakes in Iceland indicates that the search for adults should be focused north. Secondly, we should attempt sampling of *Diplostomum* spp. from Nearctic percids (species of the genera *Ammocrypta*, *Crystallaria*, *Etheostoma*, *Perca*, *Percina* and *Stizostedion*), salmonids (species of the genera *Oncorhynchus* and *Salvelinus*) and gasterosteids (species of the genera *Gasterosteus* and *Pungitius*) in order to uncover and characterise possible additional species/lineages and reveal the relationships between the Palaearctic and Nearctic forms. Finally, obtaining sequences from adult isolates of *Diplostomum* spp. parasitising fish-eating birds and their morphological assessment are critical for reaching the revisionary stage and subsequently clarifying the taxonomy of the lineages within this species rich group. This is also perhaps the weakest point in the plan, since due to ethical reasons examination of birds is difficult.

In the same vein, the ELDR strategy is currently being applied to the '*D. mergi*' species complex. Work in progress counteracted the fears of the reviewers of the first paper on *Diplostomum* spp. (Paper I) that the three lineages within the '*D. mergi*' complex are

represented by just a few isolates. We are currently analysing ample molecular and morphological evidence from abundant set of cercarial isolates from snails that confirm the distinct status of the three lineages depicted in the present moleculari study (Paper I). One of these proved to be *D. parviventosum sensu* Niewiadomska & Kiseliene (1994). But then what is *D. parviventosum* of Niewiadomska & Laskowski (2002) identified using the criteria of Niewiadomska & Kiseliene (1994) and having identical ITS1 sequence with *D. spathaceum sensu* Niewiadomska & Laskowski (2002) (both representing Clade Q, i.e. questionable, in our current treatment)? Wider sampling of larval and adult stages and careful morphological characterisation is required to solve the 'Clade Q' knot and the even more complex '*D. mergi*' knot.

Many intricately linked taxonomic questions related to the focal digenean groups remain and I look forward to addressing these in my future research.

4.4. REFERENCES

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5.1. APPENDIX 1

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Appendix 1 Summary of the novel and published sequences for diplostomid isolates from Europe and Africa, their hosts, localities and the number sequences for each marker. Hosts for each species/lineage are arranged alphabetically in the following order: snail intermediate host - fish intermediate host - definitive bird host. Shaded areas indicate re-assigned isolates as a result of the phylogenetic analyses in the present study.

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			*cox*1	ITS1-5.8S-ITS2	ITS1	
1. *D. spathaceum*						
	Radix auricularia	Germany: Lake Hengsteysee	2	2		Georgieva et al. (2013)
(as *D. paracaudum*)	*Radix auricularia*	Germany: Lake Constance	JQ639176			Behrmann-Godel (2013)
(as *D. paracaudum*)	*Coregonus lavaretus*	Germany: Lake Constance		JQ665457		Behrmann-Godel (2013)
	Gasterosteus aculeatus	Germany: Lake Hengsteysee	4			Georgieva et al. (2013)
	Gasterosteus aculeatus	Iceland: Lakes Family Park and Nordic House	6	1		Blasco-Costa et al. (2014)
(as *D.* cf. *paracaudum*)	*Leuciscus leuciscus*	Finland: Lake Huumojarvi			JF775721	Rellstab et al. (2011)
	Misgurnus anguillicaudatus	Spain: Ebro Delta	2	2		Perez-del-Olmo et al. (2014)
	Pseudochondrostoma willkommii	Spain: Villafranco del Guadiana	7	2		Perez-del-Olmo et al. (2014)
(as *D.* cf. *paracaudum*)	*Rutilus rutilus*	Finland: Lakes Vuojarvi and Kuivasjarvi			JF775723 JF775704	Rellstab et al. (2011)
	Salvelinus alpinus	Iceland: Lake Hafravatn	1	1		Blasco-Costa et al. (2014)
	Silurus glanis	Spain: Ebro Delta	1			Perez-del-Olmo et al. (2014)
	Larus argentatus	Poland: Gdańsk	1	1		Georgieva et al. (2013)
	Larus a. michahellis	Spain: Ebro Delta	3			Perez-del-Olmo et al. (2014)
	Larus cachinans	Czech Republic: Tovačov	2	2		Georgieva et al. (2013)
	Larus ridibundus	Spain: Cunit; Ebro Delta	3	1		Perez-del-Olmo et al. (2014)
2. *D. paracaudum* of Niewiadomska & Laskowski (2002)						
	Radix ovata	Poland: near Warsaw			AF419272	Niewiadomska & Laskowski (2002)
3. *D. pseudospathaceum*						
	Lymnaea stagnalis	Germany: Lakes Baldeneysee and Harkortsee	4	2		Georgieva et al. (2013)
	Lymnaea stagnalis	Poland: near Warsaw			AF419273	Niewiadomska & Laskowski (2002)
	Lymnaea stagnalis	Finland: Lake Huumojarvi			JF775760	Rellstab et al. (2011)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			*cox*1	ITS1-5.8S-ITS2	ITS1	
	Radix labiata	Germany: Lake Constance	6 isolates (JQ639170–JQ639175)			Behrmann-Godel (2013)
	Stagnicola palustris	Germany: Lakes Hengsteysee and Sorpesee	3			Georgieva et al. (2013)
(as *D.* cf. *pseudospathaceum*)	*Alburnus alburnus*	Finland: Lake Konnevesi			JF775729	Rellstab et al. (2011)
	Gasterosteus aculeatus	Germany: Lake Hengsteysee	2	1		Georgieva et al. (2013)
	Gymnocephalus cernua	Germany: Lake Constance		JQ665456		Behrmann-Godel (2013)
(as *D.* cf. *pseudospathaceum*)	*Gymnocephalus cernua*	Finland: Lake Liesvesi			JF775752	Rellstab et al. (2011)
	Larus argentatus	Poland: Gdańsk	1	1		Georgieva et al. (2013)
	Larus cachinans	Czech Republic: Tovačov	2	2		Georgieva et al. (2013)
	Larus ridibundus	Spain: Ebro Delta	1	1		Perez-del-Olmo et al. (2014)
'*D. baeri*' species complex						
4. '*D. baeri* 1 (trout)' of Georgieva et al. (2013)						
	Gobio gobio	Germany: River Ruhr (Henne)	1	1		Georgieva et al. (2013)
	Salmo trutta fario	Germany: Rivers Ruhr (Henne) and Lenne	13	3		Georgieva et al. (2013)
(as *Diplostomum* sp. 'Lin 3I')	*Salmo trutta fario*	Iceland: Lake Hafravatn	8	2		Blasco-Costa et al. (2014)
(as *Diplostomum* sp. 'Lin 3I')	*Salvelinus alpinus*	Iceland: Lake Hafravatn	6	3		Blasco-Costa et al. (2014)
5. '*D. baeri* 2 (perch)' of Georgieva et al. (2013)						
(as *Diplostomum* sp. 'Lin 4I')	*Radix peregra*	Iceland: Lake Nordic House	2	3		Blasco-Costa et al. (2014)
(as *Diplostomum* sp. 'Lin 4I')	*Gasterosteus aculeatus*	Iceland: Lakes Nordic House and Hafravatn	8	5		Blasco-Costa et al. (2014)
(as *D. baeri*)	*Perca fluviatilis*	Germany: Lake Constance	14 isolates (JQ639180 JQ639183 JQ639185-JQ639195)	1		Behrmann-Godel (2013)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			*cox*1	ITS1-5.8S-ITS2	ITS1	
***Diplostomum* sp. 'Lin 3I' or 'Lin 4I' of Blasco-Costa et al. (2014)**						
(as *D.* cf. *baeri*)	*Myxas glutinosa*	Finland: Lake Konnevesi			JF775683 JF775682	Rellstab et al. (2011)
(as *D.* cf. *baeri*)	*Radix ovata*	Finland: Lake Konnevesi			JF775685	Rellstab et al. (2011)
(as *D. baeri*)	*Perca fluviatilis*	Poland: near Warsaw			AF419274	Niewiadomska & Laskowski (2002)
(as *D.* cf. *baeri*)	*Perca fluviatilis*	Germany: Lake Constance		JQ665460		Behrmann-Godel (2013)
6. *Diplostomum* sp. 'Lin 5I'						
(as *D.* cf. *baeri*)	*Myxas glutinosa*	Finland: Lake Konnevesi			JF775680 JF775681	Rellstab et al. (2011)
	Salvelinus alpinus	Iceland: Lake Hafravatn	5	3		Blasco-Costa et al. (2014)
	Salmo trutta fario	Iceland: Lake Hafravatn	7	2		Blasco-Costa et al. (2014)
'*D. mergi*' species complex						
7. '*D. mergi* 1' of Georgieva et al. (2013)						
	Radix auricularia	Germany: Lake Hengsteysee	1	1		Georgieva et al. (2013)
8. '*D. mergi* 2' of Georgieva et al. (2013)						
	R. auricularia	Germany: Lake Hengsteysee	3	1		Georgieva et al. (2013)
(as *D. mergi*)	*Radix balthica*	Denmark: Lake Fure		JX494231		Haarder et al. (2014)
(as *D.* cf. *mergi*)	*Radix ovata*	Finland: Lake Konnevesi			JF775690	Rellstab et al. (2011)
(as *D.* cf. *mergi*)	*Alburnus alburnus*	Finland: Lake Konnevesi			JF775686	Rellstab et al. (2011)
(as *D.* cf. *mergi*)	*Rutilus rutilus*	Finland: Lake Kuivasjarvi			JF775689	Rellstab et al. (2011)
9. '*D. mergi* 3' of Georgieva et al. (2013)						
(as *D. mergi*)	*Radix balthica*	Denmark: Lake Fure		JX494233		Haarder et al. (2014)
(as *D. mergi*)	*Radix ovata*	Poland: near Warsaw			AF419279	Niewiadomska & Laskowski (2002)
	Gobio gobio	Germany: River Ruhr (Henne)	4	3		Georgieva et al. (2013)
	Salmo trutta fario	Germany: River Ruhr (Henne)	6	2		Georgieva et al. (2013)
10. *Diplostomum* sp. 'Lin 2I'						
(as *D.* cf. *pseudospathaceum*)	*Radix ovata*	Finland: Lake Konnevesi			JF775684	Rellstab et al. (2011)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			*cox*1	ITS1-5.8S-ITS2	ITS1	
	Radix peregra	Iceland: Lake Raudavatn	1	1		Blasco-Costa et al. (2014)
	Gasterosteus aculeatus	Iceland: Lakes Hafravatn and Nordic House	10	2		Blasco-Costa et al. (2014)
(as *D*. cf. *pseudospathaceum*)	*Rutilus rutilus*	Finland: Lake Kuivasjärvi		JF775755 JF775756		Rellstab et al. (2011)
	Salmo trutta fario	Iceland: Lake Hafravatn	10	3		Blasco-Costa et al. (2014)
11. *Diplostomum* sp. 'Lin 6I'						
	Radix peregra	Iceland: Lake Nordic House	5	5		Blasco-Costa et al. (2014)
	Gasterosteus aculeatus	Iceland: Lake Nordic House	7	4		Blasco-Costa et al. (2014)
12. *Diplostomum* sp. 'Clade Q' of Georgieva et al. (2013)						
(as *D. spathaceum* but as *D. mergi* in GenBank)	*Radix auricularia*	Germany: Lake Constance	JQ639179	JQ665458		Behrmann-Godel (2013)
(as *D. parviventosum*)	*Radix ovata*	Poland: near Warsaw and near Kosewo			AF419277 AF419278	Niewiadomska & Laskowski (2002)
(as *D. spathaceum*)	*Radix ovata*	Poland: near Warsaw and near Kosewo			AF419275 AF419276	Niewiadomska & Laskowski (2002)
(as *Diplostomum* sp. 'Clade Q')	*Cyprinus carpio*	Ebro Delta	1	1		Perez-del-Olmo et al. (2014)
(as *D*. cf. *parviventosum*/ *spathaceum*)	*Rutilus rutilus*	Finland: Lakes Huumojarvi and Konnevesi			JF775725 JF775727	Rellstab et al. (2011)
(as *D. spathaceum* but as *D. mergi* in GenBank)	*Rutilus rutilus*	Germany: Lake Constance	3 isolates (JQ639177–JQ639179)			Behrmann-Godel (2013)
13. '*D. huronense*' species complex						
(as *D*. cf. *pseudospathaceum*)	*Myxas glutinosa*	Finland: Lake Konnevesi			JF775757 JF775758	Rellstab et al. (2011)
14. *Diplostomum* sp. Nigeria						
	Synodontis nigrita	Nigeria	2	1		Chibwana et al. (2013)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)		Reference
			*cox*1	ITS1-5.8S-ITS2	
15. *Tylodelphys clavata*					
	Radix auricularia	Germany: Lake Hengsteysee	1		Georgieva et al. (2013)
	Radix auricularia	Germany: Lake Constance	JQ639199		Behrmann-Godel (2013)
	Radix labiata	Germany: Lake Constance	JQ639197 JQ639198		Behrmann-Godel (2013)
	Coregonus lavaretus	Germany: Lake Constance		JQ665459	Behrmann-Godel (2013)
	Perca fluviatilis	Germany: River Lippe	1		Georgieva et al. (2013)
	Perca fluviatilis	Germany: Lake Constance	6 isolates (JQ639196 JQ639200-JQ639204)		Behrmann-Godel (2013)
16. *Tylodelphys excavata*					
	Planorbarius corneus	Czech Republic: Pond Bohdaneč	1	1	Chibwana et al. (2013)
17. *Tylodelphys mashonensis* n. comb.					
	Clarias gariepinus	Tanzania: Lake Victoria	7	2	Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: River Kilombero	3		Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: River Msimbazi	4	1	Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: River Ruvu	3		Chibwana et al. (2013)
18. *Tylodelphys* sp. 1					
	Clarias gariepinus	Tanzania: Lake Victoria	6	2	Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: River Kilombero	2		Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: River Ruvu	3		Chibwana et al. (2013)
19. *Tylodelphys* sp. 2					
	Clarias gariepinus	Tanzania: River Ruvu	1	1	Chibwana et al. (2013)
	Clarias gariepinus	Tanzania: Lake Victoria	2	1	Chibwana et al. (2013)

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5.2. APPENDIX 2

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Appendix 2 Summary of the novel and published sequences for echinostomatid isolates, their hosts, localities and the number sequences for each marker. Hosts for each species/lineage are arranged alphabetically in the following order: snail intermediate host - definitive bird host. Shaded areas indicate re-assigned isolates as a result of the present phylogenetic analyses.

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			*nad*1	ITS1	28S	
1. *E. revolutum* (*s. str.*)						
	Lymnaea stagnalis	Germany: Lakes Bodensee; Baldeneysee; Hengsteysee; Pond near Krausenbechhofen	5			Georgieva et al. (2014)
	Lymnaea stagnalis	Czech Republic: Ponds Bartoňovský; Dvorecký; Hluboký u Hamru; Vlkovský	8		4	Georgieva et al. (2014)
	Lymnaea stagnalis	Finland: Lake Huumojärvi, Oulu	2			Georgieva et al. (2014)
	Radix auricularia	Germany: Lake Hengsteysee	2			Georgieva et al. (2013)
	Radix auricularia	Poland: Pond near Tomislawice	1			Georgieva et al. (2014)
	Radix peregra/*Columba livia* (exp.)	Bulgaria: Grigorevo	AY168933	AY168930		Kostadinova et al. (2003)
	Radix peregra	Iceland: Lake Mývatn	3			Georgieva et al. (2013)
	Radix peregra	Germany: Lake Hennetalsperre	5			Georgieva et al. (2013)
	Stagnicola palustris	Germany: Lake Hengsteysee	1			Georgieva et al. (2013)
	Stagnicola palustris	Czech Republic: Pond Hluboký u Hamru			1	Georgieva et al. (2014)
	Aythya fuligula	Czech Republic: Vicinities of Tovačov	1		1	Georgieva et al. (2014)
2. "*E. revolutum*" USA	*Lymnaea elodes* and *L. elodes*/*Gallus gallus* f. dom. (exp.)	USA: Indiana	34 isolates: (GQ463056-GQ463090)	3 isolates (GQ463128-GQ463130)		Detwiler et al. (2010)
	Ondatra zibethicus	USA: Virginia	JQ670862			Detwiler et al. (2012)
3. *E. bolschewense*	*Viviparus acerosus*	Slovak Republic: Danube at Gabčíkovo	16		2	Georgieva et al. (2014)
4. *E. caproni*	Laboratory isolate	Madagascar	AF025837	U58098		Morgan & Blair (1998a)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			***nad*1**	**ITS1**	**28S**	
	Rattus norvegicus	Egypt: Cairo	AJ564378	AJ564382		Marcilla et al. (Gen Bank only)
	Laboratory isolate	Cameroon	AF025838	U58104		Morgan & Blair (1998a)
	Laboratory isolate	Africa			AF026104	Mollaret et al. (1997)
5. *E. deserticum*	Laboratory isolate	Niger	AF025836	U58103		Morgan & Blair (1998a)
6. *E. miyagawai*						
(as *E. revolutum*)	*Austropeplea lessoni*	Australia: Townsville	AF026288			Morgan & Blair (1998b)
(as *E. revolutum*)	*Glyptophysa* sp.	Australia: Townsville	AF026286 AF026287			Morgan & Blair (1998b)
(as *E. revolutum*)		Europe: Germany	AF025832	U58102		Morgan & Blair (1995, 1998a,b)
(as *E. friedi*)	*Mesocricetus auratus*	Spain: Valencia	AJ564379	AJ564383	AY219700	Marcilla et al. (GenBank only; 2003)
	Planorbis planorbis	Czech Republic: Pond Loužek	14			Georgieva et al. (2014)
	Anas platyrhynchos	Poland: vicinities of Gdańsk	2			Georgieva et al. (2014)
	Aythya fuligula	Czech Republic: vicinities of Tovačov	2		1	Georgieva et al. (2014)
7. *Echinostoma nasincovae* n. sp.	*Planorbarius corneus*	Slovak Republic: Danube at Gabčíkovo	6			Georgieva et al. (2014)
	Planorbarius corneus	Czech Republic: Pond Bohdaneč	3		1	Georgieva et al. (2014)
	Planorbarius corneus	Czech Republic: Pond Vlkovský	5		2	Georgieva et al. (2014)
	Planorbarius corneus	Czech Republic: Pond Hluboký u Hamru	3		1	Georgieva et al. (2014)
	Planorbarius corneus	Czech Republic: Pond Bohumilečský	1			Georgieva et al. (2014)
8. *E. paraensei*	Laboratory isolate	Brazil	AF025834	U58100		Morgan & Blair (1998a)
	Glyptophysa sp.	Australia: Townsville	AF026282			Morgan & Blair (1998b)
9. *E. paraulum*	*Lymnaea stagnalis*	Germany: Pond near Poppenwind; Nature Reserve Mohrhof	4		1	Georgieva et al. (2014)
	Aythya fuligula	Czech Republic: vicinities of Tovačov	1		1	Georgieva et al. (2014)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			***nad*1**	**ITS1**	**28S**	
10. "*E. robustum*/*friedi*" Lineage A of Detwiler et al. (2010)	*Lymnaea elodes*	USA: Indiana	GQ463053	GQ463132		Detwiler et al. (2010)
11. "*E. robustum*/*friedi*" Lineage C of Detwiler et al. (2010)	*Lymnaea elodes*	USA: Minnesota	GQ463054			Detwiler et al. (2010)
12. "*E. robustum*/*friedi*" Lineage D of Detwiler et al. (2010)	*Biomphalaria glabrata*/*Gallus gallus* f. dom. (exp.)	Brazil	GQ463055	GQ463133		Detwiler et al. (2010)
13. *E. trivolvis* Lineage A	Laboratory isolate	North America	AF025831	U58097		Morgan & Blair (1998a)
	Helisoma trivolvis	USA: Indiana	GQ463047 GQ463048	GQ463124 GQ463125		Detwiler et al. (2010)
	Ondatra zibethicus	USA: Virginia	JQ670850			Detwiler et al. (2012)
(as *E. revolutum*)	*Physa heterostropha* (exp.)/ Adult tissue of *E. revolutum* of Olson et al. (2003)		GQ463049	GQ463126	AY222246	Detwiler et al. (2010); Olson et al. (2003)
14. *E. trivolvis* Lineage B	*Lymnaea elodes*	USA: Indiana	GQ463050	GQ463127		Detwiler et al. (2010)
	Ondatra zibethicus	USA: Virginia	9 isolates (JQ670851-JQ670859)			Detwiler et al. (2012)
15. *E. trivolvis* Lineage C	*Ondatra zibethicus*	USA: Wisconsin	GQ463051 GQ463052			Detwiler et al. (2010)
	Ondatra zibethicus	USA: Virginia	JQ670860 JQ670861			Detwiler et al. (2012)
16. *Echinostoma* sp. IG	*Radix auricularia*	Germany: Lake Hengsteysee	2		1	Georgieva et al. (2013)
	Radix peregra	Iceland: Lake Nordic House	1			Georgieva et al. (2013)
(as *E.* cf. *friedi*)	*Radix peregra*	UK: Lake Pwll Penarth	AY168937			Kostadinova et al. (2003)
17. *Echinostoma* sp. NZ-Ad	*Branta candensis*	New Zealand	AF026289			Morgan & Blair (1998b)

Species	Host	Locality	Number of new sequences (GenBank ID for other published sequences)			Reference
			***nad*1**	**ITS1**	**28S**	
18. *Petasiger islandicus*	*Gyraulus* cf. *laevis*	Iceland: Lake Mývatn	3			Georgieva et al. (2012)
	Podiceps auritus	Iceland: Lake Mývatn	1			Georgieva et al. (2012)
19. *Petasiger* sp. 1	*Gyraulus albus*	Germany: Lake Hennetalsperre	2		2	Selbach et al. (2014)
	Planorbis planorbis	Czech Republic: Pond Černiš			1	Selbach et al. (2014)
20. *Petasiger* sp. 2	*Gyraulus albus*	Germany: Lake Hennetalsperre	2		1	Selbach et al. (2014)
21. *Petasiger* sp. 3	*Gyraulus albus*	Germany: Lake Hennetalsperre	2		2	Selbach et al. (2014)
	Gyraulus albus	Germany: Lake Hengsteysee	1		1	Selbach et al. (2014)
	Planorbis planorbis	Germany: Lake Kleiner Plöner See	2		1	Selbach et al. (2014)
22. *Petasiger* sp. 4	*Gasterosteus aculeatus*	Canada: Lake Gosling	1		1	Selbach et al. (2014)

5.3. APPENDIX 3

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VERSITA

Fasciola hepatica miracidia: Lectin binding and stimulation of *in vitro* miracidium-to-sporocyst transformation

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Abstract

The lectin binding properties of *Fasciola hepatica* miracidia were studied by a panel of fluorescein- and gold-conjugated lectins (ConA, LCA, WGA, LEA, SBA, HPA and UEA-I). The presence of mannose and/or glucose residues was demonstrated with ConA and LCA as weak diffuse fluorescence of the miracidial surface, which was more intense at the anterior part of the larva. The N-acetylglucosamine-binding lectins WGA and LEA reacted intensely with the whole miracidial surface. No labelling with N-acetylgalactosamine and/or galactose-specific (SBA and HPA) and fucose-specific UEA-I lectins was observed. The possibility that the specific recognition of the miracidial surface carbohydrates by lectins may initiate the process of transformation of the miracidia into sporocysts was examined *in vitro* in physiological saline for *Galba truncatula*. Incubation in the presence of ConA and WGA resulted in facilitation of the transformation process. Facilitation was absent in the presence of inhibitor sugars. Incubation in the presence of SBA or UEA-I had no effect. The results suggested a possible impact of carbohydrate-lectin interactions in transformation of miracidia of *F. hepatica* to sporocysts *in vivo*.

Keywords

Fasciola hepatica, miracidia, lectin binding, lectin-carbohydrate interactions, *in vitro* miracidium-to-sporocyst transformation

Introduction

The surface carbohydrates of trematode larvae have long attracted attention due to the important role of glycan-binding proteins (lectins) among the non-self recognition mechanisms of the intermediate gastropod host (Horák and van der Knaap 1997, Lockyer *et al*. 2004, Loker 2010). One traditional way to characterize carbohydrate surface epitopes is using panels of commercially available lectins with different sugar specificities. Extensive studies on larval stages of representatives of the family Schistosomatidae have drawn the attention to the occurrence of stage- and species-specific differences in the lectin-binding capacity. This also concerns miracidia (Coles *et al*. 1988). Notably, several species (*Schistosoma mansoni*, *S. margrebowiei*, *Trichobilharzia ocellata* and *T. szidati*) were shown to undergo drastic changes in their surface carbohydrates and lose a number of their surface epitopes while sloughing their ciliated epithelia during miracidium-to-sporocyst transformation (Yoshino *et al*. 1977, Gerhardus *et al*. 1991, Daniel *et al*. 1992, Horák 1995, Peterson *et al*. 2009).

Fasciola hepatica, a helminth of unquestionable economic importance, has attracted little attention in this respect. This motivated our interest, and we have previously shown stage-specific lectin reactivities in sporocysts and rediae (Georgieva *et al*. 2005, 2007). The present study is directed to the characterization of the lectin-binding capacity of miracidia.

While sporocysts and rediae throughout their life span are in contact with snail effector mechanisms, including hemolymph lectins, the possible role of species specificity of miracidial surface carbohydrates and the related specific lectin binding capacity is less clear. One possibility is that this might be responsible for the recognition of the specific host by the miracidia prior to the penetration of miracidia into the snail. Specificity at this level of the host-parasite interaction is however quite questionable. For instance, using a set of four digenean (echinostomatid and schistosomatid) and five snail species, Sapp and Loker (2000) failed to confirm host selectivity at the stage of miracidium-snail attachment or penetration. Rather, over half of the miracidia of each parasite species attached and attempted to penetrate into both compatible and incompatible snails.

Another possibility is that the species-specific miracidial carbohydrate disguise may be related with triggers of their developmental into a next intramolluscan larval stage upon con-

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Morphometric and molecular characterisation of specimens of *Lepidapedon* Stafford, 1904 (Digenea: Lepidapedidae) from the deep-sea fish *Mora moro* (Risso) (Teleostei: Moridae) in the western Mediterranean

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Received: 25 January 2013 / Accepted: 22 May 2013

Abstract In a study of the parasites of the deep-sea fish *Mora moro* (Risso) (Gadiformes: Moridae) off the Mediterranean coasts of Catalonia and the Balearic Islands (Spain), we were able to distinguish two morphs of specimens belonging to *Lepidapedon* Stafford, 1904 (Digenea: Lepidapedidae). This material is herein described and illustrated. Comparative sequence analyses using partial mitochondrial *nad*1 sequences revealed that the material assigned to one of these morphs can be considered conspecific with the material identified as *Lepidapedon desclersae* Bray & Gibson, 1995 from the same host. However, the published *nad*1 sequence for *L. desclersae* was generated from a specimen ex *M. moro* from the North East Atlantic. Examination of the voucher specimens associated with this sequence revealed that both the North East Atlantic and the Mediterranean specimens ex *M. moro* differ from *L. desclersae* as described from its type-host, *Lepidion eques* (Günther), in the anterior extent of the vitelline fields which is further posterior, reaching only to the posterior margin of the external seminal vesicle in *L. desclersae*, versus being at the mid-level of this organ and reaching the posterior margin of the ventral sucker. Therefore, we have tentatively assigned the material characterised here, both morphologically and molecularly as *Lepidapedon* sp. Acquisition of additional sequences for both *nad*1 mitochondrial and 28S rRNA genes of *L. desclersae* from material ex *Lepidion* spp. is required in order to determine whether the observed morphometric variation reflects host-related or interspecific differences. The second morph of *Lepidapedon* from *M. moro* is described and distinguished on morphometric grounds, such as the position of the most anterior vitelline follicles, which reach to the anterior margin of the ventral sucker. Its identity is commented upon, but, in view of the fact that there were few specimens and no molecular data available, it is not named.

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A new species of *Saturnius* Manter, 1969 (Digenea: Hemiuridae) from Mediterranean mullet (Teleostei: Mugilidae)

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Received: 9 December 2013 / Accepted: 27 December 2013

Abstract A new hemiurid digenean, *Saturnius gibsoni* n. sp., is described from the stomach lining of *Mugil cephalus* L. off Oran, Mediterranean coast of Algeria. Characteristic morphological features of the new species include small size of the body which is comprised of six pseudosegments, small ventral sucker, weakly developed mound-shaped flange at the level of the ventral sucker, and eggs being large in relation to the size of the body. *Saturnius gibsoni* n. sp. resembles *S. minutus* Blasco-Costa, Pankov, Gibson, Balbuena, Raga, Sarabeev & Kostadinova, 2006 and two unidentified *Saturnius* spp. in the small size of the body and most metrical features. However, in spite of the presence of five transverse septa resulting in six pseudosegments and the range overlap of some metrical features, the ventral sucker in *S. minutus* is much larger, the ventral sucker muscular flange is more prominent, the last pseudosegment is narrower in relation to body width and more rounded, and the eggs are smaller (mean 21 × 10 *vs* 25 × 12 μm). Furthermore, the partial sequences of the 28S rRNA gene region (domains D1–D3; 1,195 nt) obtained from two isolates of *S. gibsoni* n. sp. differed by 11 nt (0.9%) from that of *S. minutus*. Both unidentified forms of *Saturnius* are clearly distinguishable from *S. gibsoni* n. sp. by the presence of six stout, transverse muscular septa, forming seven pseudosegments (*vs* five septa forming six pseudosegments). Bayesian inference analysis of partial 28S rDNA sequences based on a total of 15 species from the families Hemiuridae and Lecithasteridae depicted the Bunocotylinae Dollfus, 1950 as a strongly supported basal clade, with *Bunocotyle progenetica* (Markowski, 1936) as the closest sister taxon to *Saturnius* spp.

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Introduction

Saturnius Manter, 1969 is a small genus of bunocotyline hemiurids specific to mullets (Mugilidae) which currently contains eight species: *S. segmentatus* Manter, 1969 (type-species); *S. mugilis* (Yamaguti, 1970); *S. belizensis* Fischthal, 1977; *S. maurepasi* Overstreet, 1977; *S. papernai* Overstreet, 1977; *S. dimitrovi*

5.4. APPENDIX 4

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CURRICULUM VITAE

SIMONA GEORGIEVA

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EDUCATION

2011–2014: PhD student, Department of Parasitology, Faculty of Science, University of South Bohemia in České Budějovice & Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic. PhD thesis: "An integrative taxonomic approach to the study of trematode diversity and life-cycles in freshwater ecosystems" (Supervisor: Dr. Aneta Kostadinova)

2008: MSc, Faculty of Biology, Sofia University St. Kliment Ohridski, Sofia, Bulgaria. MSc thesis: "Morphology, taxonomy and ecology of monogeneans of the genus *Ligophorus* Euzet & Suriano, 1977 (Monogenea: Dactylogyridae) parasities of *Liza aurata* (Teleostei:Mugilidae) along the Bulgarian Black Sea coast" (Supervisor: Dr. Aneta Kostadinova)

2006: BSc, Faculty of Biology, Sofia University St. Kliment Ohridski, Bulgaria

RESEARCH EXPERIENCE

2011–present: Research Assistant, Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic

2010–2011: Research Fellow Grade 3, Institute for Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria

2009–2010: Research Fellow Grade 3 Institute for Experimental Pathology and Parasitology, Bulgarian Academy of Sciences, Sofia Bulgaria

2006–2009: Biologist, Institute for Experimental Pathology and Parasitology, Bulgarian Academy of Sciences, Sofia, Bulgaria

RESEARCH GRANTS

2012–2018: ECIP - European Centre of Ichtyoparasitology. Center of Excellence, Czech Science Foundation (505/12/G112; Coordinator: Dr. Milan Gelnar; Co-PI: Professor Tomáš Scholz)

2010–2014: Trematode communities in molluscs as a model system to forecast the impact of climate change in freshwater systems in Central Europe. Czech Science Foundation (P505/10/1562; PI: Dr. Anna Faltýnková)

2004–2008: Evaluating the effect of an invasive species on local mullet communities in the Mediterranean: A parasite community approach (FP6 INTAS 03-51-5998. Coordinator J.A. Balbuena; Co-PI Aneta Kostadinova)

FELLOWSHIPS & AWARDS

2014: Visiting Research Trainee at the Laboratory of Helminthology, National Autonomous University of Mexico, Mexico City (Supervisor: Professor Gerardo Pérez Ponce de León). Funded by the Grant Agency of the University of South Bohemia (GAJU) (project 04-135/2010), the University of South Bohemia and the European Centre of Ichtyoparasitology (grant 505/12/G112) (1 month)

2014: Student Scolarship for the attendence of the 13th International Congress of Parasitology (ICOPA XIII), Mexico City, Mexico, August 10–15, 2014

2013: Visiting Research Trainee at the Concordia University, Montreal, Canada: "Diversity of *Diplostomum* spp. in snails from St. Lawrence River" (Supervisors: Dr David Marcogliese & Professor Daniel McLaughlin). Funded by the Grant Agency of the University of South Bohemia (GAJU) (project 04-135/2010), the University of South Bohemia and the European Centre of Ichtyoparasitology (grant 505/12/G112) (1 month)

2013: Marc Dresden Student Travel Award for the attendence of the 88th Annual Meeting of the American Society of Parasitologists, Québec City, Canada, June 26–29, 2013

2012: Travel Grant from the European Molecular Biology Organization (EMBO) for the attendance of EMBO Practical course on Computational Molecular Evolution, Heraklion, Greece, 29 April–10 May 2012

2011: EDIT (European Distributed Institute of Taxonomy) award to participate the EDIT training course "Integrative taxonomy and taxonomic expertise in the framework of the DNA- barcoding initiative", Paris, 7–11 February 2011

2011–2014: PhD scholarship, Government of the Czech Republic

MENTORING

2012–2014: Training of Jana Zikmundová, BSc and MSc student (University of South Bohemia, Czech Republic)

2011: Consultant of Jana Zikmundová, BSc Thesis "Is there a soldier caste in freshwater echinostome trematodes?" (University of South Bohemia, Czech Republic)

2014: Training of David Pérez í García, visiting PhD student (Autonomous University of Barcelona, Spain); Sarah Madache, visiting PhD student (University of Annaba, Algeria)

2013: Training of Chistian Selbach, visiting PhD student (University of Duisburg-Essen, Germany); Rym Antar, visiting PhD student (University of Tunis El Manar, Tunisia); Sara Dallarés, visiting MSc student (Autonomous University of Barcelona, Spain) and Francesca Barbieri, visiting BSc student (University of Bologna, Italy)

2012: Training of Maria Cristina Piras, Erasmus visiting PhD student (University of Sassari, Italy); Salvatore Mele, Erasmus visiting PhD student (Univeristy of Sassari, Italy) and Veronika Michálková, visiting MSc student (Masaryk University, Brno, Czech Republic)

2011: Training of Fred Chibwana, visiting PhD student (University of Dar es Salaam, Tanzania)

PUBLICATIONS SUMMARY

Papers in international ISI journals: 14
Conference proceedings: 14
Citations (without self-citations): 14 (four cited papers)

PUBLICATIONS LIST

Papers in international ISI journals

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