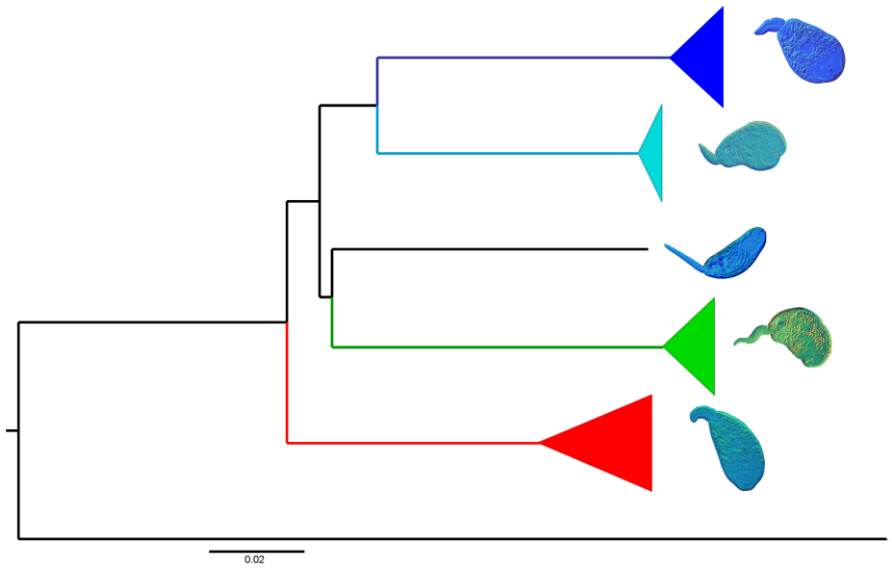
University of South Bohemia in České Budějovice
Faculty of Science

MOLECULAR AND MORPHOLOGICAL CHARACTERISATION OF SPECIES OF *PLAGIORCHIS* LÜHE, 1899 (DIGENEA: PLAGIORCHIIDAE) IN LYMNAEID SNAILS FROM FRESHWATER ECOSYSTEMS IN CENTRAL EUROPE



Master thesis

Bc. Jana Zikmundová

Supervisor: Aneta Kostadinova, PhD

Consultant: Miroslava Soldánová, PhD
Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic

České Budějovice
2014

Zikmundová, J., 2014: Molecular and morphological characterisation of species of *Plagiorchis* Lühe, 1899 (Digenea: Plagiorchiidae) in lymnaeid snails from freshwater ecosystems in central Europe. Mgr. Thesis, in English – 41 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

ANNOTATION

This study applies molecular and morphological approaches addressing the identification of morphologically similar larval stages (cercariae) of *Plagiorchis* spp. (Digenea: Plagiorchiidae) parasitising lymnaeid snail populations in the freshwater ecosystems of central Europe. Five morphologically homogeneous and genetically distinct lineages of *Plagiorchis* spp. were identified *via* matching molecular data for the mitochondrial *cox*1 gene with detailed morphometric data. Phylogenetic and comparative sequence analyses using partial 28S rDNA and ITS1-5.8S-ITS2 sequences allowed molecular identification of three species (*P. elegans*, *P. maculosus* and *P. koreanus*) *via* matching sequences from larval and adult digenean stages. A key for the identification of the cercariae of *Plagiorchis* spp. parasitising lymnaeid populations in central Europe is provided.

DECLARATION

Prohlašuji, že svoji diplomovou 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 diplomové práce, a to v nezkrácené podobě 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, 14 April, 2014 Jana Zikmundová

ACKNOWLEDGEMENTS

First I would like to express my gratitude to my supervisor, Dr. Aneta Kostadinova, for her excellent teaching and patient guidance during this study. I am also very grateful to my consultant, Dr. Miroslava Soldánová, for all her very important comments and help, and to Simona Georgieva for introducing me to molecular methods and phylogenetic analyses and checking the morphometric data. Professor Tomáš Scholz is gratefully acknowledged for giving me the opportunity to become a member of his team and for his critical comments and important suggestions during the interpretation of the results. Sincere thanks go to Blanka Škoríková who kindly helped me with the preparation of digital images for this thesis. I also thank all colleagues in the laboratory for creating such a pleasant and friendly working environment. Finally, I would like to express my gratitude to my family and Tomáš Roháč for their constant support in the course of my study.

FINANCIAL SUPPORT

This study was funded by the Czech Science Foundation (grant P505/10/1562 to Dr Anna Faltýnková) and the Institute of Parasitology (RVO 60077344).

TABLE OF CONTENTS

1. INTRODUCTION

The family Plagiorchiidae Lühe, 1901 consists of an extremely large number of digenetic trematodes parasitic in tetrapods worldwide. These parasites can be localised in all parts of the digestive tract as well as bile-ducts, gall-bladder, liver, lungs, uteres and kidneys of their hosts. Despite the enormous literature on the Plagiorchiidae this family is perhaps characterised by the most complex taxonomic history and controversial content among digeneans. At different times about 150 genera have been included in the Plagiorchiidae, the majority subsequently being either synonymised or transferred to other families (Tkach, 2008). According to Tkach (2008) the family Plagiorchiidae (*sensu stricto*) should include only digeneans from tetrapods with a well-developed cirrus-sac and a bipartite seminal receptacle, a uterus that passes between the testes or slightly overlaps them, a Y-shaped excretory bladder with short arms, a flame-cell formula 2[(3+3+3)+(3+3+3)]=36, and utilising arthropods as their intermediate hosts.

The type-genus *Plagiorchis* Lühe, 1899 is perhaps the most speciose genus within the family Plagiorchiidae with c.190 species described from different parts of the world, many of them on the basis of a few specimens and a substantial part without data on the life-cycle (Kharoo, 2011). Until recently, the type-species of the genus, *Plagiorchis vespertilionis* (Müller, 1780), was also associated with the controversies concerning the concept of *Plagiorchis.* Due to the inadequate initial description and high morphological similarities between different species belonging to the "*Plagiorchis vespertilionis*" group, almost all *Plagiorchis* spp. found in bats throughout Europe and other parts of the Holarctic region were incorrectly identified as *P. vespertilionis*. Since morphological examination alone has not provided sufficient resolution to solve the problem of the systematic status of *Plagiorchis* spp. occurring in bats in Europe, Tkach et al. (2000b) used molecular data (ITS1+5.8S+ITS2 nuclear rDNA sequences) for three species [*P. vespertilionis, Plagiorchis muelleri* Tkach & Sharpilo, 1990 and *Plagiorchis koreanus* (Ogata, 1938)] to test their validity. Their results confirmed the distinct species status of the three species. Due to the combination of molecular and morphological approaches these authors were able to provide a key for identification of *Plagiorchis* spp. occurring in European bats. An important outcome of the study of Tkach et al. (2000b) is the availability of modern morphological descriptions of the three species based on adult worms that are associated with molecular data.

The use of molecular data in association with detailed morphological descriptions represents a powerful approach to revealing the actual species diversity and testing the

validity of species (reviewed in Nolan & Cribb, 2005 and Olson & Tkach, 2005). As indicated above, this combined approach may be especially effective in distinguishing of closely related species with similar morphologies i.e. cryptic or sibling species as well as in clarifying the relationships within the genus *Plagiorchis* and the Plagiorchiidae (see Tkach, 2008).

Species of *Plagiorchis* are known to utilise a three-host life-cycle using lymnaeid snails as first intermediate hosts that are passively infected with miracidia hatching from the operculate eggs only in the intestine of molluscs. The xiphidiocercariae released from the second generation of the sporocysts undergo encystment within aquatic insects and freshwater crustaceans that serve as second intermediate hosts. The final hosts are birds and mammals, accidentally amphibians and reptiles (Galaktionov & Dobrovolskij, 2003; Gorman, 1980).

Larval stages of *Plagiorchis* spp. are both ubiquitous and ecologically important 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) reported high prevalences of infections with *P. elegans* in *L. stagnalis* in Lake Kuivasjärvi in northern Finland and in Lake Kuuhankavesi in central Finland (10.3 and 17.3%, respectively). Faltýnková (2005) studied parasites of 11 gastropod and one bivalve mollusc species in three small ponds (Kořenský, Motovidlo and Hluchá Bašta) near České Budějovice and identified cercariae of two species of *Plagiorchis*, both parasitising *L. stagnalis*: *P. elegans* (prevalence range 2.5–4.7%) and *P. maculosus* (Rudolphi, 1802) (prevalence 1.1%).

Plagiorchis elegans was also the most prevalent species (overall prevalence 4.7% in *L. stagnalis*) reported by Faltýnková & Haas (2006) in a survey on larval trematodes in 28 mollusc species conducted in southeast Germany. These authors also recorded another unidentified species of *Plagiorchis* in *L. stagnalis* (overall prevalence 0.2%) and *Plagiorchis neomidis* Brendow, 1970 in *Radix peregra* (Müller) (overall prevalence 0.8%). In a study on trematode infections in different age (size) groups of *L. stagnalis* in Lake Jeziorak in northern Poland, Żbikowska et al. (2006) reported *P. elegans* as one of the two most common trematode (overall prevalence 24.0%) detected in all monthly samples. Żbikowska (2007) examined the biodiversity of digenean larval stages in populations of *L. stagnalis* from 29 water bodies in northern and central Poland and found infections with *P. elegans* in 25 populations of *L. stagnalis* (prevalence ranging from <1 to 19%). In the same host she also recorded *P. maculosus* (in 10 water bodies; prevalence ranging from <1 to 4%).

Faltýnková et al. (2007) summarised the data from an extensive survey of trematodes in *L. stagnalis* conducted in five countries in central Europe (Austria, Czech Republic, southeast Germany, Poland and Slovak Republic) and reported *P. elegans* as a dominant species (overall prevalence 3.9%).

These authors revised the previous records of larval trematodes in *L. stagnalis* in Europe and provided an illustrated key for identification of cercariae which includes four plagiorchioidean species. These include, in addition to cercariae of *P. elegans* and *P. maculosus*, *P. laricola* (Skrjabin, 1924), a species occasionally recorded in *L. stagnalis* (Žďárská, 1966; Gelnar, 1980; Samnaliev et al., 1983) and the cercaria of *Neoglyphe sobolevi* (Shaldybin, 1953) (Digenea: Omphalometridae) which is morphologically very similar to *Plagiorchis* spp. The latter species has been recorded in *L. stagnalis* in the Czech Republic by Našincová et al. (1989) and Našincová (1992). The most important distinguishing features used by Faltýnková et al. (2007) for identification of the cercaraie of the four plagiorchioidean species in *L. stagnalis* are: the number of penetration glands, the presence/absence of thickening and/or "column" at the base of the stylet, and the presence/absence of "fat inclusions" in body parenchyma. According to the key of Faltýnková et al. (2007), both *P. elegans* and *N. sobolevi* possess seven penetration glands on one side and eight on the other side of body, but *P. elegans* is characterised by the lack of thickening at the base of the stylet (*vs* thickening present in *N. sobolevi*); the posterior penetration glands in the latter species are also paraacetabular. Cercariae with seven pairs of penetration glands on each side of the body, "fat inclusions" in body parenchyma and stylet without column corespond to *P. maculosus*. *Plagiorchis laricola* shares the number of penetration glands with *P. maculosus*, but can be differentiated by the lack of "fat inlusions" in body parenchyma and the presence of "column" in the stylet. In summary, the faunistic studies in Europe listed above have recognised *P. elegans* as either "dominant" (Faltýnková & Haas, 2006; Faltýnková et al., 2007), "most common" (Żbikowska et al., 2006) and "most frequent" (Żbikowska, 2007).

Furthermore, recent community studies on larval parasites in *L. stagnalis* have identified *P. elegans* as one of the three species that contributes substantially to the structure of component communities and patterns of parasite flow in euthrophic fishponds in the Czech Republic (Soldánová et al., 2011). This is probably associated with the fact that infections overwinter in the snail hosts and this gives the species a competitive advantage over the other trematodes due to the fast re-start of cercarial emergence and transmission in early spring (Soldánová et al., 2011). Furthermore, *P. elegans* was found to exhibit the highest rates of colonisation of the snail populations probably due to its passive dispersal *via*

environmentally resistant eggs that accumulate in the habitat and in which the miracidia can remain infective for 5-6 months (Galaktionov & Dobrovolskij, 2003; Soldánová & Kostadinova, 2011).

However, although the identification of the cercariae of *P. elegans* may appear straightforward (e.g. Faltýnková et al., 2007), the numerous records of a single species may represent underestimation of parasite diversity due to difficulties in distinguishing the morphologically similar larval stages (cercariae) used for species identification of *Plagiorchis* spp. parasitising lymnaeid snails. For example, a recent checklist of bird parasites in the Czech Republic and Slovakia reports eight species of *Plagiorchis* (see Table 1 for details). Although identification of the adult stages of *Plagiorchis* spp. is also constrained by the high morphological variability, low host-specificity and poor original descriptions (see Tkach et al., 2000b), the fact that occasionally cercariae of different *Plagiorchis* spp. have been identified (as detailed above), supports the suggestion of higher diversity of *Plagiorchis* spp. in the snail populations in central Europe.

Table 1 Species of *Plagiorchis* Lühe, 1899 and their bird hosts reported in the Czech Republic and Slovakia by Sitko et al. (2006)

Trematode species	**Host species**
Plagiorchis arcuatus Shtrom, 1924	*Corvus corone* L.
Plagiorchis elegans (Rudolphi, 1802)	*Accipiter gentilis* (L.); *A. nisus* (L.); *Acrocephalus arundinaceus* (L.); *Aegithalos caudatus* (L.); *Anas platyrhynchos* L.; *Apus apus* (L.); *Aythya ferina* (L.); *Corvus cornix* L.; *Crex crex* (L.); *Cuculus canorus* L.; *Delichon urbica* (L.); *Dendrocopos major* (L.); *D. medius* (L.); *Falco subbuteo* L.; *F. tinnunculus* L.; *Ficedula albicollis* (Temminck); *Motacilla alba* L.; *Nucifraga caryocatactes* (L.); *Parus caeruleus* L.; *P. major* L.; *Passer domesticus* (L.); *P. montanus* (L.); *Phylloscopus collybita* (Vieillot); *Pica pica* (L.); *Podiceps cristatus* (L.); *Tachybaptus ruficollis* (Pallas); *Turdus merula* L.; *Vanellus vanellus* (L.)
Plagiorchis fastuosus Szidat, 1924	*Aythya fuligula* (L.)
Plagiorchis laricola Skrjabin, 1924	*Aythya fuligula* (L.); *Chlidonias niger* (L.); *Larus argentatus* Pontoppidan; *L. ridibundus* (L.); *Mergus merganser* L.; *Stercorarius parasiticus* (L.); *S. skua* (Brünnich); *Sterna hirundo* L.

Table 1 Continued

Trematode species	Host species
Plagiorchis maculosus (Rudolphi, 1802)	*Anthus pratensis* (L.); *A. trivialis* (L.); *Apus apus* (L.); *Delichon urbica* (L.); *Dendrocopos major* (L.); *D. medius* (L.); *Emberiza citrinella* L.; *Fringilla coelebs* L.; *Hirundo rustica* L.; *Motacilla alba* L.; *M. cinerea* Tunstall; *Muscicapa striata* (Pallas); *Parus major* L.; *Pica pica* (L.); *Riparia riparia* (L.)
Plagiorchis moravicus Sitko, 1993	*Larus ridibundus* (L.)
Plagiorchis multiglandularis Semenov, 1927	*Cuculus canorus* L.; *Parus major* L.
Plagiorchis nanus (Rudolphi, 1802)	*Actitis hypoleucos* (L.); *Arenaria interpres* (L.); *Calidris alpina* (L.); *C. minuta* (Leisler); *Numenius arquatus* (L.); *Phalaropus lobatus* (L.); *Tringa erythropus* (Pallas); *T. glareola* L.; *T. nebularia* (Gunnerus); *T. ochropus* L.

The results presented in this Master thesis served as the basis for a publication in *Systematic Parasitology* (2014), 88 (1), 37–54.

2. AIM AND OBJECTIVES

The study aimed to apply comparative morphological and molecular approaches to the diversity and identification of *Plagiorchis* spp. in central Europe using a relatively large sample of isolates of *Plagiorchis* spp. infecting two lymnaeid snails, *Lymnaea stagnalis* and *Radix auricularia* (L.), in five central European freshwater ecosystems. The goal of the project was two-fold: (i) to delineate species based on both molecular and morphological evidence; and (ii) to achieve identification of the cercarial isolates *via* matching life-cycle stages (cercariae and adults) using molecular data.

OBJECTIVES

(i) To characterise morphologically isolates of cercariae of *Plagiorchis* spp. and morphologically similar cercariae and to perform initial identification based on morphology using literature data.

(ii) To obtain partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (*cox*1) gene for the isolates and test if the species identified using a few larval distinguishing features in published keys represent genetically distinct lineages.

(iii) To obtain morphometric data for the isolates and carry out statistical analyses to test if the genetically distinct lineages are morphologically homogeneous and to reveal their relationships in the multivariate morphometric space.

(iv) To assess the morphometric variability and provide additional cercarial morphological features useful for species delimitation in the group studied.

(v) To obtain partial sequences of the 28S rRNA gene or the ITS1-5.8S-ITS2 cluster in order to achieve molecular identification *via* matching the distinct *cox*1 lineages with adult isolates of *Plagiorchis* spp. that have been identified by expert taxonomists and for which sequences are available.

(vi) To construct a key for the identification of the cercariae of *Plagiorchis* spp. studied.

3. MATERIALS AND METHODS

3.1. LOCALITIES AND SAMPLE COLLECTION

Cercarial isolates of *Plagiorchis* spp. were collected from *L. stagnalis* as part of a wider sampling programme in four localities in central Europe: Ponds Bohdanečský (50°05'33"N, 15°40'1"E; 16 isolates), Hluboký u Hamru (49°09'37"N, 14°46'20"E; 3 isolates) and Vlkovský (49°08'54"N, 14°43'49"E; 4 isolates) in the Czech Republic, and at three sampling sites in blind arms of Danube near Gabčíkovo in Slovakia (47°53'24"N, 17°30'59"E; 47°54'31"N, 17°27'11"E; 47°54'09"N, 17°26'40"E; 13 isolates) (Fig. 1). *Radix auricularia* infected with larval *Plagiorchis* sp. were collected from a pond Černiš in the nature reserve Vrbenské ponds (49°00'28"N, 14°26'23"E; 2 isolates) near České Budějovice (Czech Republic) (Fig. 1). A total of 2,189 snails was collected and examined for parasites (Table 2). Snails were identified using Glöer (2002). In the laboratory, snails were placed individually into plastic containers with dechlorinated tap water and cercarial emergence was stimulated under light source for at least three days. Live cercariae were identified using the key of Faltýnková et al. (2007) and other relevant publications (e.g. Žďárská, 1966; Brendow, 1970; Théron, 1976; Bušta & Našincová, 1986; Bock & Janssen, 1987; Našincová et al., 1989). After preliminary identification, two samples of cercariae per isolate (individual snail) were fixed, one in molecular grade ethanol for DNA isolation and one in 4% cold formaldehyde solution for obtaining morphometric data. A total of 38 isolates was subjected to molecular and morphological characterisation.

Table 2 Sampling localities, number of snails examined and overall prevalence of *Plagiorchis* spp. *Abbreviations*: CZ, Czech Republic; SK, Slovak Republic

	***Lymnaea stagnalis* (2010–2013)**			***Radix auricularia* (2010–2013)**		
Pond	**Examined**	**Infected**	**Prevalence (%)**	**Examined**	**Infected**	**Prevalence (%)**
Bohdanečský (CZ)	813	163	20.05	–	–	–
Černiš (CZ)	7	1	14.29	108	4	3.70
Gabčíkovo (SK)	789	91	11.53	28	–	0
Hluboký u Hamru (CZ)	188	19	10.11	1	–	0
Vlkovský (CZ)	255	8	3.14	–	–	–
Total	**2,052**	**282**	**13.74**	**137**	**4**	**3.70**

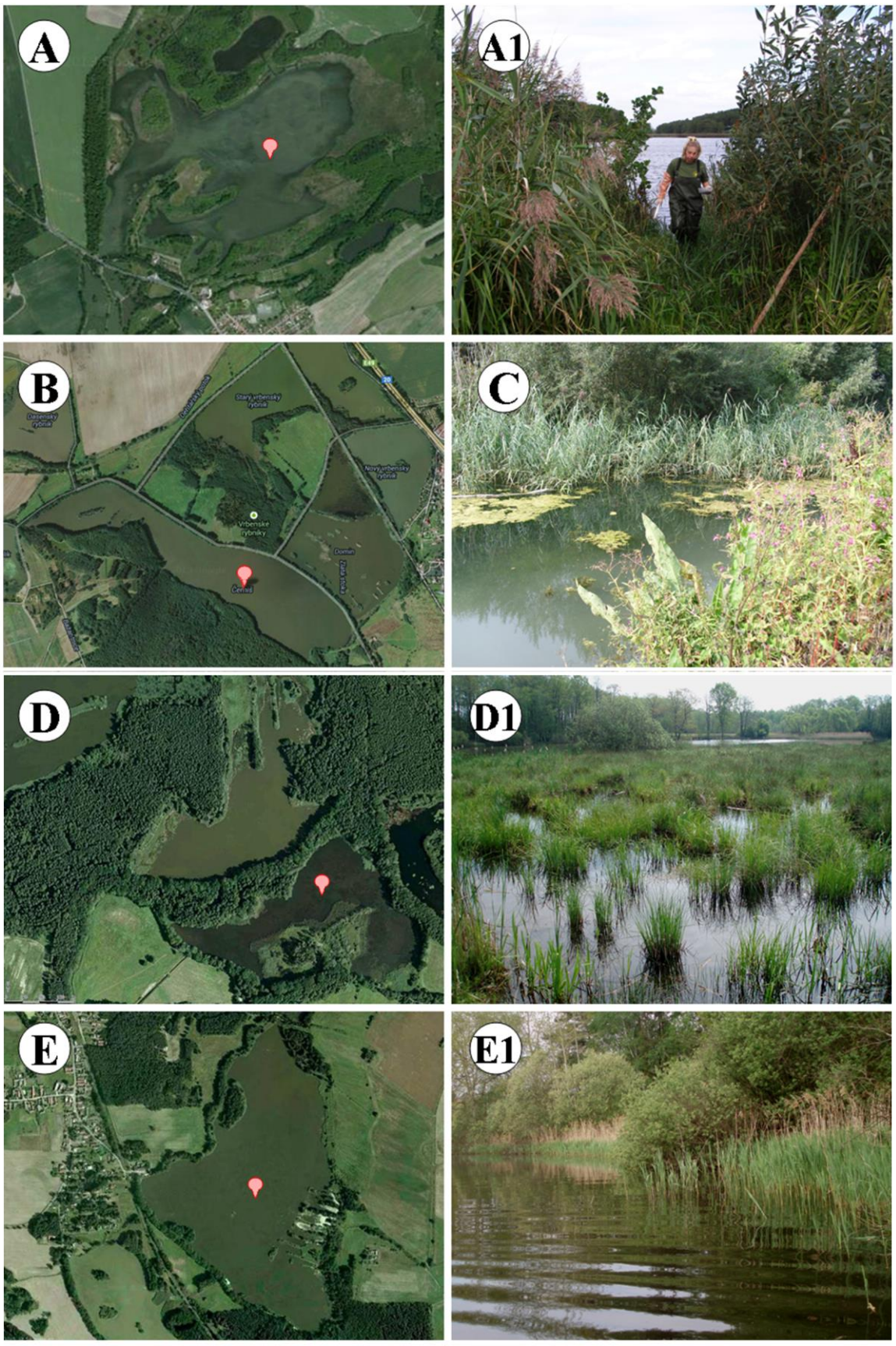


Fig. 1 Localities (left column) and sampling sites (right column) of collection: A, A1, Pond Bohdanečský; B, Pond Černiš in the Nature Reserve Vrbenské Ponds; C, Gabčíkovo**;** D, D1, Pond Hluboký u Hamru; E, E1, Pond Vlkovský

3.2. DNA ISOLATION AND SEQUENCING

Total genomic DNA was isolated from ethanol-fixed samples (20–50 pooled cercariae emerged 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 16,000 g for 10 min. Sequences of partial fragments of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene, 28S ribosomal RNA gene (domains D1-D3; *c*.1,200 nt) for a subset of isolates and complete fragments of the ribosomal ITS1-5.8S-ITS2 gene cluster for two isolates identified as *P. maculosus* were amplified using specific primers (see Table 3 for primer definitions). Polymerase chain reaction (PCR) amplifications were performed in a total volume of 25 µl using Ready-To-Go-PCR Beads (GE Heathcare, UK) containing *c*.2.5 units of puReTaq DNA polymerase, 10 mM Tris-HCL (pH 9.0), 50 mM KCl , 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. PCR reaction profiles used for individual gene fragment amplifications using different primer combinations are shown in Fig. 2.

Agarose gel electrophoresis (*c.*45 min at 100V) was performed to visualise the results of PCR amplifications in 1% agarose gels stained with GelRed. The gels were immediately exposed to UV light and digital images taken with the aid of Kodak Digital Science 1D computer software.

PCR amplicons were purified directly using Qiagen QIAquick™ PCR Purification Kit (Qiagen Ltd, UK) and DNA quantification (ng/µl) was carried out with NanoDrop 1000 Spectrophotometer using the programme ND1000. PCR amplicons were sequenced from both strands using the PCR primers and additional sequencing primers for 28S and ITS1-5.8S-ITS2 (see Table 3). Sequencing was performed on an ABI Prism 3130x1 automated DNA sequencer using ABI Big Dye chemistry (ABI Perkin-Elmer, UK) according to the manufacturer's instructions. Contiguous sequences were assembled using Mega v5 (Tamura et al., 2011) and submitted to GenBank (accession numbers shown in Table 4).

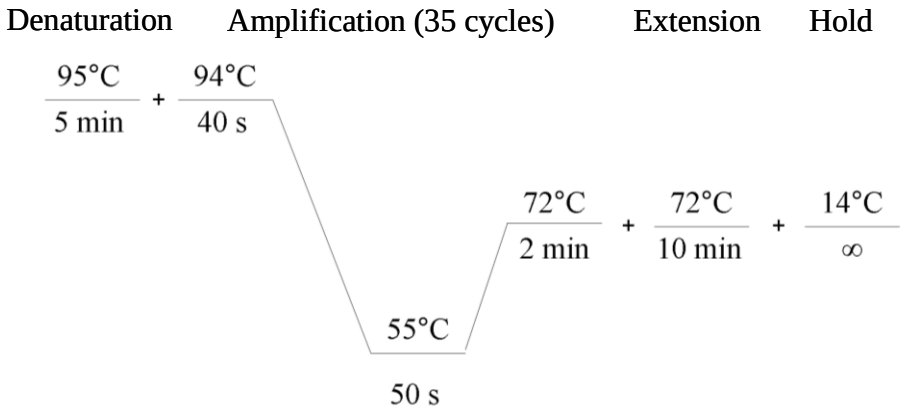
Thirty-eight newly-generated *cox*1 sequences for *Plagiorchis* spp. and a sequence for *Haematoloechus longiplexus* Stafford, 1902 used as an outgroup (León-Règagnon, 2010) were aligned using Muscle implemented in Mega v5 with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code (Telford et al., 2000). The alignment included no insertions or deletions. The alignment of the 28S rDNA sequences included seven newly-obtained and all published sequences for *Plagiorchis* spp. [*P. elegans* (AF151911; JX522535; KF556678), *P. muelleri* (AF184250), *P. koreanus*

(AF151930), and *P. vespertilionis* (AF151931) (Tkach et al., 1999, 2000a, 2001b; Greiman et al., 2013)] and a sequence for *Neoglyphe sobolevi* (AF300329) (Tkach et al., 2001a) as an outgroup. ITS1-5.8S-ITS2 sequences were aligned with the available sequences for *Plagiorchis* spp. [*P. elegans* (JX522536; AF151952), *P. maculosus* (AF316152), *P. muelleri* (AF151948; AF151947), *P. koreanus* (AF151946; AF151945; AF151944), and *P. vespertilionis* (AF151951, AF151950, AF151949) (Tkach et al., 2000b; Snyder & Tkach, 2001)] and a sequence for *H. longiplexus* (AF133193; León-Règagnon et al., 1999) as an outgroup. Uncorrected 'p' pairwise distance matrices were calculated for each gene using Mega v5.

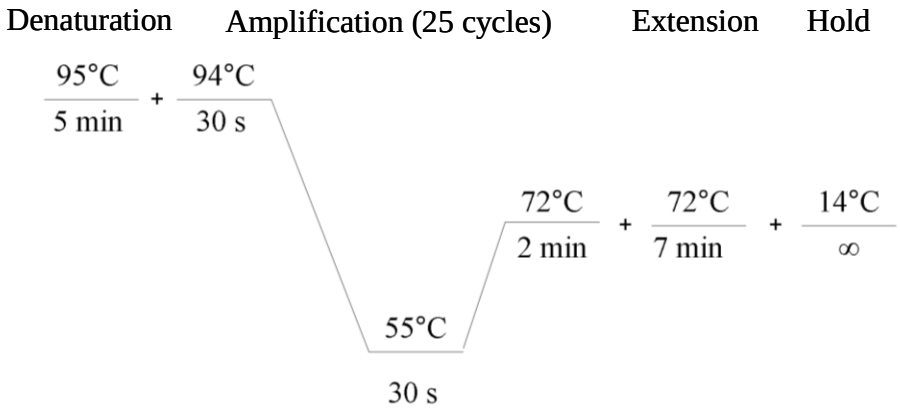
Table 3 Primers used for gene fragment amplification (PCR) and/or sequencing (Seq).

Gene fragment/ Primer	**Sequence (5'→3')**	**Direction**	**Application**	**Source**
***cox*1**				
JB3	TTTTTTGGGCATCCTGAGGTTTAT	Forward	PCR+Seq	Bowles et al. (1995)
JB4.5	TAAAGAAAGAACATAATGAAAATG	Reverse	PCR+Seq	Bowles et al. (1995)
28S				
LSU5'	TAGGTCGACCCGCTGAAYTTAAGCA	Forward	PCR+Seq	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	Reverse	PCR+Seq	Tkach et al. (1999)
ECD2	CTTGGTCCGTGTTTCAAGACGGG	Reverse	Seq	Littlewood et al. (2000)
900F	CCGTCTTGAAACACGGACCAAG	Rorward	Seq	
300R	GTTCATGGCACTCCCTTTCAAC	Reverse	Seq	Littlewood & Olson (2001)
1200R	GCATAGTTCACCATCTTTCGG	Reverse	Seq	
ITS1-5.8S-ITS2				
Br	GTAGGTGAACCTGCAGG	Forward	PCR+Seq	Tkach et al. (2000b)
digl1	GTGATATGCTTAAGTTCAGC	Reverse	PCR+Seq	Tkach et al. (2000b)
5.8Sr	TGTCGATGAAGAGCGCAGC	Forward	Seq	Tkach et al. (2000b)
5.8S2	TAAGCCGACCCTCGGACAGG	Reverse	Seq	Tkach et al. (2000b)

***cox*1 (primers JB and JB4.5)**



28S rRNA gene (variable domains D1-D3; primers LSU5' and 1500R)



ITS1-5.8S-ITS2 cluster (primers br and digl1)

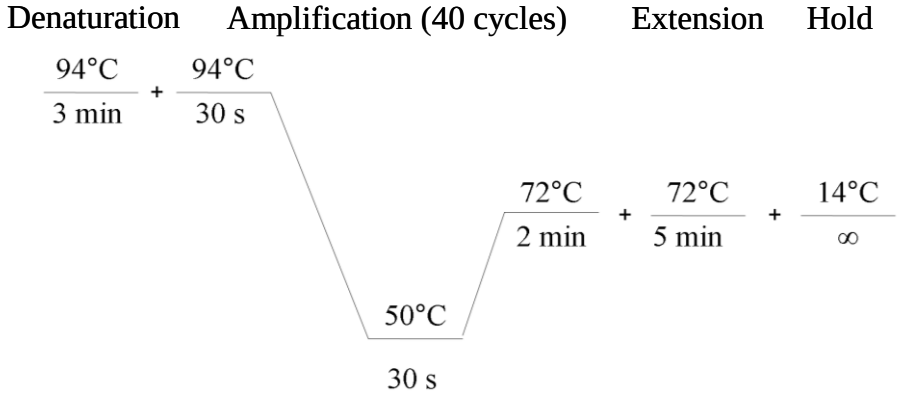


Fig. 2 PCR thermocycle profiles used for amplification of the three genetic markers

Table 4 Summary data for the isolates of *Plagiorchis* spp. from *Lymnaea stagnalis* and *Radix auricularia* used for generation of the new mitochondrial (*cox*1) and ribosomal (28S and ITS1-5.8S-ITS2) DNA sequences

Final identification	Preliminary identification	Isolate	Snail host	Locality	GenBank accession number (*cox*1/28S/ITS)
Plagiorchis elegans	*P. elegans*	LSG1	*L. stagnalis*	Danube near Gabčíkovo	KJ533399
Plagiorchis elegans	*Neoglyphe sobolevi*	LSG2	*L. stagnalis*	Danube near Gabčíkovo	KJ533400
Plagiorchis elegans	*P. laricola*	LSG3	*L. stagnalis*	Danube near Gabčíkovo	KJ533401/ KJ533392
Plagiorchis elegans	*P. elegans*	LSG4	*L. stagnalis*	Danube near Gabčíkovo	KJ533402
Plagiorchis elegans	*P. laricola*	LSG5	*L. stagnalis*	Danube near Gabčíkovo	KJ533403
Plagiorchis elegans	*P. elegans*	LSG6	*L. stagnalis*	Danube near Gabčíkovo	KJ533404
Plagiorchis elegans	*P. laricola*	LSB1	*L. stagnalis*	Pond Bohdanečský	KJ533405
Plagiorchis elegans	*P. elegans*	LSB2	*L. stagnalis*	Pond Bohdanečský	KJ533406
Plagiorchis elegans	*P. elegans*	LSB3	*L. stagnalis*	Pond Bohdanečský	KJ533407
Plagiorchis elegans	*Neoglyphe sobolevi*	LSB4	*L. stagnalis*	Pond Bohdanečský	KJ533408
Plagiorchis elegans	*P. elegans*	LSB5	*L. stagnalis*	Pond Bohdanečský	KJ533409
Plagiorchis elegans	*P. elegans*	LSB6	*L. stagnalis*	Pond Bohdanečský	KJ533410
Plagiorchis elegans	*P. elegans*	LSB7	*L. stagnalis*	Pond Bohdanečský	KJ533411
Plagiorchis elegans	*P. elegans*	LSB8	*L. stagnalis*	Pond Bohdanečský	KJ533412
Plagiorchis elegans	*P. laricola*	LSB9	*L. stagnalis*	Pond Bohdanečský	KJ533413
Plagiorchis elegans	*P. elegans*	LSHH1	*L. stagnalis*	Pond Hluboký u Hamru	KJ533414/ KJ533393
Plagiorchis elegans	*P. elegans*	LSHH2	*L. stagnalis*	Pond Hluboký u Hamru	KJ533415
Plagiorchis elegans	*P. elegans*	LSHH3	*L. stagnalis*	Pond Hluboký u Hamru	KJ533416
Plagiorchis koreanus	*P. elegans*	RAV1	*R. auricularia*	Pond Vrbenský	KJ533417/ KJ533394
Plagiorchis koreanus	*P. elegans*	RAV2	*R. auricularia*	Pond Vrbenský	KJ533417
Plagiorchis maculosus	*P. maculosus*	LSB10	*L. stagnalis*	Pond Bohdanečský	KJ533419/ KJ533395/ KJ533390
Plagiorchis maculosus	*P. maculosus*	LSB11	*L. stagnalis*	Pond Bohdanečský	KJ533420
Plagiorchis maculosus	*P. maculosus*	LSB12	*L. stagnalis*	Pond Bohdanečský	KJ533421/ KJ533396/ KJ533391
Plagiorchis maculosus	*P. maculosus*	LSB13	*L. stagnalis*	Pond Bohdanečský	KJ533422
Plagiorchis maculosus	*P. maculosus*	LSB14	*L. stagnalis*	Pond Bohdanečský	KJ533423

Table 4 Continued

Final identification	Preliminary identification	Isolate	Snail host	Locality	GenBank accession number (*cox*1/28S/ITS)
Plagiorchis maculosus	*P. maculosus*	LSB15	*L. stagnalis*	Pond Bohdanečský	KJ533424
Plagiorchis maculosus	*P. maculosus*	LSB16	*L. stagnalis*	Pond Bohdanečský	KJ533425
Plagiorchis maculosus	*P. maculosus*	LSV1	*L. stagnalis*	Pond Vlkovský	KJ533426
Plagiorchis maculosus	*P. maculosus*	LSV2	*L. stagnalis*	Pond Vlkovský	KJ533427
Plagiorchis maculosus	*P. maculosus*	LSV3	*L. stagnalis*	Pond Vlkovský	KJ533428
Plagiorchis neomidis	*P. neomidis*	LSG7	*L. stagnalis*	Danube near Gabčíkovo	KJ533429
Plagiorchis neomidis	*P. neomidis*	LSG8	*L. stagnalis*	Danube near Gabčíkovo	KJ533430
Plagiorchis neomidis	*P. neomidis*	LSG9	*L. stagnalis*	Danube near Gabčíkovo	KJ533431
Plagiorchis neomidis	*P. neomidis*	LSG10	*L. stagnalis*	Danube near Gabčíkovo	KJ533432
Plagiorchis neomidis	*P. neomidis*	LSG11	*L. stagnalis*	Danube near Gabčíkovo	KJ533433
Plagiorchis neomidis	*P. neomidis*	LSG12	*L. stagnalis*	Danube near Gabčíkovo	KJ533434/ KJ533397
Plagiorchis neomidis	*P. neomidis*	LSG13	*L. stagnalis*	Danube near Gabčíkovo	KJ533435
Plagiorchis sp. CR	*Plagiorchis* sp.	LSV4	*L. stagnalis*	Pond Vlkovský	KJ533436/ KJ533398

3.3. PHYLOGENETIC ANALYSES

Phylogenetic relationships were assessed *via* neighbour-joining (NJ) based on Kimura-2-parameter distances, Bayesian inference (BI) and Maximum Likelihood (ML) analyses. Prior to BI and ML analyses, the best-fitting models were estimated with jModelTest 2.1.1 (Guindon & Gascuel, 2003; Darriba et al., 2012) using the Akaike Information Criterion (AIC). The models GTR+Γ, GTR+I and GTR+Γ were estimated as the best fitting for *cox*1, 28S and ITS alignments, respectively. Bayesian inference (BI) analyses were carried out in MrBayes 3.2 (Ronquist et al., 2012) using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains during 10,000,000 generations, sampling trees every 1,000 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 (Huelsenbeck et al., 2001). Maximum likelihood analyses were performed in PhyML 3.0 (Guindon et al., 2010) with a non-parametric bootstrap validation based on 100 replicates.

3.4. MORPHOLOGICAL AND MORPHOMETRIC STUDY

Series of digital photographs of live cercariae for each isolate were taken using a digital camera of an Olympus BX51 microscope. Measurements (in micrometres) for live and fixed cercariae were taken from the digital images with the aid of QuickPHOTO CAMERA 2.3 image analysis software. Prior to measuring, formalin-fixed cercariae were stained with iron acetocarmine, dehydrated in an ethanol series, cleared in dimethyl phthalate and measured on temporary mounts; it was impossible to measure cercarial stylets in all fixed specimens. The following abbreviations for the morphometric variables included in the analyses used in the text and tables are given in Table 5 (see also Fig. 3 for abundance/size of the refractile granules).

Table 5 Abbreviations for the morphometric variables used in the analyses, text and tables

Abbreviation	**Variable**
BL	Body length
BW	Maximum body width
TL	Tail length
TW	Tail width
OSL	Oral sucker length
OSW	Oral sucker width
VSL	Ventral sucker length
VSW	Ventral sucker width
SL	Stylet length
SWbt	Stylet width at base/basal thickening
SWabt	Stylet width above the basal thickening
SWantt	Stylet width at the anterior thickening
OSW/VSW	Oral sucker width to ventral sucker width ratio
TL/BL (%)	Tail length as a percentage of body length
SWantt/SL (%)	Stylet width at anterior thickening as a percentage of stylet length

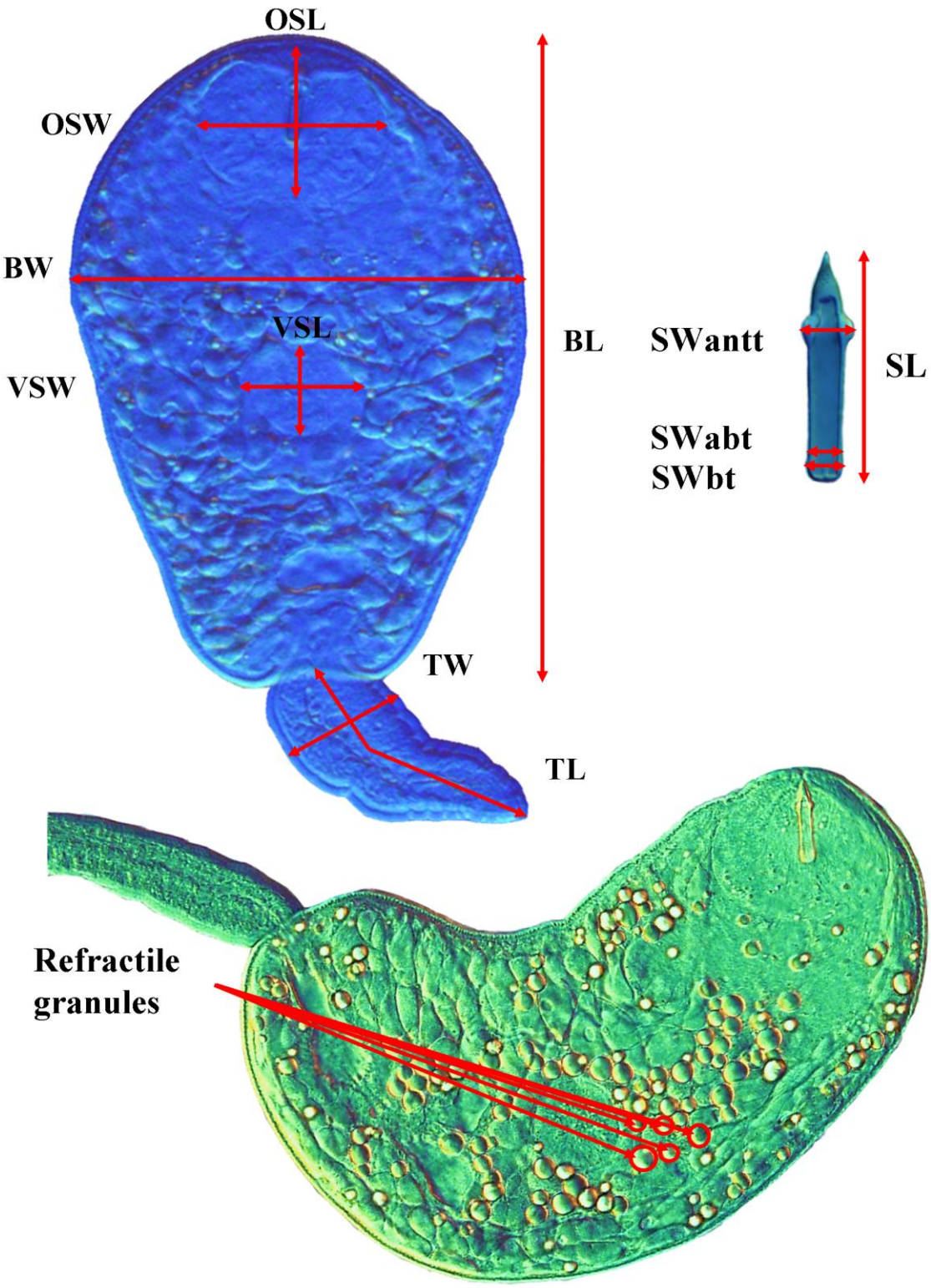


Fig 3 Variables measured for each cercaria (see definitions in Table 5) and abundance/size of the refractile granules

3.5. MORPHOMETRIC STATISTICAL ANALYSES

Analysis of principal components (PCA) was initially applied in order to reveal the multivariate relationship between the cercarial isolates subjected to sequencing. PCA is multivariate statistical ordination technique based on a correlation matrix (a symmetrical table of the correlation coefficients between the variables) that reduces the number of dimensions (variables) in a set of specimens and finds linear combinations of the variables that explain substantial proportion of the variance. The analysis assigns a component score to each individual and uses these scores to produce a two-dimensional graphical representation of the multivariate relationships between the specimens (Sokal & Rohlf, 1981).

Subsequently, linear discriminant analysis (LDA) was carried out in order to select the variables yielding optimal separation between the groups identified in the phylogenetic analysis based on *cox*1 sequences. LDA is a multivariate statistical technique that is a useful taxonomic tool for detecting variables that allow best discrimination between different natural groups (e.g. species) of specimens. LDA algorithm computes linear functions (discriminant functions) of the variables used to characterise the sets of individuals which are based on the equations of the lines cutting across the cluster of points representing individuals of pre-defined groups in the dataset in the multivariate space. LDA algorithm also computes classification functions and classification scores for each individual in each group. The latter are used to classify specimens as belonging to *a priori* defined groups. The resulting classification matrix shows the number of specimens that were correctly classified and those that were misclassified.

Two datasets were used in the multivariate morphometric analyses: (i) live cercariae (12 metrical variables representing body and stylet measurements recorded for a total of 37 isolates); (ii) fixed cercariae (8 metrical variables representing body measurements recorded for a total of 76 cercariae). In LDA the specimens were distributed into four *a priori* groups corresponding to their clustering in the *cox*1 analysis (one group represented by a single isolate was excluded in this analysis). Metrical data were log-transformed prior to analyses. All analyses were carried out using Statistica 6.0 (StatSoft, Inc., Tulsa, OK, USA).

4. RESULTS

4.1. MOLECULAR CHARACTERISATION OF *PLAGIORCHIS* SPP.

The preliminary identification using the key in Faltýnková et al. (2007) resulted in assigning the 38 cercarial isolates to six species: *Plagiorchis elegans* (12 isolates ex *L. stagnalis* and 2 isolates ex *R. auricularia*); *P. maculosus* (10 isolates ex *L. stagnalis*); *P. laricola* (4 isolates ex *L. stagnalis*); *Plagiorchis* sp. (1 isolate ex *L. stagnalis*); *P. neomidis* (7 isolates ex *L. stagnalis*); and *Neoglyphe sobolevi* (2 isolates ex *L. stagnalis*).

The aligned *cox*1 dataset for these isolates was comprised of 423 nt positions. All analyses resulted in trees with similar topologies. Figure 4 presents the phylogenetic relationships between isolates inferred from genetic distances and BI and ML analyses. These fall into four strongly supported reciprocally monophyletic lineages: (i) Clade 1 (18 isolates; 12 identified as *P. elegans*, 4 as *P. laricola* and 2 as *N. sobolevi*); (ii) Clade 2 (2 isolates identified as *P. elegans*); (iii) Clade 3 (7 isolates identified as *P. neomidis*); and (iv) Clade 4 (10 isolates identified as *P. maculosus*). The sequence for an isolate (LSV4) identified as *Plagiorchis* sp. did not join any of these clusters. Intra-lineage divergence (p-distance; overall range 0–4.3%) was consistently lower than inter-lineage divergence levels (range 11.1–16.5%; see Table 6 for details). The somewhat higher upper divergence limit within Clade 4 was due to the pairwise comparisons with isolate LSB10 (Fig. 4). Isolate LSV4 differed from all other isolates by 11.8–15.1%.

In order to achieve molecular identification of the lineages depicted in the analysis of *cox*1 dataset, partial sequences for the 28S rRNA gene were obtained for two isolates of Clade 1, one isolate of Clades 2–4 each, plus the two isolates exhibiting higher inter- or intralineage divergence (LSV4 and LSB10, respectively). These were aligned with all available sequences for species of *Plagiorchis* based on adult isolates from birds and bats in Europe (Tkach et al., 1999, 2000a, 2001b). One otherwise unpublished sequence for adult isolate of *P. elegans* ex *Apodemus sylvaticus* from the UK and one sequence for *P. elegans* ex *L. stagnalis* from the USA (Greiman et al., 2013) were also added to the alignment, which was comprised of 1,255 nt positions.

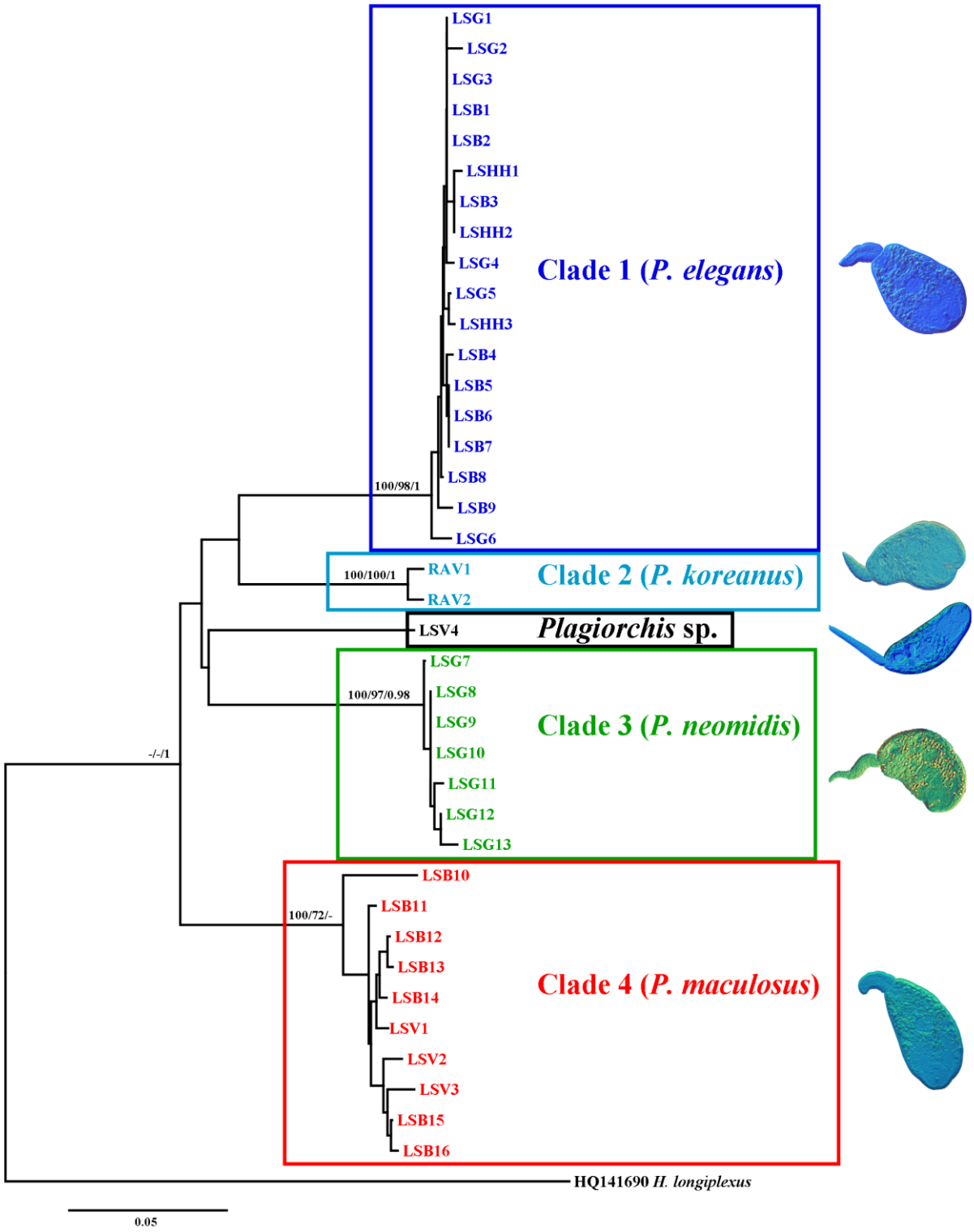


Fig. 4 Phylogenetic relationships of *Plagiorchis* spp. isolated from *Lymnaea stagnalis* and *Radix auricularia* inferred by neighbour joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analysis of the *cox*1 sequence data. Isolate codes indicate host, locality, isolate number (see Table 4 for details). Bootstrap values associated with the branches are listed as NJ/ML/BI; values < 70 (NJ, ML) and 0.95 (BI) indicated by a dash. The scale-bar indicates the expected number of substitutions per site

Table 6 Pairwise nucleotide sequence comparisons (p-distance in %) between taxa for the *cox*1 (interspecific divergence below the diagonal; intraspecific divergence highlighted in the diagonal) and 28S rDNA sequences (above the diagonal)

	Clade 1 *P. elegans*	Clade 2 *P. koreanus*	Clade 3 *P. neomidis*	Clade 4 *P. maculosus*	Isolate LSV4 *Plagiorchis* sp. CR
Clade 1	**0–1.7**	1.7–1.8	0.4	0.3	1.4
Clade 2	11.1–12.1	**0.9**	1.8	1.7	1.7
Clade 3	13.0–14.9	12.3–13.2	**0–0.9**	0.4	1.5
Clade 4	12.5–14.7	15.1–16.5	13.9–15.6	**0.2–4.3**	1.6
Isolate LSV4	13.0–13.9	11.8–12.1	12.3–13.0	13.7–15.1	**–**

Comparative sequence analysis revealed five unique genotypes among the newly-generated 28S sequences; these corresponded to the four main clades plus the isolate LSV4, which showed high divergence in the *cox*1 dataset. Divergence in the 28S between species of *Plagiorchis* ranged from 0.3 to 1.8% (4–23 nt) (see Table 6 for details). The two representative sequences for Clade 1 were identical with the three sequences for *P. elegans* and that for Clade 2 was identical with the sequence for *P. koreanus*. The remaining four new sequences did not share any of the *Plagiorchis* spp. genotypes used in the comparison. The tree depicting relationships between species of *Plagiorchis* in Fig. 5 strongly supports the distinct status of the four clades identified in the *cox*1 analyses and the isolate LSV4 and confirms the conspecificity of Clade 1 and *P. elegans* and of Clade 2 and *P. koreanus*. *Plagiorchis elegans* and Clades 3 and 4 exhibited a close association, whereas the support for (and relationships within) the second main cluster, comprised of the remaining *Plagiorchis* spp. was low.

Since no published 28S rDNA sequence was available for *P. maculosus* based on adult material, the alignment comprising complete ITS1-5.8S-ITS2 sequences for two of the cercarial isolates identified as *P. maculosus* and the isolates of *Plagiorchis* spp. used for obtaining 28S rDNA sequences by Tkach et al. (2000b). The phylogenetic relationships estimated from NJ, BI and ML provided congruent strong support for all five *Plagiorchis* spp. (Fig. 6). The newly-obtained sequences from cercarial isolates were identical with that for the adult isolate of *P. maculosus* thus confirming their conspecificity. The interspecific divergence in the ITS1-5.8S-ITS2 was between 2.8 and 9.7% (p-distance) in contrast with the lack of intraspecific variation (as in Tkach et al., 2000b). In summary, the analyses of the molecular data provided congruent strong evidence for the existence of five species of *Plagiorchis* in the two lymnaeid hosts examined in central Europe: *P. elegans* (Clade 1), *P. koreanus* (Clade 2), *P. neomidis* (Clade 3), *P. maculosus* (Clade 4), and *Plagiorchis* sp. CR (isolate LSV4).

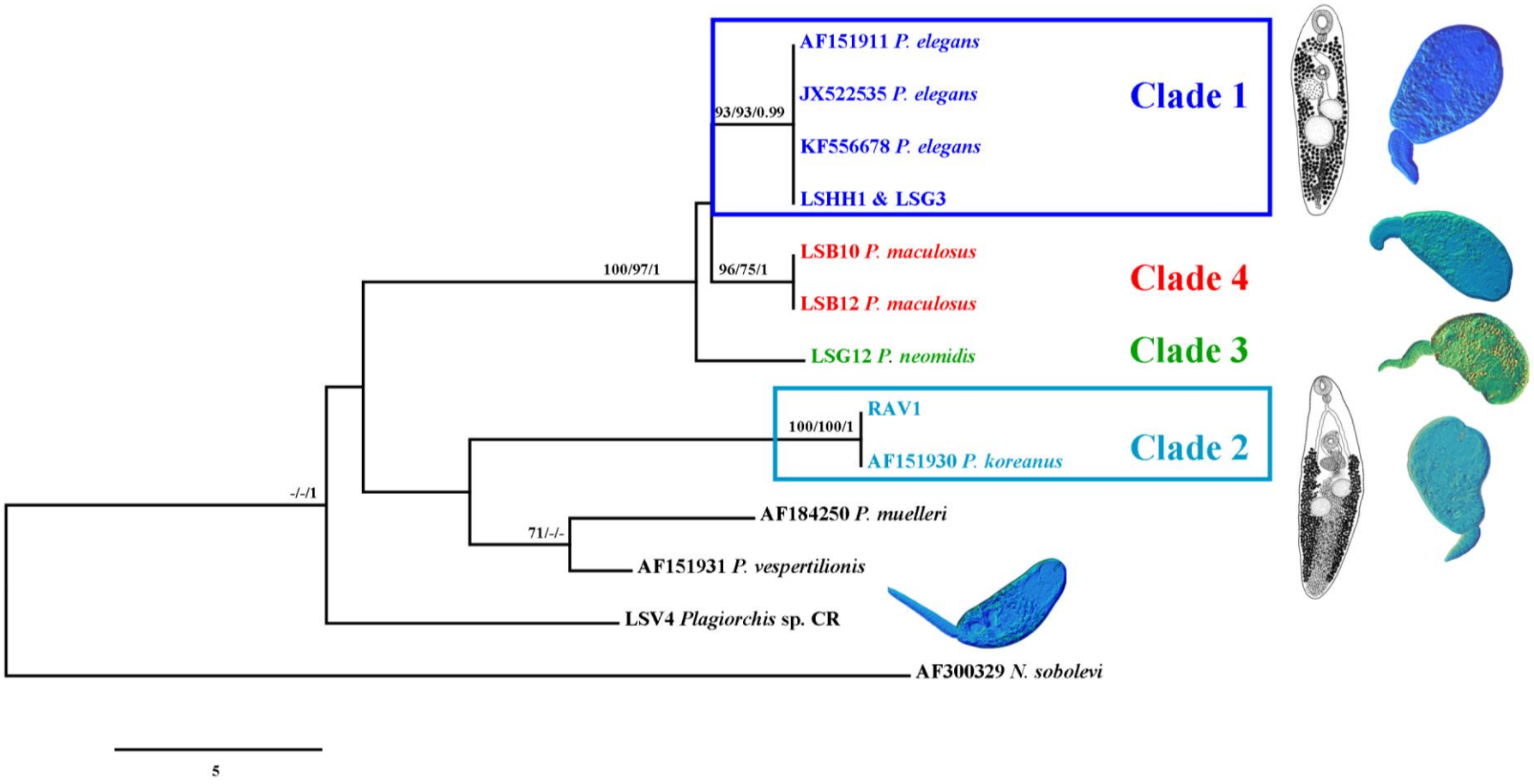


Fig. 5 Phylogenetic relationships of *Plagiorchis* spp. isolated from *Lymnaea stagnalis* and *Radix auricularia* inferred by neighbour joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analysis of the 28S sequence data. Isolate codes indicate host, locality, isolate number (see Table 4 for details). Bootstrap values associated with the branches are listed as NJ/ML/BI; values < 70 (NJ, ML) and 0.95 (BI) indicated by a dash. The scale-bar indicates the expected number of substitutions per site

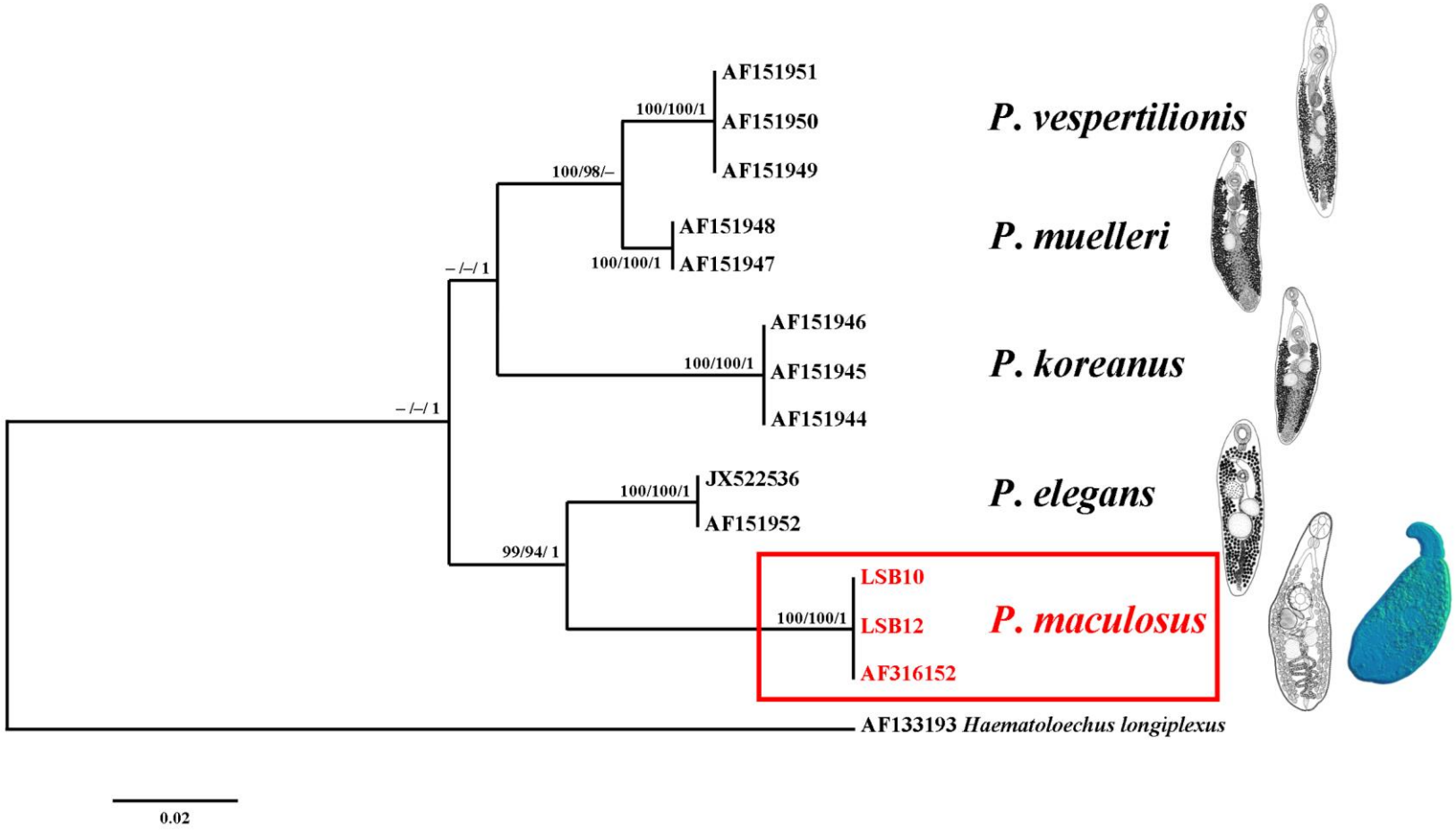


Fig. 6 Phylogenetic relationships of *Plagiorchis* spp. inferred by neighbour joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analysis of the ITS1-5.8S-ITS2 sequence data. Isolate codes indicate host, locality, isolate number (see Table 4 for details). Bootstrap values associated with the branches are listed as NJ/ML/BI; values < 70 (NJ, ML) and 0.95 (BI) indicated by a dash. The scale-bar indicates the expected number of substitutions per site

4.2. MORPHOMETRIC CHARACTERISATION OF *PLAGIORCHIS* SPP.

The first two principal components of the PCA run on the correlation matrix between 12 metrical variables of the live cercariae (n = 37: 13 *P. elegans*, 10 *P. maculosus*, 11 *P. neomidis*, 2 *P. koreanus*, and 1 *Plagiorchis* sp.) of the five species explained 62.6% of the variation in the data-set. The size of the stylet (length and width at base) and of the oral sucker had the highest coefficients on the first component, whereas tail length and width at base had most important contributions to the second principal component. A plot of the specimens in the first plane of the PCA (Fig. 7A) shows that the four species formed a gradient along the first axis in which cercariae of *P. neomidis*, possessing the largest stylets and oral suckers, were clearly separated from the cercariae of *P. maculosus* and *P. koreanus*, possessing the smallest stylets. There was a clear separation of *Plagiorchis* sp. CR, possessing longer and narrower tail along the second axis. Specimens of *P. elegans* showed an intermediate position. LDA run on the same metrical variables separated the four species (excluding the single isolate of *Plagiorchis* sp. CR) with 100% accuracy (Fig. 7B) (Wilk's Lambda = 0.0039; approximate $F_{(36, 59)} = 9.189$, $p < 0.0001$). The discriminatory power of the model was associated with only four variables: the length of the oral sucker, the width of the ventral sucker, the length of the stylet, and the width at its anterior thickening.

Since it was impossible to measure cercarial stylets in fixed material for all species, we performed PCA and LDA on a much larger dataset of fixed cercariae (8 metrical variables for a total of 76 cercariae). The first two principal components of the PCA explained 79.7% of the variation in this dataset; the first component associated with the size of the body and the width of the ventral sucker accounting for most of the variation (Fig. 8A), whereas the length of the tail and the width of the oral sucker had most important contributions to the second principal component. The plot of the specimens in the first plane of the PCA exhibited a very similar pattern to that observed in live cercariae (compare with Fig. 7A) but with a higher overlap between samples of *P. maculosus* and *P. koreanus* (due to the increased number of specimens) and a similar dispersion within *P. elegans*. The same pattern was obtained in the LDA (Wilk's Lambda = 0.011; approximate $F_{(24, 189)} = 29.724$, $p < 0.0001$), which separated the four species with an accuracy of 100% (Fig. 8B).

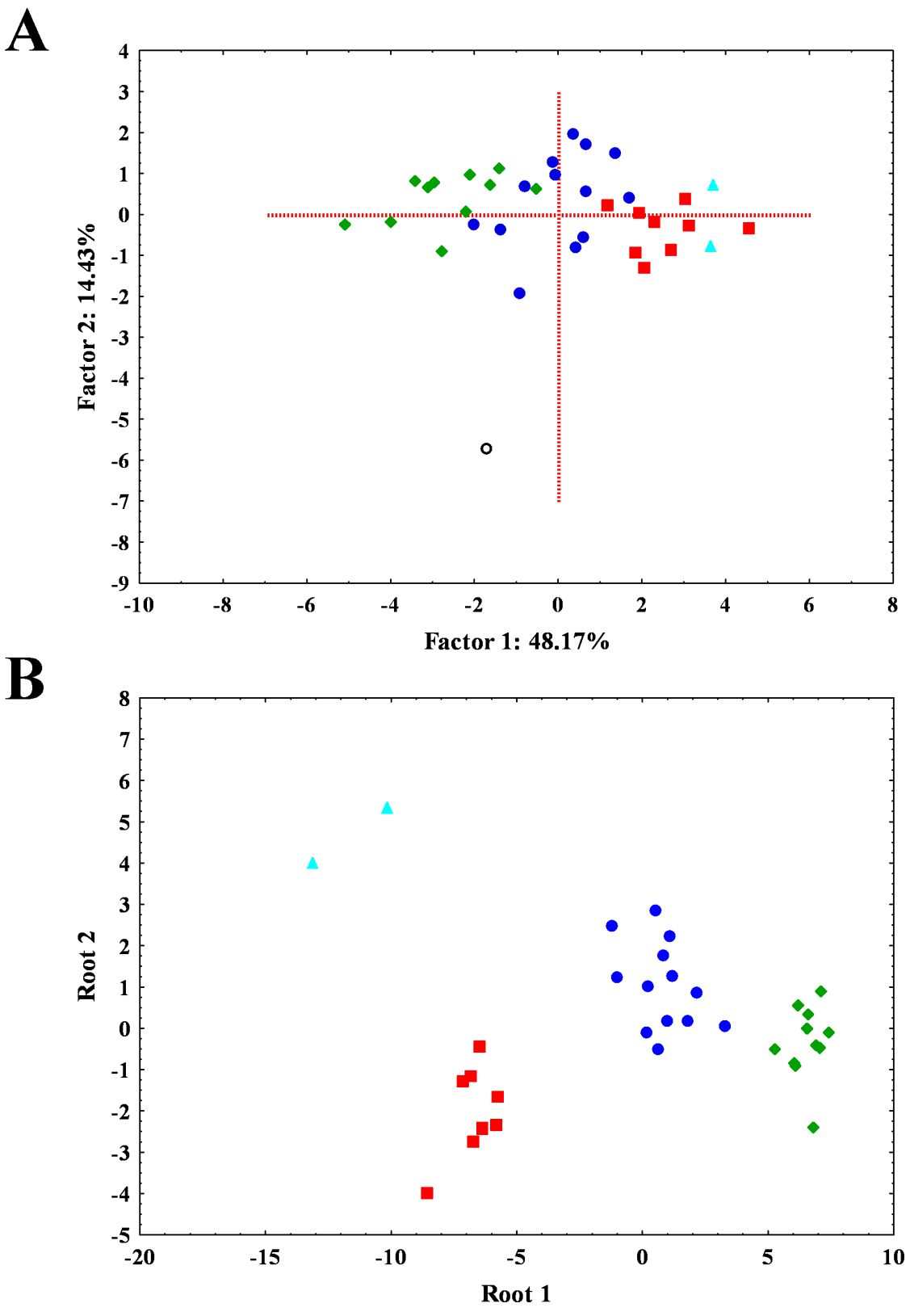


Fig. 7 Plot of the 37 live cercariae from isolates of *Plagiorchis* spp. used for obtaining *cox*1 sequences in the first plane of the PCA (A) and against the first and second canonical discriminant functions (LDA) (B). *Key to species*: *P. elegans*, filled circles; *P. maculosus*, squares; *P. neomidis*, diamonds; *P. koreanus*, triangles; *Plagiorchis* sp., open circle

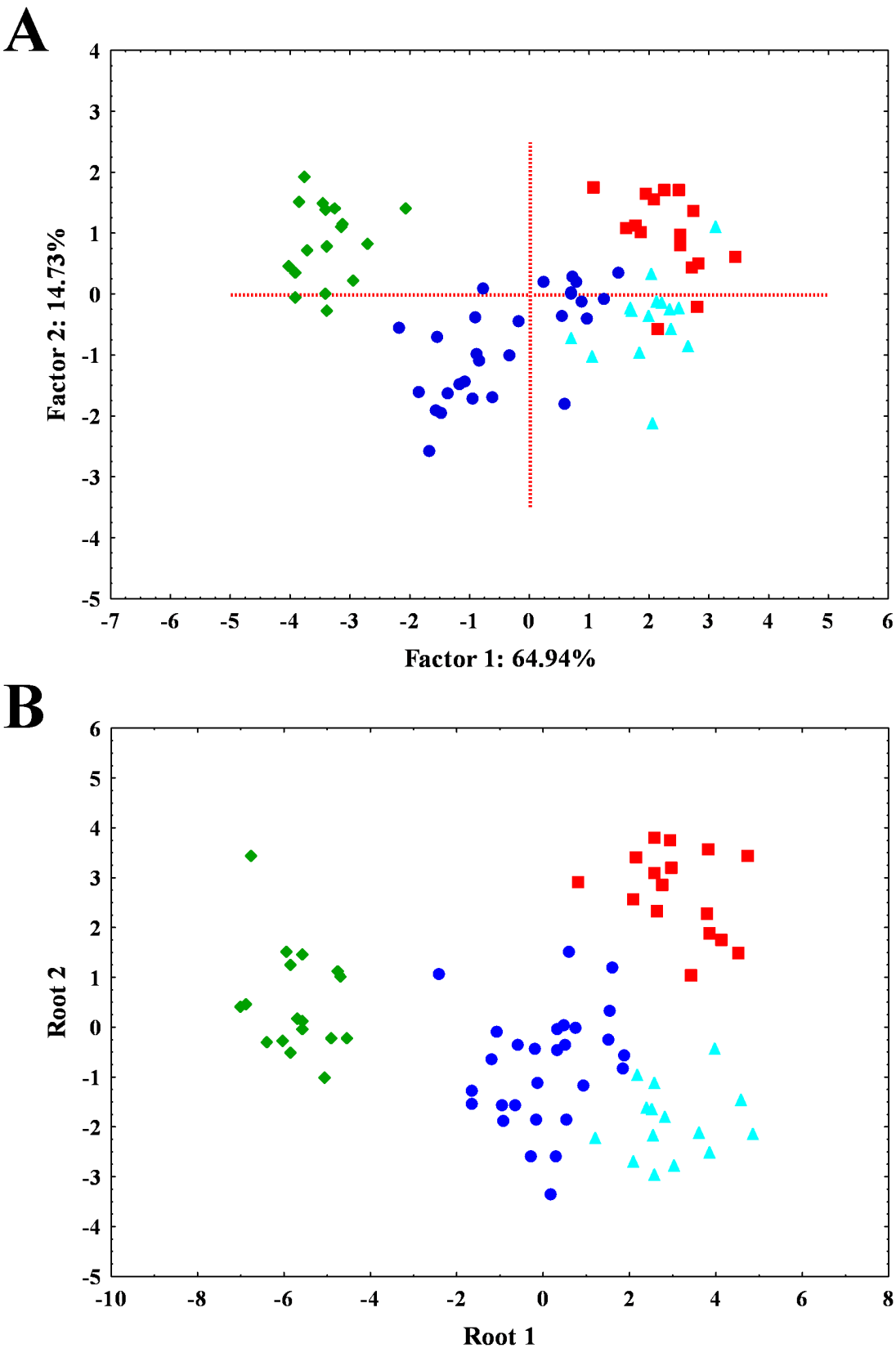


Fig. 8 Plot of the 76 fixed cercariae from isolates of *Plagiorchis* spp. used for obtaining *cox*1 sequences in the first plane of the PCA (A) and against the first and second canonical discriminant functions (LDA) (B). *Key to species*: *P. elegans*, filled circles; *P. maculosus*, squares; *P. neomidis*, diamonds; *P. koreanus*, triangles

4.3. COMPARATIVE MORPHOLOGY AND PRACTICAL IDENTIFICATION OF CERCARIAE

The cercariae of the five species of *Plagiorchis* share the following characteristics (Figs. 9–12): stylet with developed anterior thickening; ventral sucker just postequatorial, distinctly smaller than oral sucker; penetration gland-cells in two groups of 7–8 on each side of the body; numerous cystogenous gland-cells obscuring internal structures; variable number of refractile spherical granules [called "fat inclusions" by Faltýnková et al. (2007) and "spherical fat droplets" by Bušta & Našincová (1986)] scattered throughout body; tail simple, without finfolds, much shorter than body; excretory vesicle Y-shaped; flame cell formula 2[(3+3+3) + (3+3+3)] = 36.

The detailed molecular and morphometric characterisation achieved in this study shows a relatively good agreement with the preliminary identification of the isolates based on the keys in Faltýnková et al. (2007). However, 21% of the isolates were misidentified, i.e. four *P. elegans* as *P. laricola*, two *P. elegans* as *N. sobolevi* and two *P. koreanus* as *P. elegans*. *Plagiorchis laricola* was identified mainly due to the presence of a slight invagination in the middle of the stylet base (Fig. 11B) ["column" of Žďárská (1966) and Faltýnková et al. (2007); "columella" of Bušta & Našincová (1986)] which has been considered a differentiating feature of the cercaria of *P. laricola* (see Žďárská, 1966; Faltýnková et al., 2007). The morphology of the four isolates misidentified as *P. laricola* agreed well with that of cercariae of *P. elegans* in the present study; none of the isolates was misidentified in the LDA analysis. Two isolates were preliminary identified as *N. sobolevi* based on the slight thickening at the base of the stylet (Fig. 11C). These clustered within Clade 1 (*P. elegans*) and within the cluster of *P. elegans* based on morphometric data. Furthermore, detailed examination of the stylets of the molecularly identified isolates of *P. elegans* revealed that the slight basal thickening is a characteristic feature of the cercariae of this species (Fig. 11A). Basal thickening was also present in *P. neomidis* (Fig. 12A) but absent in *P. maculosus*, *P. koreanus* and *Plagiorchis* sp. CR (Fig. 12B–D).

The cercariae of the five species studied here also differ with respect to the number and size of the refractile granules scattered throughout body parenchyma. These are most numerous and prominent in *P. neomidis* (Fig. 9B), fewer and very small in *P. koreanus* (Fig. 9D) and *Plagiorchis* sp. CR (Fig. 10), whereas *P. elegans* (Fig. 9A) and *P. maculosus* (Fig. 9C) occupy intermediate position (see also key below).

Post-hoc analysis of the morphometric data (Tables 7–10) revealed generally smaller dimensions of the cercariae fixed in cold formalin and an overlap in most metrical variables for live and fixed materials compared separately. With respect to the mean values for body

size and the correlated metrical variables, the cercariae of the four species for which abundant material was examined, follow a similar rank order: (i) live: *P. neomidis* > *P. maculosus* > *P. elegans* > *P. koreanus*; (ii) fixed: *P. neomidis* > *P. elegans* > *P. maculosus* > *P. koreanus*. The rank order for the stylet measurements was: *P. neomidis* > *P. elegans* > *P. maculosus*, *P. koreanus*, *Plagiorchis* sp. The cercaria of the latter species, which was examined on live material only, is characterised by having both long and narrow body and tail; the length of the tail also represents a larger proportion of body length (Table 10). Below, we provide a key for the identification of the cercariae of the five species characterised molecularly in our study.

Table 7 Comparative metrical data for cercariae of *Plagiorchis elegans* and *Neoglyphe sobolevi*

Species	***Plagiorchis elegans***				***N. sobolevi***
Source	**Present study**		**Našincová (1992)**	**Styczyńska-Jurewicz (1962)**	**Našincová et al. (1989)**
	Live (n = 10)	**Fixed (cold formalin; n = 31)**	**Fixed (hot formalin)**	**Killed in hot water**	**Fixed (hot formalin)**
BL	218–283 (253)	139–178 (159)	180–221 (195)	240–280	138–218 (168)
BW	134–171 (154)	77–115 (96)	67–110 (94)	108–122	69–98 (87)
TL	86–141 (114)	78–131 (107)	126–163 (163)	108–122	–
TW	31–49 (43)	25–39 (30)	20–25 (23)	–	–
OSL	54–71 (63)	31–44 (35)	38–52 (44)	50–53	30–42 (37)
OSW	63–76 (69)	37–48 (43)	42–50 (46)	51–57	38–45 (41)
VSL	32–44 (37)	20–28 (24)	29–36 (33)	32–34	–
VSW	36–47 (40)	27–34 (30)	25–35 (32)	36	–
OSW/VSW	1.6–1.9 (1.7)	1.2–1.7 (1.4)	–	–	–
TL/BL (%)	35.0–58.1 (44.9)	49.4–93.6 (67.1)	–	–	–
SL	31–37 (34)	–	31–35 (33)	28–30	27–30 (28)
SWantt	7–9 (8)	–	–	–	–
SWabt	5–6 (5)	–	–	–	–
SWbt	5–8 (6)	–	–	–	–
SWantt/SL (%)	20.6–25.8 (23.6)	–	–	–	–

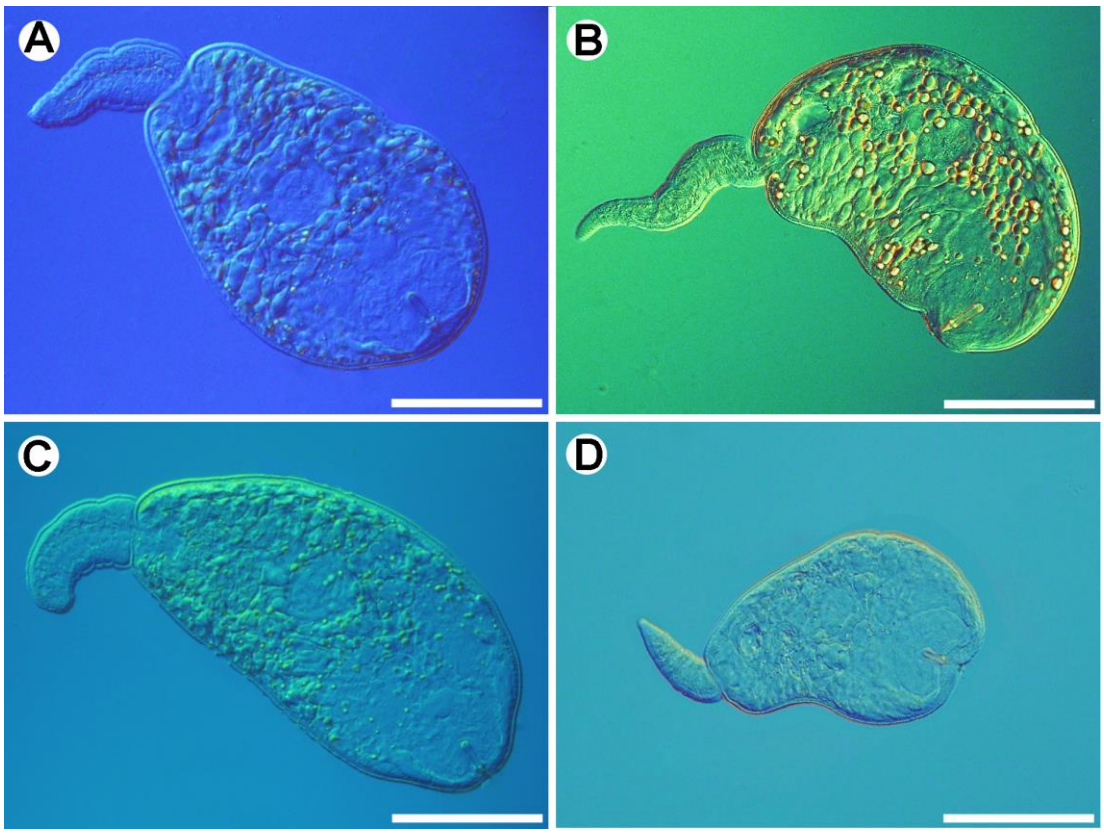


Fig. 9 Photomicrographs of live cercariae of *Plagiorchis* spp. showing the relative body size and abundance/size of the refractile granules. A, *P. elegans*; B, *P. neomidis*; C, *P. maculosus*; D, *P. koreanus*. *Scale-bars*: 100 μm

Fig. 10 Photomicrograph of live cercariae of *Plagiorchis* sp. showing few, very small refractile gnaules in body parenchyma. *Scale-bar*: 100 μm

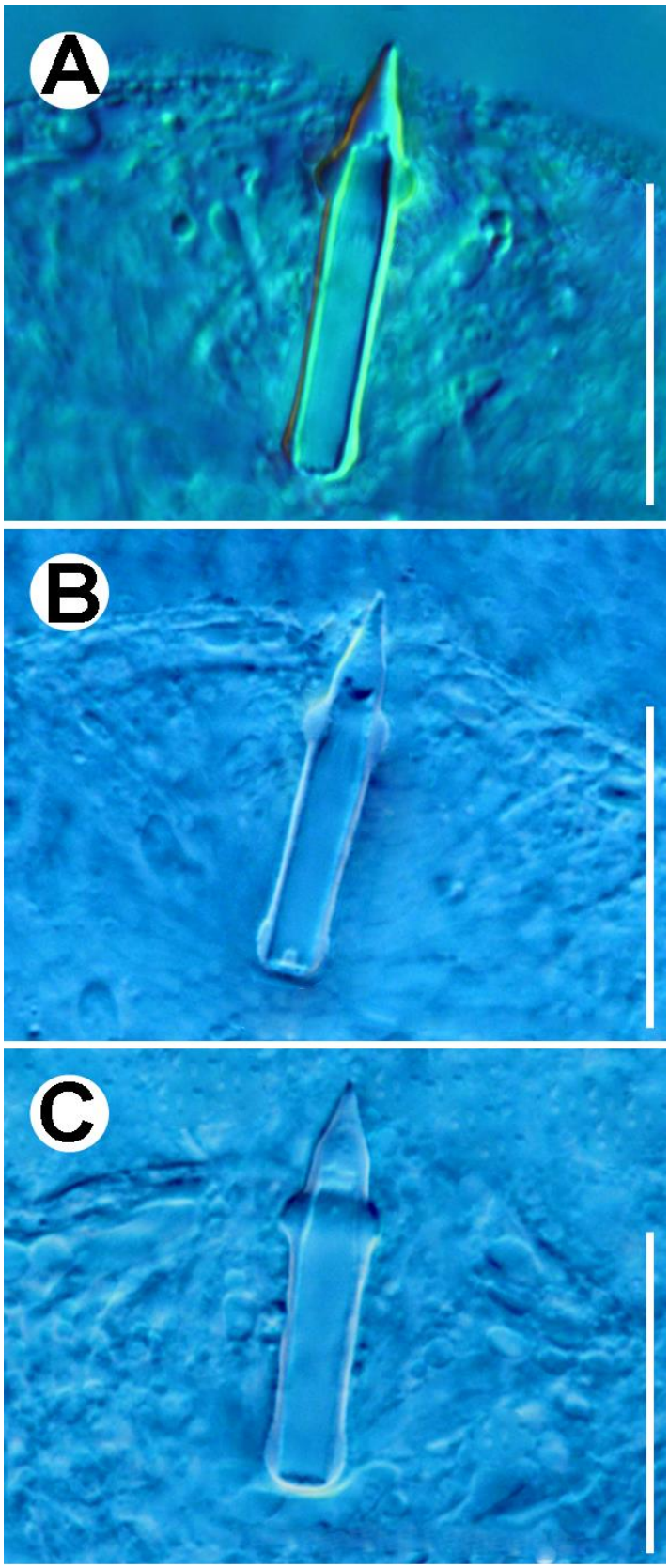


Fig. 11 Photomicrographs of stylets of live cercariae of *P. elegans*. A, Isolate identified as *P. elegans*; B, Isolate initially identified as *P. laricola*; C, Isolate initially identified as *Neoglyphe sobolevi*. *Scale-bars*: 25 µm

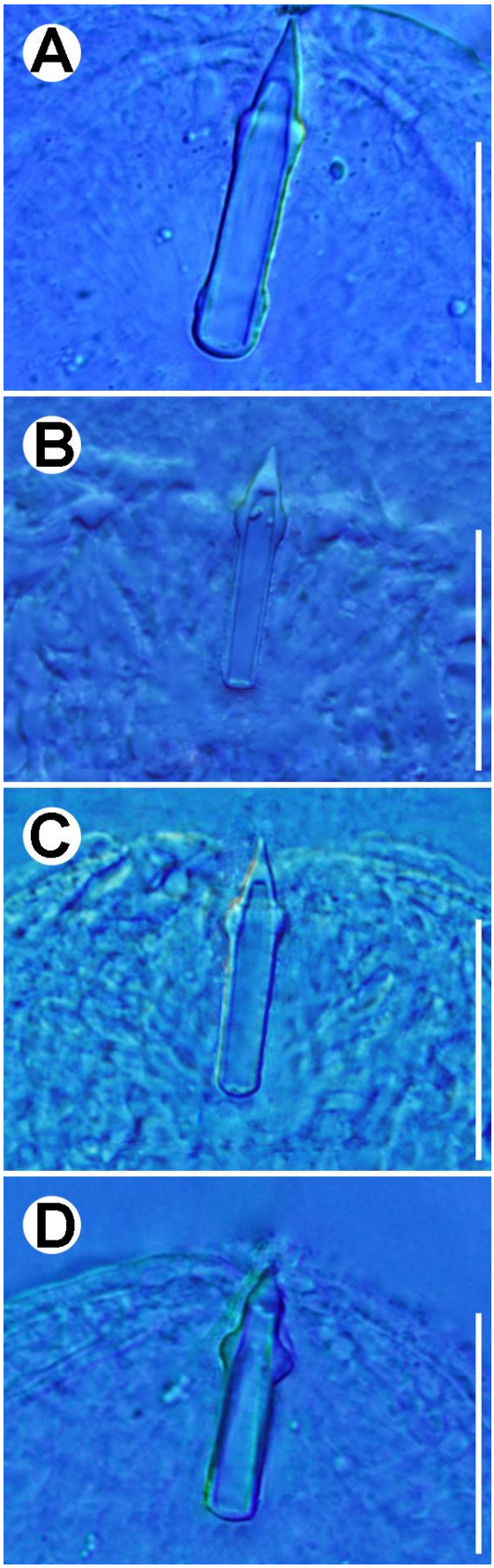


Fig. 12 Photomicrographs of stylets of live cercariae of *Plagiorchis* spp. A, *P. neomidis*; B, *P. maculosus*; C, *P. koreanus*; D, *Plagiorchis* sp. *Scale-bars*: 25 µm

Table 8 Comparative metrical data for cercariae of *Plagiorchis maculosus*

Source	Present study		Bock & Janssen (1987)	Našincová (1992)	Strenzke (1952)
	Live (n = 8)	Fixed (cold formalin; n = 16)	Killed in hot water	Fixed (hot formalin)	Killed in hot water
BL	233–322 (282)	129–156 (139)	207–279 (242)	148–207 (169)	250–300
BW	129–161 (143)	62–83 (74)	79–95 (85)	75–100 (84)	120–140
TL	105–128 (115)	74–140 (116)	137–172 (153)	72–148 (113)	260
TW	41–49 (45)	20–27 (24)	22–29 (27)	20–24 (22)	30–35
OSL	55–62 (58)	30–36 (33)	35–47 (43)	32–41 (37)	–
OSW	50–65 (58)	30–35 (33)	35–47 (42)	30–41 (36)	60
VSL	31–45 (37)	20–26 (23)	29–35 (32)	23–32 (28)	36
VSW	37–42 (40)	26–30 (27)	29–35 (32)	25–35 (28)	–
OSW/VSW	1.3–1.6 (1.5)	1.0–1.4 (1.2)	–	–	–
TL/BL (%)	33.5–47.6 (41.0)	53.2–107.8 (84.0)	–	–	–
SL	25–28 (27)	–	26–29 (27)	25–28 (26)	23–27
SWantt	5–6 (6)	–	6–7 (6.5)	–	–
SWabt	4–5 (5)	–	–	–	–
SWbt	4–5 (5)	–	4–5	4–5	–
SWantt/SL (%)	20.0–23.1 (22.2)	–	–	–	–

Table 9 Comparative metrical data for cercariae of *Plagiorchis neomidis*

Species	Present study		Brendow (1970)	Théron (1976)	Našincová (1992)	Bušta & Našincová (1986)
	Live (n = 11)	Fixed (cold formalin; n = 17)	Live	Live	Fixed (hot formalin)	Killed in hot water
BL	207–364 (290)	177–233 (210)	348–396	270–340 (312)	176–246 (199)	276–334 (307)
BW	150–193 (171)	99–132 (112)	162–192	115–140 (128)	88–125 (103)	113–151 (136)
TL	94–150 (129)	127–168 (148)	204–240	140–160 (150)	120–188 (156)	–
TW	39–55 (49)	29–36 (31)	–	35–45 (38)	18–27 (23)	–
OSL	66–85 (72)	39–54 (44)	64–76	–	41–53 (47)	50–65 (55)
OSW	68–95 (75)	37–47 (43)	60–75	50–65 (60)	42–56 (49)	51–65 (59)
VSL	31–50 (40)	26–33 (30)	42–54	–	29–40 (35)	34–42 (38)
VSW	35–54 (44)	28–36 (33)	42–48	35–50 (42)	30–42 (36)	35–42 (40)
OSW/VSW	1.6–2.0 (1.7)	1.1–1.4 (1.3)	–	1.4	–	–
TL/BL (%)	35.4–62.2 (45.2)	60.5–83.5 (70.7)	–	–	–	–
SL	37–40 (38)	–	28–34	30	29–32 (30)	29–32 (30)
SWantt	7–9 (8)	–	–	–	–	–
SWabt	5–7 (6)	–	–	–	–	–
SWbt	6–8 (7)	–	–	–	5–6	6
SWantt/SL (%)	18.9–23.1 (21.0)	–	–	–	–	–

Table 10 Comparative metrical data for cercariae of *Plagiorchis koreanus* and *Plagiorchis* sp. CR

Species	***Plagiorchis koreanus***		***Plagiorchis* sp. CR**
	Live (n = 2)	**Fixed (cold formalin; n = 15)**	**Live (n = 1)**
BL	206–207 (207)	121–155 (140)	312
BW	142–153 (148)	67–92 (81)	129
TL	100–105 (103)	64–87 (75)	230
TW	31–32 (32)	19–29 (24)	29
OSL	57 (57)	30–38 (34)	60
OSW	56–63 (60)	34–42 (39)	60
VSL	35–38 (37)	20–26 (23)	42
VSW	33–35 (34)	25–32 (27)	46
OSW/VSW	1.6–1.9 (1.8)	1.3–1.6 (1.4)	1.3
TL/BL (%)	48.5–50.7 (49.6)	41.3–60.6 (53.6)	73.7
SL	24–27 (26)		28
SWantt	7 (7)		10
SWabt	4–5 (5)		8
SWbt	5 (5)		8
SWantt/SL (%)	25.9–29.2 (27.5)		35.7

4.4. KEY TO CERCARIAE OF MOLECULARLY CHARACTERISED *PLAGIORCHIS* SPP.

1a Stylet > 30 μm in length, with faint thickening at its base 2

1b Stylet < 30 μm in length, lacking thickening at its base 3

2a Stylet length 37–40 μm; large number (100–160) of large (*c.*10 μm) and small (< 5 μm) refractile granules scattered throughout body; in *Lymnaea stagnalis* .... *P. neomidis*

2b Stylet length 31–37 μm; small number (40–50) of small refractile granules scattered throughout body; in *Lymnaea stagnalis* ..... *P. elegans*

3a Width of stylet at anterior thickening < 7 μm (5–6 μm); large number (70–80) of small refractile granules scattered throughout body; in *Lymnaea stagnalis* .... *P. maculosus*

3b Width of stylet at anterior thickening > 7 μm (7–10 μm); small number (< 40) of small refractile granules scattered throughout body 4

4a Stylet length 24–27 μm, width at anterior thickening 7 μm (26–30% of stylet length); oral sucker distinctly larger than ventral sucker (OSW/VSW = 1.6–1.9); tail short (TL/BL < 50%); 10–40 refractile granules scattered throughout body; in *Radix auricularia* ..... *P. koreanus*

4b Stylet length 28 μm, width at anterior thickening 10 μm (36% of stylet length); oral sucker slightly larger than ventral sucker (OSW/VSW = 1.3); tail long (TL/BL = 74%); less than 10 refractile granules scattered throughout body; in *Lymnaea stagnalis* ..... *Plagiorchis* sp. CR

5. DISCUSSION

To the best of our knowledge this study is the first to use morphological and molecular data in conjunction to distinguish between morphologically similar larval stages of *Plagiorchis* spp. and the first to apply *cox*1 'barcoding' to prospect for *Plagiorchis* spp. in natural snail populations. The cercariae of all five species were clearly distinguishable with respect to the novel molecular (*cox*1 and 28S sequences) and morphometric data gathered in the study. This has enabled us to define the morphological traits that can be used in a key for the identification of *Plagiorchis* spp. cercariae in freshwater snails in Europe.

Our study has largely profited from the molecular data provided by the studies of Tkach et al. (1999, 2000a, 2001b) because they made it possible to establish links for larval and adult isolates of three species, *P. elegans*, *P. maculosus* and *P. koreanus*, and to confirm the lack among the samples studied by us of two other species for which molecular data are available, i.e. *P. muelleri* and *P. vespertilionis*. The phylogenetic analyses of the 28S rDNA data also provided support for the distinct species status of *P. maculosus* and *P. neomidis* as revealed by the *cox*1 data and confirmed the identification of these species based on cercarial morphology. The ITS1-5.8S-ITS2 data provided further support for the identification of the cercarial isolates of *P. maculosus*.

Although species distinction of the present material was relatively straightforward, comparisons with published data revealed a dearth of problems. First, very few reliable sources dealing with the life-cycles of some of the species identified here exist (Tables 7–9). Some of these descriptions provide limited data for the morphology and morphometry of the cercariae and the descriptions of *P. elegans* and *P. maculosus* by Našincová (1992) although more detailed, are in fact not published. Secondly, the descriptions are based on live [*P. neomidis* (see Brendow, 1970; Théron, 1976)] or differently fixed specimens, measurements either taken from cercariae killed by heat but not fixed [*P. elegans* (see Styczyńska-Jurewicz, 1962); *P. maculosus* (see Strenzke, 1952; Bock & Janssen, 1987); *P. neomidis* (see Bušta & Našincová, 1986)] or from cercariae fixed in hot formalin [*P. elegans*, *P. maculosus*, *P. neomidis* (see Našincová, 1992)] (see also comparative data in Tables 7–9). Finally, in the past measurements were taken from wet temporary mounts under the slight pressure of a cover slide; this procedure is longer compared with taking digital images and may have resulted in measurements taken from differently flattened specimens as a result of evaporation of the liquid (water or formalin).

Overall, the comparative data provided in Tables 7–9 show that, irrespective of species, cercariae killed in hot water have much larger dimensions and those fixed in hot

formalin exhibit somewhat higher upper limits of variation for some of the measurements than the cercariae fixed in cold formalin (present study). It is worth noting that we did not apply fixation with hot formalin since work with hot formalin is forbidden in many universities and laboratories. Thus, we hope that our detailed morphometric data can serve in future comparative studies using both live and fixed in cold formalin specimens.

Table 7 contains the limited data available to date for the cercariae of *N. sobolevi* illustrating a range overlap of the size of the body and oral sucker with cercariae of *P. elegans*. The presence of a slight thickening at stylet base was a feature used in the preliminary identification that resulted in misidentifications of *P. elegans* as *N. sobolevi*. In fact, our observations revealed that the thickening at stylet base is consistently present in cercariae of *P. elegans*. Therefore, this character should not be used for differentiation of cercariae of *P. elegans* and *N. sobolevi*, thus leaving the size of the stylet as the sole distinguishing feature (see Faltýnková et al., 2007). However, the range of variation of stylet length in *N. sobolevi* is not known and the reported ranges (27–30 μm; see Našincová et al., 1989) are really close to that in *P. elegans* (31–37 μm; present study). The life-cycle of *N. sobolevi* has been completed experimentally by Našincová et al. (1989) who have found natural infections in *L. stagnalis* and *Stagnicola corvus* (Gmelin). Therefore, although there is experimental evidence that lymnaeid populations (and especially those of *S. corvus*) in central Europe are parasitised by *N. sobolevi*, identification of cercariae should be based on molecular evidence; the availability of a sequence for this species based on the adult stage represents an advantage (Tkach et al., 2001b).

Of the four species for which comparative data are available, the most problematic identification appears that of *P. neomidis*. The descriptions based on live specimens provide higher upper limits or means for body and tail length (Table 9); the width of the oral sucker described by Théron (1976) is distinctly smaller and this results in a smaller sucker width ratio (1:1.4 *vs* 1:1.6–2.0). On the other hand, the materials killed in hot water have much higher upper limits and means than the present cercariae fixed in cold formalin, whereas those fixed in hot formalin have lower means for body size and tail width (Table 9). In addition to these variations, the data for the size of the stylet in the previous descriptions of *P. neomidis* are below the lower range of variation observed by us (28–34 *vs* 37–40 μm). Whether this is indication of specific distinction or intraspecific variation can be assessed after obtaining sequences from adults of this species. This also applies to *Plagiorchis* sp. described here based on the characters of the cercariae and molecular data.

The discovery of *P. koreanus* among the isolates studied was somewhat surprising. However, after careful consideration of the morphology of the two cercarial isolates, we

conclude that the provisional identification was apparently erroneous. The two isolates provisionally identified as *P. elegans* were well separated from the samples of the other four species in the multivariate analyses, especially in those based on analyses of live cercariae that included stylet measurements. Furthermore, although there is a range overlap in the morphometric data (but with mean values always lower in *P. koreanus*, see Tables 7 and 10), a number of distinguishing features between the cercaria of *P. koreanus* and *P. elegans* were revealed during post-hoc comparisons: the length of the stylet (24–27 *vs* 31–37 μm), the thickening at stylet base (absent *vs* present); the relative size of the anterior thickening of the stylet (SWantt/SL 26–29 *vs* 21–26%); the number of the refractile granules in *P. koreanus* (< 40 *vs* 40–50). The molecular identification of *P. koreanus* achieved in our study helped elucidate the first intermediate host of this species (*R. auricularia*). This is the first record of *P. koreanus* in the Czech Republic and central Europe.

Although statistical comparisons revealed separation of the five species of *Plagiorchis* in the multivariate morphometric space which was in agreement with the molecular data, very few features could be used directly for practical identification of field samples; this is reflected in the key to cercariae provided above. On the other hand, our study has shown that two morphological features used to differentiate cercariae of *Plagiorchis* spp. (see Faltýnková et al., 2007) are questionable and should not be used. The first is the lack of thickening at stylet base (see above), and the second is the presence of column (slight invagination in the middle of stylet base) in *P. laricola* (see Žďárská, 1966; Faltýnková et al., 2007). However, this feature is not unique for *P. laricola*, e.g. it was described in 96% out of 40 cercariae of *P. elegans* examined by Gorman (1980), in *P. neomidis* (see Bušta & Našincová, 1986) and in *P. peterborensis* Kavelaars & Bourns, 1968 (see Kavelaars & Bourns, 1968). Further, comparative sequence analysis has clearly shown that the cercarial isolates in which a column was documented belong to *P. elegans*.

The nature of the refractile granules of the cercariae is not known. However, Galaktionov & Dobrovolskij (2003) suggested that these represent excretory lipids stored in the parenchyma to form a kind of hydrostatic apparatus. The authors also suggested that this feature may have an adaptive character as a means of increasing the buoyancy of the cercariae, so that they would not sink too low during its repose. Therefore, the different degree of lipid storage in cercarial parenchyma observed in the species studied here, may reflect differential microhabitats in the water column where infection of the second intermediate hosts takes place.

Recent extensive faunistic and ecological surveys have reported either single (*P. elegans*) or two species (*P. elegans* and *P. maculosus*) parasitic in *L. stagnalis* in central

Europe (Faltýnková, 2005; Faltýnková & Haas, 2006; Żbikowska et al., 2006; Faltýnková et al., 2007; Żbikowska, 2007; Brown et al., 2011; Soldánová et al., 2011; Soldánová & Kostadinova, 2011; Soldánová et al., 2012). This is in contrast with the results of our study which provides molecular and morphological evidence for five species. It is possible that the combined approach to the identification of *Plagiorchis* spp. would reveal more species in this and other lymnaeid hosts that have not been subject of intensive surveys (species of *Galba*, *Radix* and *Stagnicola*). We believe that our study would thus serve as a starting point in the development of an integrated molecular and morphological assessment of the diversity of larval *Plagiorchis* spp.

6. CONCLUSIONS

The application in the present study of both morphological and molecular comparative approaches to the examination of a large number of larval isolates provided novel information on the species richness and morphological characterisation of *Plagiorchis* spp. parasitising *L. stagnalis* and *R. auricularia* in central Europe. The following conclusions can be drawn as a result of the present study:

6.1 The first application of *cox*1 'barcoding' to prospect for *Plagiorchis* spp. in natural snail populations in association with sequence data for 28S rRNA gene revealed that at least five genetically distinct species complete their life-cycles in the freshwater habitats of central Europe.

6.2 Molecular identification of three species was achieved *via* the links established between larval and adult stages in the phylogenetic analyses of the 28S (*P. elegans* and *P. koreanus*) and ITS1+5.8S+ITS2 rDNA data (*P. maculosus*). The phylogenetic analyses also provided support for the distinct species status of *P. maculosus*, *P. neomidis* and *Plagiorchis* sp. CR and confirmed their identification based on cercarial morphology.

6.3 The molecular identification of *P. koreanus* achieved in the study helped elucidate the first intermediate host of this species, *R. auricularia*, and to provide the first record of this parasite of bats in the Czech Republic and central Europe.

6.4. The multivariate statistical approaches which facilitate an assessment of the morphometric variation in both live and fixed cercariae, proved to be very useful for species discrimination based on cercarial morphology. The good agreement between the two methods applied (PCA and LDA) and the consistency with the molecular results support this suggestion.

6.5 Although the molecular and morphometric data obtained agreed relatively well with the preliminary identifications based on the key of Faltýnková et al. (2007), one fifth of the isolates were misidentified. Two morphological features used to differentiate cercariae of *Plagiorchis* spp. in the key are questionable and should not be used: the lack of thickening at stylet base in *P. elegans* and the presence of "column" in the middle of stylet base in *P. laricola*.

6.6 A key to cercariae based on the novel morphological data obtained was constructed for the five molecularly charactersied species.

7. REFERENCES

Bock, D. & Janssen, O. (1987) The life-cycle of *Plagiorchis maculosus* (Rudolphi, 1802) Braun, 1902 (Trematoda: Plagiorchiidae), a parasite of swallows (Hirundinidae). *Systematic Parasitology, 9,* 203–212.

Bowles, J., Blair, D. & McManus, D. P. (1995) A molecular phylogeny of the human schistosomes. *Molecular Phylogenetics and Evolution, 4,* 103–109.

Brendow, V. (1970) A contribution to the fauna of the Soricidae in the Giessen Area and in the Naturpark Hoher Vogelsberg. Part. I. *Zeitschrift für Parasitenkunde, 33,* 282–313.

Brown, R., Soldánová, M., Barrett, J. & Kostadinova, A. (2011). Small-scale to large-scale and back: Larval trematodes in *Lymnaea stagnalis* and *Planorbarius corneus* in Central Europe. *Parasitology Research, 108,* 137–150.

Bušta, J. & Našincová, V. (1986) Record of *Plagiorchis neomidis* Brendow, 1970 (Trematoda: Plagiorchidae) in Czechoslovakia and studies on its life cycle. *Folia Parasitologica, 33,* 123–129.

Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods, 9,* 772.

Faltýnková, A. (2005) Larval trematodes (Digenea) in molluscs from small water bodies near České Budějovice, Czech Republic. *Acta Parasitologica, 50,* 49–55.

Faltýnková, A. & Haas, W. (2006) Larval trematodes in freshwater molluscs from the Elbe to Danube rivers (Southeast Germany): before and today. *Parasitology Research, 99,* 572–582.

Faltýnková, A., Našincová, V. & Kablásková, L. (2007) Larval trematodes (Digenea) of the great pond snail, *Lymnaea stagnalis* (L.), (Gastropoda, Pulmonata) in Central Europe: a survey of species and key to their identification. *Parasite, 14,* 39–51.

Galaktionov, K. V. & Dobrovolskij, A. A. (2003) *The biology and evolution of trematodes. An essay on the biology, morphology, life cycles, transmissions, and evolution of digenetic trematodes.* Dordrecht: Kluwer Academic Publishers, 592 pp.

Gelnar, M. (1980) *Taxonomie a morfologie vývojových stádií motolic*. MSc thesis, University of Jana Evangelisty Purkyně v Brně, Česká Republika, 189 pp.

Glöer, P. (2002) *Die Süßwassergastropoden Nord- und Mitteleuropas. Bestimmungsschlüssel, Lebensweise, Verbreitung. 2, neubearbeitete Auflage*. Hackenheim: ConchBooks, 327 pp.

Gorman, A. M. (1980) Studies on the biology of *Plagiorchis elegans* (Rudolphi, 1802), (Trematoda: Digenea) in its mammalian and molluscan hosts. PhD thesis, University of Leeds, UK, 220 pp.

Greiman, S. E., Tkach, V. V. & Vaughan, J. A. (2013) Transmission rates of the bacterial endosymbiont, *Neorickettsia risticii*, during the asexual reproduction phase of its digenean host, *Plagiorchis elegans*, within naturally infected lymnaeid snails. *Parasites & Vectors*, 6, 303.

Guindon, S. & Gascuel, O. (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*, *52*, 696–704.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, *59*, 307–321.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001) Bayesian Inference of phylogeny and its impact on evolutionary biology. *Science*, *294*, 2310–2314.

Kavelaars, J. & Bourns, T. (1968) *Plagiorchis peterborensis* sp. n. (Trematoda: Plagiorchiidae), a parasite of *Lymnaea stagnalis appressa*, reared in the laboratory mouse, *Mus musculus*. *Canadian Journal of Zoology*, *46*, 135–140.

Kharoo, V. K. (2011). Studies on the family Plagiorchiidae Lühe, 1901 with redescription of *Plagiorchis bulbulii* Mehra, 1937 a digenetic trematode collected from two different hosts in Kashmir, India. *Indian Journal of Fundamental and Applied Life Sciences*, *1*, 119–126.

León-Règagnon, V. (2010) Evidence of new species of *Haematoloechus* (Platyhelminthes: Digenea) using partial cox1 sequences. *Mitochondrial DNA*, *21*, 12–17.

León-Règagnon, V., Brooks, D. R. & Pérez-Ponce de León, G. (1999) Differentiation of Mexican species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiformes): molecular and morphological evidence. *Journal of Parasitology*, *85*, 935–946.

Littlewood, D. T. J. & Olson, P. D. (2001) Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood, D.T.J. & Bray, R.A. (Eds) *Interrelationships of the Platyhelminthes*. London: Taylor and Francis Inc., pp. 262–278.

Littlewood, D. T. J., Curini-Galletti, M. & Herniou, E. A. (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution*, *16*, 449–66.

Našincová, V. (1992) Trematode developmental stages in Czech aquatic snails and life-cycles of selected species of the family Omphalometridae and Echinostomatidae.

PhD Thesis, Institute of Parasitology, Czechoslovak Academy of Sciences, České Budějovice, 268 pp. (In Czech).

Našincová, V., Bušta, J. & Krasnolobova, T. A. (1989) Contribution to the developmental cycle and taxonomy of *Neoglyphe sobolevi* Shaldybin, 1953 (Trematoda: Omphalometridae). *Folia Parasitologica*, *36*, 313–320.

Nolan, M. J. & Cribb, T. H. (2005) The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. *Advances in Parasitology*, *60*, 101–163.

Olson P. D. & Tkach V. V. (2005) Advances and trends in the molecular systematics of the parasitic Platyhelminthes. *Advances in Parasitology*, *60*, 165–243.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542.

Samnaliev, P., Dimitrov, V. & Bušta, J. (1983) Argentophilic integumentary structures of *Plagiorchis laricola* Skrjabin, 1924 cercariae. *Folia Parasitologica*, *30*, 329–334.

Sitko, J., Faltýnková, A. & Scholz, T. (2006). Checklist of trematodes (Digenea) of birds of the Czech and Slovak Republics. Praha: Academia. 112 pp.

Snyder, S. D. & Tkach,V. V. (2001) Phylogenetic and biogeographical relationships among some holarctic frog lung flukes (Digenea: Haematoloechidae). *Journal of Parasitology*, *87*, 1433–1440.

Sokal R.R. & Rohlf F.J. (1995) *Biometry. The Principles and Practices of Statistics in Biological Research*, 3rd edn. New York: W. H. Freeman and Company, 887 pp.

Soldánová, M. & Kostadinova, A. (2011) Rapid colonisation of *Lymnaea stagnalis* by larval trematodes in eutrophic ponds in central Europe. *International Journal for Parasitology*, *41*, 981–990.

Soldánová, M., Faltýnková, A., Scholz, T. & Kostadinova, A. (2011) Parasites in a man-made landscape: Contrasting patterns of trematode flow in a fishpond area in Central Europe. *Parasitology*, *138*, 789–807.

Soldánová, M., Kuris, A. M., Scholz, T. & Lafferty, K. D. (2012). The role of spatial and temporal heterogeneity and competition in structuring trematode communities in the great pond snail *Lymnaea stagnalis* (L.). *Journal of Parasitology*, *98*, 460–471.

Strenzke, K. (1952) Der Wirtswechsel von *Plagiorchis maculosus*. *Zeitschrift für Parasitenkunde*, *15*, 369–391.

Styczyńska-Jurewicz, E. (1962) The life cycle of *Plagiorchis elegans* (Rud., 1802) and the revision of the genus *Plagiorchis* Lühe, 1889. *Acta Parasitologica Polonica, 10,* 419–445.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28,* 2731–2739.

Telford, M. J, Herniou, E. A., Russell, R. B. & Littlewood, D. T. J. (2000) Changes in mitochondrial genetic codes as phylogenetic characters: Two examples from the flatworms. *Proceedings of the National Academy of Sciences of the United States of America, 97,* 11359–11364.

Théron, A. (1976) Le cycle biologique de *Plagiorchis neomidis* Brendow, 1970, digène parasite de *Neomys fodiens* dans les Pyrénées. Chronobiologie de l'émission cercarienne. *Annales de Parasitologie Humaine et Comparée, 51,* 329–340.

Tkach, V. V. (2008). Family Plagiorchiidae Lühe, 1901. In Bray, R. A., Gibson, D.I. & Jones, A. (Eds.), *Keys to the Trematoda*. *Volume 3*. CAB International, Cambrodge, U.K., pp. 295–325.

Tkach, V. V., Grabda-Kazubska, B., Pawlowski, J. & Swiderski, Z. (1999) Molecular and morphological evidences for close phylogenetic affinities of the genera *Macrodera*, *Leptophallus*, *Metaleptophallus* and *Paralepoderma* (Digenea, Plagiorchioidea). *Acta Parasitologica, 44*, 170–179.

Tkach, V. V., Pawlowski, J. & Mariaux, J. (2000a) Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *International Journal for Parasitology, 30*, 83–93.

Tkach, V. V., Pawlowski, J. & Sharpilo, V. P. (2000b) Molecular and morphological differentiation between species of the *Plagiorchis vespertilionis* group (Digenea, Plagiorchiidae) occurring in European bats, with a re-description of *P. vespertilionis* (Müller, 1780). *Systematic Parasitology, 47,* 9–22.

Tkach,V., Grabda-Kazubska,B. & Swiderski, Z. (2001a) Systematic position and phylogenetic relationships of the family Omphalometridae (Digenea, Plagiorchiida) inferred from partial lsrDNA sequences. *International Journal for Parasitology, 31*, 81–85.

Tkach,V. V., Pawlowski, J., Mariaux, J. & Swiderski, Z. (2001b). Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. In: Littlewood,

D. T. J. & Bray, R. A. (Eds) *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 186–193.

Väyrynen, T., Siddal, R., Valtonen, E. T. & Taskinen, J. (2000) Patterns of trematode parasitism in lymnaeid snails from northern and central Finland. *Annales Zoologici Fennici*, *37*, 189–199.

Żbikowska, E. (2007) Digenea species in chosen populations of freshwater snails in northern and central part of Poland. *Wiadomości Parazytologiczne*, *53*, 301–308.

Żbikowska, E., Kobak, J., Żbikowski, J. & Kąklewski, J. (2006) Infestation of *Lymnaea stagnalis* by digenean flukes in the Jeziorak Lake. *Parasitology Research*, *99*, 434–439.

Žďárská, Z. (1966) Der Entwicklungszyklus des Trematoden *Plagiorchis laricola* (Skrjabin, 1924). *Acta Societatis Zoologicae Bohemoslovenicae*, *20*, 179–184.