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Exploring species diversity of lissorchiid trematodes (Digenea: Lissorchiidae) associated with the gravel snail, *Lithoglyphus naticoides*, in European freshwaters

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Research Paper

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Asymphylodora spp; cercariaeum; karyotype; life cycle; molecular phylogeny; *Palaeorchis incognitus*; *Rutilus rutilus*

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Abstract

Comparative analysis using complete ITS2 and partial 28S rDNA sequence data revealed that cercariaeum developing in rediae in *Lithoglyphus naticoides* represent two different lissorchiid species. One morphotype of cercariaeum is conspecific with adult *Palaeorchis incognitus* from European roach, *Rutilus rutilus*. The other cercariaeum is attributable to the genus *Asymphylodora*, but the species identity is not yet determined. We also generate the first rDNA sequences for *Asymphylodora progenetica* based on new collections from *Bithynia tentaculata* from Lithuania. Phylogenetic analyses of the newly generated sequences, together with information for other lissorchiids available on GenBank, showed that all representatives of Lissorchiidae form a strongly supported clade. Three monophyletic lineages, *Asymphylodora*, *Palaeorchis* and *Lissorchis*, were recognized at the generic level. Karyological analysis of the chromosome set of larval *P. incognitus* revealed a diploid number of $2n = 20$. Its karyotype with subtelocentric chromosomes prevailing can be regarded as comparatively 'primitive', which is consistent with the basal position of *P. incognitus* in the 28S tree relative to the representatives of the genus *Asymphylodora*. The present study adds significant new information for establishing species-specific markers for the confident characterization of different developmental stages of lissorchiid species and clarification of their life cycles.

Introduction

The gravel snail, *Lithoglyphus naticoides* (Pfeiffer, 1828), is native to the western part of the Black Sea, from where it colonized Europe in the second half of the 19th century (Bij de Vaate *et al.*, 2002). Now, this species is a common component of various ecosystems of rivers, water reservoirs and lakes. In Lithuania, *L. naticoides* was mainly found in running waters, mostly in sites at the Nemunas River and also at some of its tributaries not far upstream from their confluences. The lentic sites where this species was detected are located in the Curonian Lagoon, and Kaunas and Elektrėnai water reservoirs (Butkus *et al.*, 2014). In Hungary, *L. naticoides* is widely distributed in the Danube River and its main tributaries (Bódis *et al.*, 2012).

Lithoglyphus naticoides is known to serve as first intermediate host for several trematode species (Zdun, 1961; Odening, 1971; Chernogorenko, 1983; Stanevičiūtė *et al.*, 2008). Some parasite species, namely *Nicolla skrjabini* (Iwanitzky, 1928), *Apophallus muehlingi* (Jagerskiold, 1898) and *Apophallus* (=*Rossicotrema*) *donicus* (Skrjabin & Lindtrop, 1919), are highly specific to their first intermediate host, *L. naticoides*, and disperse together with snails (Zhokhov & Pugacheva, 2001; Tyutin & Slynko, 2010). However, a number of cercariae developing in this snail are still not associated with adult stages of digeneans and their definitive hosts are unknown.

Morphologically different cercariae of cercariaeum type (without a tail) were found in *L. naticoides* snails by different authors (Zdun, 1961; Odening, 1971; Chernogorenko, 1983). They were described under different provisional names (see also the checklist provided by Cichy *et al.*, 2011) and their identification and comparison based on morphological characters is very complicated due to the incomplete and sometimes erroneous descriptions. Frequent instances of repeatedly describing the same species have caused difficulties when attempting to compare data from various authors. The main consequence of using many synonyms is an unclear view of the digenean biodiversity in the European populations of molluscs.

While studying the larval trematodes associated with *L. naticoides* inhabiting different water bodies in Central and East Europe, we detected two morphologically different forms of lissorchiid cercariaeum developing in rediae. One of them, found in Kaunas reservoir and Elektrėnai reservoir, Lithuania, and in Lake Balaton, Hungary, was identified as *Palaeorchis* sp., and the other, obtained from snails from the Danube River, Hungary, conformed to the morphological characteristics of the genus *Asymphylodora*. The taxonomic status and actual diversity of the

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cercariaeum species developing in *Lithoglyphus* snails is not clear and deserves more comprehensive phylogenetic studies using molecular and/or karyological markers.

Herein, we provide new rDNA reference sequences for two different cercariaeum developing in *L. naticoides* and compare this information with the existing data for lissorchiid species. Sequences of adult *Palaeorchis incognitus* Szidat, 1943 obtained from the fish host, *Rutilus rutilus*(Linnaeus, 1758) and *Asymphylodora progenetica* (Sercova & Bychovsky, 1940) from the prosobranch snail *Bithynia tentaculata* (Linnaeus, 1758) were generated for the first time and used for comparative analysis. The chromosome set structure of larval *Palaeorchis* was studied. Our results provide new insights into the phylogenetic relationships of lissorchiid genera that have not been extensively characterized with respect to their position on molecular phylogenetic trees. The developmental stages of *P. incognitus* in the first intermediate host were verified with the aid of molecular evidence.

Material and methods

Specimens

Gravel snails were collected by hand in Kaunas (54°51′38″N, 24°09′07″E) and Elektrėnai (54°77′99″N, 24°65′87″E) water reservoirs, Lithuania, and in Lake Balaton (46°58′72″N, 17°57′44″E) and the Danube River (47°49′17″N, 19°00′43″E), Hungary. In the laboratory, snails were dissected and intramolluscan stages were examined alive unstained under slight coverslip pressure. Adult specimens of *P. incognitus* were recovered from the intestine of European roach, *R. rutilus*, caught in the Kaunas water reservoir, placed in saline solution, and identified *in vivo*. The morphology of the adult worms agreed well with the morphological characteristics of this species provided in the relevant identification keys to parasites of fish (Bykhovskaya-Pavlovskaya & Kulakova, 1987; Niewiadomska, 2003). Photomicrographs of live adult and larval (redial and cercarial) isolates were taken with a digital camera mounted on an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan)(fig. 1a–c) and used for further identification. Drawing of the cercaria is based on digital photographs of live cercariae. Measurements are in micrometres and are presented as a range, followed by a mean in parentheses. Progenetic specimens of *A. progenetica* were obtained from *B. tentaculata*, collected in two nameless ponds (54°74′80″N, 25°27′77″E and 54°75′24″N, 25°29′22″E) in Vilnius, Lithuania. For molecular analyses of intramolluscan stages, cercariae and rediae were pooled from one single infected snail host and fixed in 96% ethanol.

Karyological analysis

Samples of *Palaeorchis* sp., used for karyological analysis, were obtained from gravel snails collected in the Kaunas reservoir. Living snails were incubated in 0.01% colchicine in well water for 3–5 h at room temperature. Tissues containing parthenitae (rediae and cercariaeum) were dissected and treated in distilled water for hypotony for 40–50 min. The material was then fixed in fixative (ethanol:glacial acetic acid, 3:1) with three changes, 20 min each. Slide preparations of mitotic chromosomes were made using an air-drying technique (Petkevičiūtė *et al.*, 2015). Slides were stained with 4% Giemsa solution (pH 6.8) for 30–40 min and examined with an Olympus BX51 microscope using a 100× oil immersion objective. Chromosome measurements were made of the seven best mitotic spreads out of 36 analysed cells chosen from parthenitae from three infected snails. Measurements (absolute length in micrometres, relative length in percentage and centromeric indices) are given as mean values and standard deviations. The terminology used to describe the centromere position follows that of Levan *et al.* (1964).

DNA isolation, polymerase chain reaction (PCR) amplification and DNA sequencing

Genomic DNA for molecular analysis was extracted from ethanol-fixed helminths following the protocol of Stunžėnas *et al.* (2011), with a slight modification described in Petkevičiūtė *et al.* (2014). DNA fragments spanning the 3′ end of the 5.8S rRNA gene, the complete internal transcribed spacer 2 region (ITS2) and a small section at the 5′ end of the 28S gene were amplified using universal primers for flatworms, the forward primer 3S (5′-CGG TGG ATC ACT CGG CTC GTG-3′) (Bowles *et al.*, 1995) and the reverse primer ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) (Cribb *et al.*, 1998). Primer pair AlJe-F (5′-GTC TGG CTT GGC AGT TCT A-3′) and AlJe-R (5′-CTG CCC AAT TTG ACC AAG C-3′), designed for species of the Allocreadiidae (Petkevičiūtė *et al.*, 2018b)), was suitable to amplify the end of the internal transcribed spacer 1 (ITS1), the complete 5.8S rDNA and ITS2, and also a small section at the 5′ end of the 28S gene of *A. progenetica*. A fragment at the 5′ end of the 28S rRNA gene was amplified using the forward primers Digl2 (5′-AAG CAT ATC ACT AAG CGG-3′) (Tkach *et al.*, 2003) or ZX-1 (5′-ACC CGC TGA ATT TAA GCA TAT-3′) (Bray *et al.*, 2009) and the reverse primers L0 (5′-GCT ATC CTG AG (AG) GAA ACT TCG-3′) (Tkach *et al.*, 1999) or 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Olson *et al.*, 2003; Tkach *et al.*, 2003). Amplification protocols are as described in Petkevičiūtė *et al.* (2014). PCR products were purified and sequenced in both directions at BaseClear B.V. (Leiden, Netherlands) using the PCR primers. Contiguous sequences were assembled using Sequencher 4.10.1 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences generated in this study have been deposited in the GenBank database (see accession numbers in table 1).

Molecular phylogenetic analyses

For the phylogenetic analyses, both the ITS2 and 28S datasets were aligned independently using ClustalW (Thompson *et al.*, 1994) with an open gap penalty of 15 and gap extension penalty of 6.66. The best-fit model of sequence evolution for phylogenetic analysis was estimated using jModeltest version 0.1.1 software (Posada, 2008). Maximum likelihood (ML) phylogenetic trees were obtained and analysed using MEGA version 6 (Tamura *et al.*, 2011). Branch support was estimated by bootstrap analyses with 1000 pseudoreplicates. The ML trees were obtained using the general time reversible model with a gamma distribution rate and a proportion of invariant sites (GTR + G + I) for both the ITS2 and the 28S gene datasets. The value for gamma and the number of invariant sites were estimated from the data. Parsimony analysis based on subtree pruning and regrafting was used with default parsimony settings. If two or more sequences belonged to one species, they were collapsed into one branch, except those newly obtained in this study. Estimates of mean evolutionary divergence over sequence

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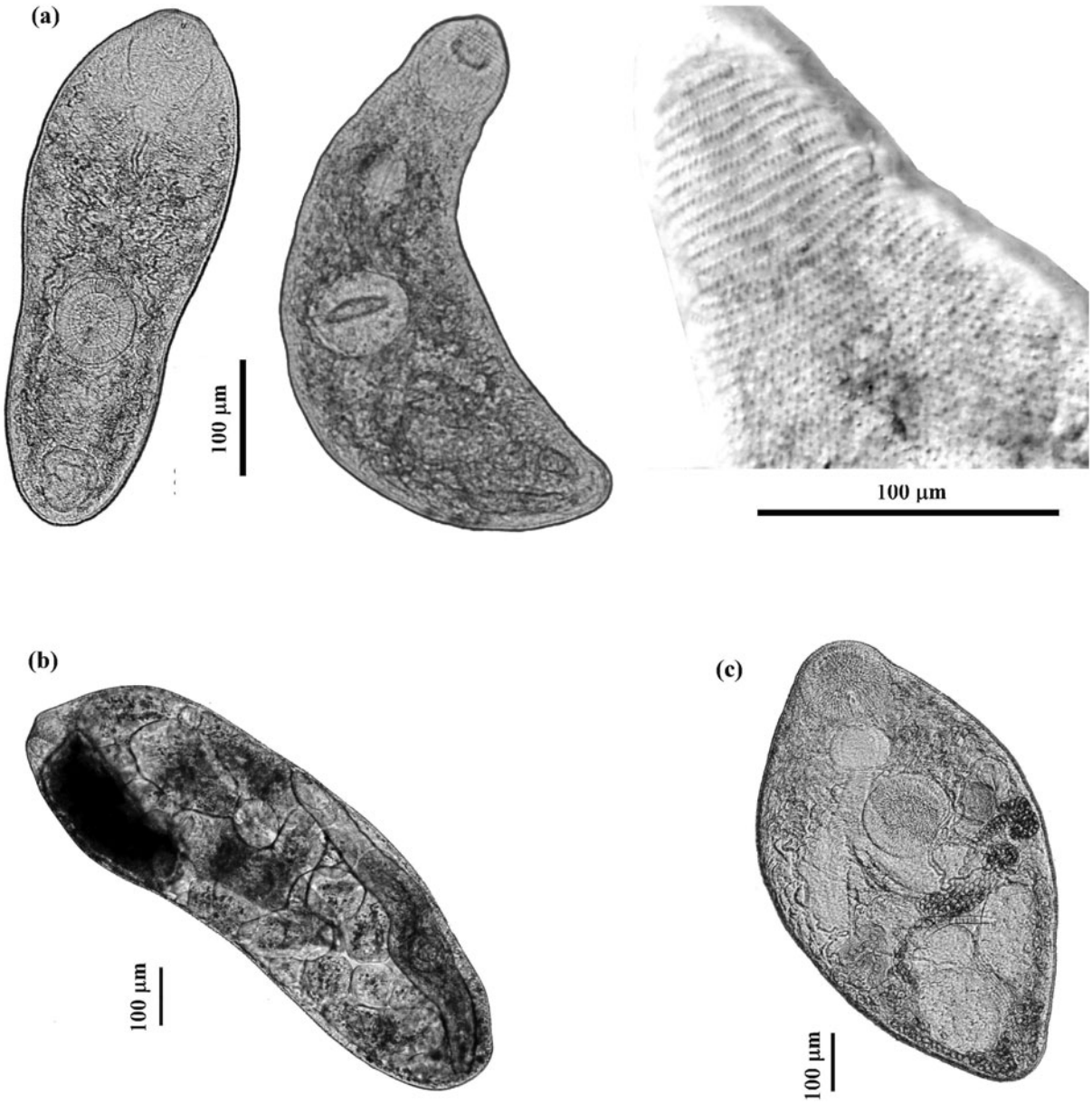

Fig. 1. Photomicrographs of live developmental stages of *Palaeorchis incognitus*: (a) two specimens of cercariae and tegumental spines of the anterior part of cercarial body; (b) rediae; (c) adult.

pairs within and between groups were calculated using MEGA version 6.

Results

Morphology of cercaria

Palaeorchis incognitus Szidat, 1943 cercaria (figs 1a and 2)

Measurements based on nine specimens. Cercariae developing in elongate-saccular rediae without locomotor appendages (fig. 1b), containing several germ balls and few (mostly three or four) fully developed cercariae. Live cercariae are mobile and contractile. Cercarial body tailless, elongate, oval, with maximum width at level of acetabulum, and measured 390–535 × 160–193 (474 × 174). The body surface is armed with transversal rows of scale-like tegumental spines, covering the forebody, becoming smaller and scarcely distributed posteriorly (up to the post-acetabulum level). Oral sucker round, subterminal, 67–78 × 70–81 (73 × 76), with tiny stylet embedded in the anterior part of the rim. Prepharynx conspicuous; pharynx globular, muscular, 33–37 × 35–41 (35 × 38). Oesophagus long and sinuous, bifurcating at level of anterior border of acetabulum; intestinal caeca terminating blindly in the third quarter of the body. Ventral sucker almost equal in size to oral sucker, round, 68–77 × 70–75 (72 × 71), situated somewhat behind the middle of body. The excretory vesicle oval, thick-walled, with primary collecting ducts extending up above ventral sucker where they form loops and divide into anterior and posterior collecting ducts. Flame cell formula difficult to establish. Subtegumental gland cells large, with large nuclei scattered beneath tegument on

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Table 1. Species subjected to molecular phylogenetic analysis with information for hosts, localities and GenBank accession numbers.

			GenBank number and source if it is not from this study	
Species	Host	Locality	28S	5.8S-ITS2-28S
Asymphylodora perccotti	*Perccottus glenii*	Primorsky region, Russian Far East	FR822731 Besprozvannykh *et al.* (2012)	
Asymphylodora sp.	*Lithoglyphus naticoides*	Danube River, Hungary	**MT153916, MT153917**	**MT153914, MT153915**
Asymphylodora progenetica	*Bithynia tentaculata*	Jeruzalė pond, Vilnius, Lithuania	**MT103401, MT103402, MT103403**	**MT103396, MT103398, MT103399**
A. progenetica	*B. tentaculata*	Verkiai pond, Vilnius, Lithuania	**MT103400**	**MT103397**
Palaeorchis incognitus	*Rutilus rutilus*	Kaunas water reservoir, Lithuania	**MT103408**	**MT103406**
P. incognitus	*L. naticoides*	Kaunas water reservoir, Lithuania	**MT103409**	
P. incognitus	*L. naticoides*	Elektrėnai water reservoir, Lithuania	**MT103407**	**MT103404**
P. incognitus	*L. naticoides*	Balaton Lake, Hungary	**MT103410**	**MT103405**
Lissorchis kritskyi	*Carpiodes cyprinus*	USA	AY222250 Olson *et al.* (2003)	
L. kritskyi	*Minytrema melanops*	USA	EF032689 Curran *et al.* (2006)	
Provitellus turrum	*Pseudocaranx dentex*	Australia	AY222253 Olson *et al.* (2003)	
Provitellus infrequens	*Gnathanodon speciosus*	Australia		MK501981 Wee *et al.* (2019)
Monorchis monorchis	*Diplodus vulgaris*	Near Corsica	AF184257 Tkach *et al.* (2001)	
Monorchis lewisi	*Acanthopagrus australis*	Moreton Bay, Queensland, Australia		MF503313 Cribb *et al.* (2018)
Allocreadium neotenicum	*Hydroporus rufifrons*	Lake District, Cumbria, UK	JX977132 Bray *et al.* (2012)	
Phyllodistomum angulatum	*Sander lucioperca*	Rybinsk water reservoir on the Volga River, Russia	KX957735 Stunžėnas *et al.* (2017)	

Sequences generated in the present study are indicated in bold.

both sides, especially numerous at level of acetabulum. Reproductive system poorly developed, anteriorly to excretory vesicle. In some specimens, the cirrus pouch and metraterm could be seen posterolateral to the acetabulum.

Taxonomic summary

First intermediate host. *Lithoglyphus naticoides* (Pfeiffer, 1828) (Gastropoda: Hydrobiidae).

Site of infection: Digestive gland, gonads and intestinal surface.

Prevalence: 0.47% (May–September 2019, $n = 635$).

Locality: Kaunas water reservoir, Lithuania (54°51′38″N, 24°09′07″E).

GenBank accession numbers: MT103409 (28S), MT103404 (5.8-ITS2-28S).

Karyotype structure: $2n = 20 = 8st + 4st\text{-}sm + 8sm$.

Karyotype of *P. incognitus*

Most dividing mitotic cells (35 from 36 examined) of larval *P. incognitus* had 20 chromosomes in the diploid complements (fig. 3). One cell was aneuploid, with 19 chromosomes. The chromosomes are comparatively small and gradually decrease in length from 4.86 to 1.49 µm (table 2). The mean total length of the haploid complement is 26.7 µm. Chromosomes with subterminally located centromeres prevail in the karyotype. According to centromere position, only the last four pairs of small chromosomes – 7, 8, 9 and 10 – have submedially located centromeres and are classified as submetacentric. Secondary constriction in pericentromeric region of short arms of the chromosomes constituting pair 9 was observed in most metaphase plates; this constriction is most likely physically associated with the nucleolar organizer and contains rRNA genes.

Molecular analysis and phylogeny

The newly obtained sequences of two different regions of rDNA (ITS2 region and partial 28S gene) of lissorchiid and additional relevant rDNA sequences of lissorchiid and monorchiid trematode species and outgroup taxa were downloaded from

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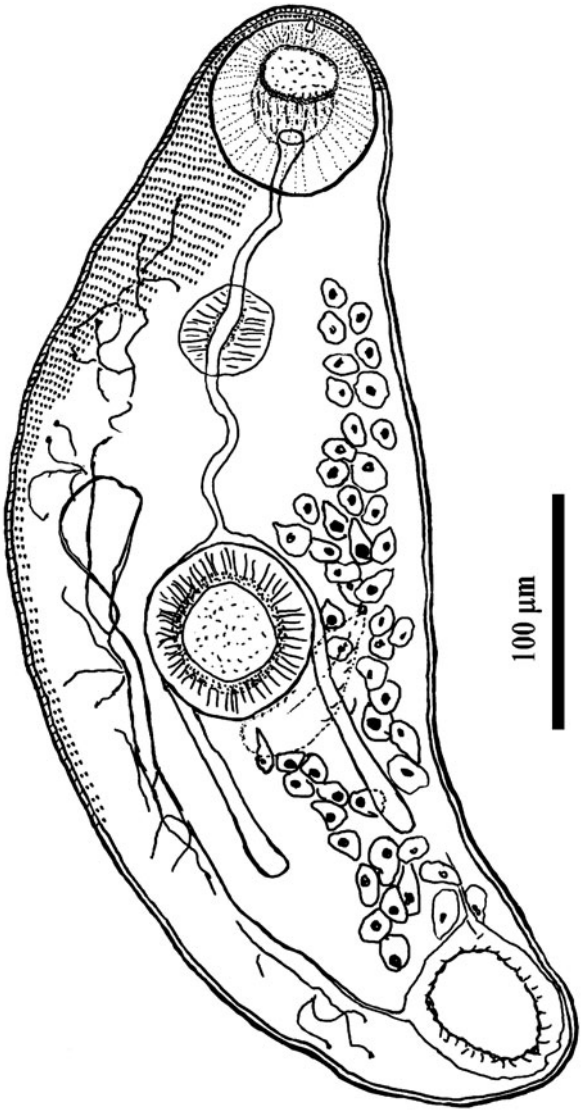

Fig. 2. Cercaria of *Palaeorchis incognitus*.

GenBank and included in pairwise sequence comparisons and phylogenetic analyses (table 1). An alignment of the ITS2 and partial 28S data sets yielded 654 and 1113 characters for analysis, respectively; a couple sequence of the Allocreadiidae and the Gorgoderidea were used as outgroup. The phylogenetic trees made with the new sequences and the sequences deposited in GenBank are shown in figs 4 and 5. Two genetically different lissorchiid cercariaeum were revealed from *L. naticoides*. The cercariaeum from Hungary (Balaton Lake) and Lithuania (Elektrėnai and Kaunas water reservoirs) matched exactly the sequences of adult *P. incognitus ex* European roach, *R. rutilus*. The other cercariaeum isolate from *L. naticoides* was detected only in one location, in the Danube River, Hungary. The species of this cercariaeum was identified as *Asymphylodora* sp. based on the results of molecular data. Differences between two lissorchiids from *L. naticoides* are 8% (38 nucleotides of 472) and 6.8% (73 of 1159), in ITS2 and partial 28S sequences, respectively. *Asymphylodora* sp. *ex L. naticoides* is similar to *A. progenetica ex B. tentaculata*, collected in Lithuania; diverging 7.8% (39 of 500) and 5.9% (68 of 1159) in ITS2 and partial 28S datasets, respectively. However, it appeared as sister clade to *A. progenetica* and *Asymphylodora perccotti* Besprozvannykh, Ermolenko & Atopkin, 2012 in the 28S tree (fig. 4), while the sequences of *P. incognitus* form distinct robustly supported lineage. In this tree, *Lissorchis kritskyi* Barnhart & Powell, 1979 forms a separate branch, which clustered with other lissorchiids in one well-supported higher clade. The topography of the ITS2 tree (fig. 5) is identical; however, it contains less species.

Discussion

In Europe, the Lissorchiidae includes the genera *Asymphylodora* Looss, 1899 and *Palaeorchis* Szidat, 1943. These trematodes are common and widely distributed parasites of freshwater fish in the Palaearctic region (Bray, 2008), and are well known from fish hosts in Lithuania and neighbouring countries (Rauckis, 1988; Niewiadomska, 2003; Kirjušina & Vismanis, 2007). In older publications, the species of these genera are assigned to the family Monorchiidae Odhner, 1911. Later, Shimazu (1992) gave a clear summary of features distinguishing members of Lissorchiidae from monorchiids. Molecular evidence in Tkach *et al.* (2001) confirmed the independent status of the Lissorchiidae.

Despite the fact that our current knowledge of life cycles is relatively good for trematodes parasitic in European freshwater fish (Faltýnková *et al.*, 2016), the vast majority of helminth species are known only from their adult stage (Blasco-Costa & Poulin, 2017). Life cycles are known for some freshwater lissorchiid species, which infect pulmonate (heterobranch) and prosobranch gastropods as first intermediate hosts (Stunkard, 1959; Lambert, 1976; van den Broek & de Jong, 1979; Našincová & Scholz, 1994; Besprozvannykh, 2005; Besprozvannykh *et al.*, 2012). Numerous other putative but unidentified freshwater lissorchiid cercariae (non-oculate cercariae of cercariaeum type in rediae in snails) are known from different gastropods.

Similarly sized (morphometric) parameters and the absence of contrasting characters in rediae and cercariae of closely related species are sometimes compounded by contradicting descriptions by different authors. In many cases, it is impossible to correlate the morphological data on intramolluscan stages with those of their adults; moreover, sometimes it was even difficult to establish the attribution of particular larvae to any trematode family. Thus, the widespread enigmatic species *Cercariaeum crassum* Wesenberg-Lund, 1934, developing in rediae in the sphaeriid clam *Pisidium amnicum* (Müller, 1774), was incorrectly assigned to the lissorchiid genus *Palaeorchis* as *Palaeorchis* sp. or *Palaeorchis crassus* in some publications (see Zhokhov & Pugacheva, 1995; Holopainen *et al.*, 1997; Rantanen *et al.*, 1998). Subsequent comprehensive morphological and then karyological and molecular analysis corroborate the allocation of *C. crassum* to *Allocreadium* Looss, 1900 in the Allocreadiidae (Niewiadomska & Valtonen, 2007; Petkevičiūtė *et al.*, 2012).

Recent molecular tools in the form of DNA sequence data provide reliable markers for species identification and are fast dominating in the studies of digenean life cycles. However, not a single life cycle of European *Asymphylodora* or *Palaeorchis* species has been proven by molecular methods, and there are many controversies in the data on lissorchiid life cycles known from experimental infections.

Two species of the genus *Palaeorchis* are known from fish hosts (mainly cyprinids) in Lithuania and neighbouring countries: *P. incognitus* and *Palaeorchis unicus* Szidat, 1943

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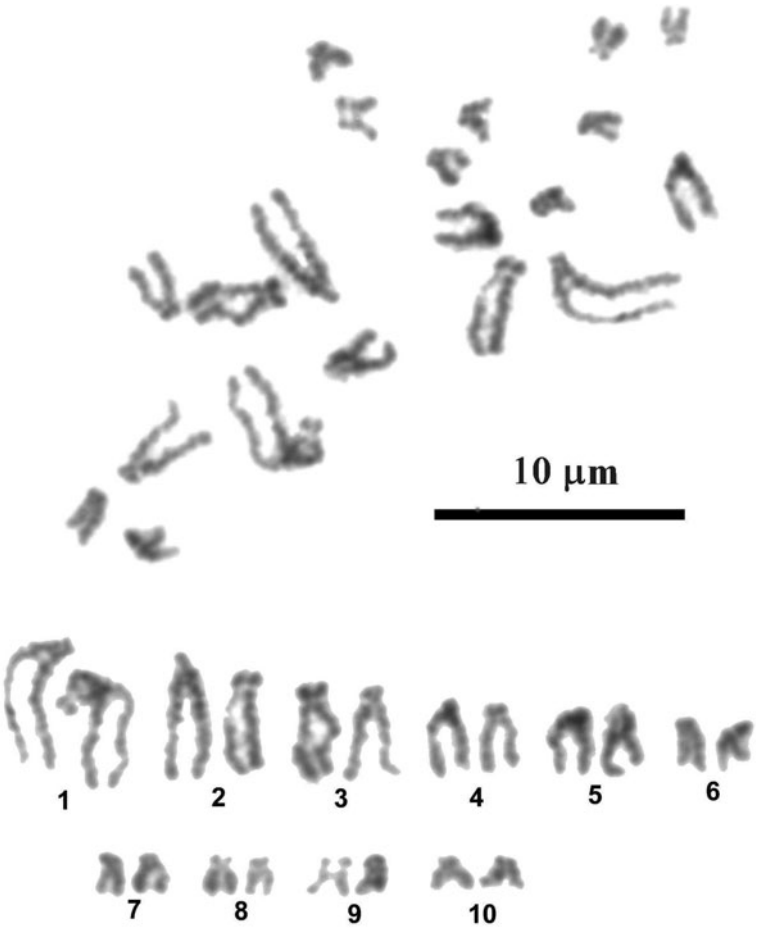

Fig. 3. Mitotic metaphase and karyotype of *Palaeorchis incognitus*.

Table 2. Measurements (means ± standard deviation) and classification of chromosomes of *Palaeorchis incognitus*.

Chromosome number	Absolute length (µm)	Relative length (%)	Centromeric index	Classification
1	4.86 ± 1.86	18.51 ± 0.46	13.00 ± 2.24	st
2	4.14 ± 1.61	15.71 ± 1.19	14.77 ± 1.46	st
3	3.48 ± 1.33	12.84 ± 1.26	20.73 ± 2.56	st
4	2.90 ± 0.92	10.79 ± 0.66	21.61 ± 3.07	st
5	2.51 ± 0.85	9.41 ± 0.52	23.6 ± 3.20	st-sm
6	2.13 ± 0.56	7.84 ± 0.43	24.63 ± 3.88	st-sm
7	1.87 ± 0.58	7.15 ± 0.80	28.37 ± 4.33	sm
8	1.72 ± 0.53	6.27 ± 0.38	29.89 ± 3.54	sm
9	1.67 ± 0.52	5.97 ± 0.62	31.41 ± 3.92	sm
10	1.49 ± 0.46	5.49 ± 0.50	30.64 ± 2.49	sm

sm, submetacentric chromosomes; st, subtelocentric chromosomes.

(Rauckis, 1988; Niewiadomska, 2003; Kirjušina & Vismanis, 2007). Their life cycles are not elucidated. Szidat (1943) assumed that *Cercariaeum paludinae impurae* f. *armatum* de Filippi, 1854 from *B. tentaculata* is the larval stage of *P. incognitus*. This assumption was based on morphological similarity and was not confirmed under experimental conditions or other evidence. Szidat (1943) declared that the larvae of *Palaeorchis* certainly belong in the 'Helveticum' group of Dubois (1929) (these larvae develop in rediae; they have two testes; the excretory vesicle is tubular and flexible). In his study of trematodes using the snail *L. naticoides* as first intermediate host in Lake Müggelsee (Berlin), Odening (1971) described cercariaeum developing in rediae identified as '? *Cercariaeum papillosum* Mödlinger, 1934', which closely resembles our cercariaeum of *P. incognitus* in having the tailless body and two suckers of equal size. The oral sucker bears a tiny stylet. The anterior part of the body is armed with

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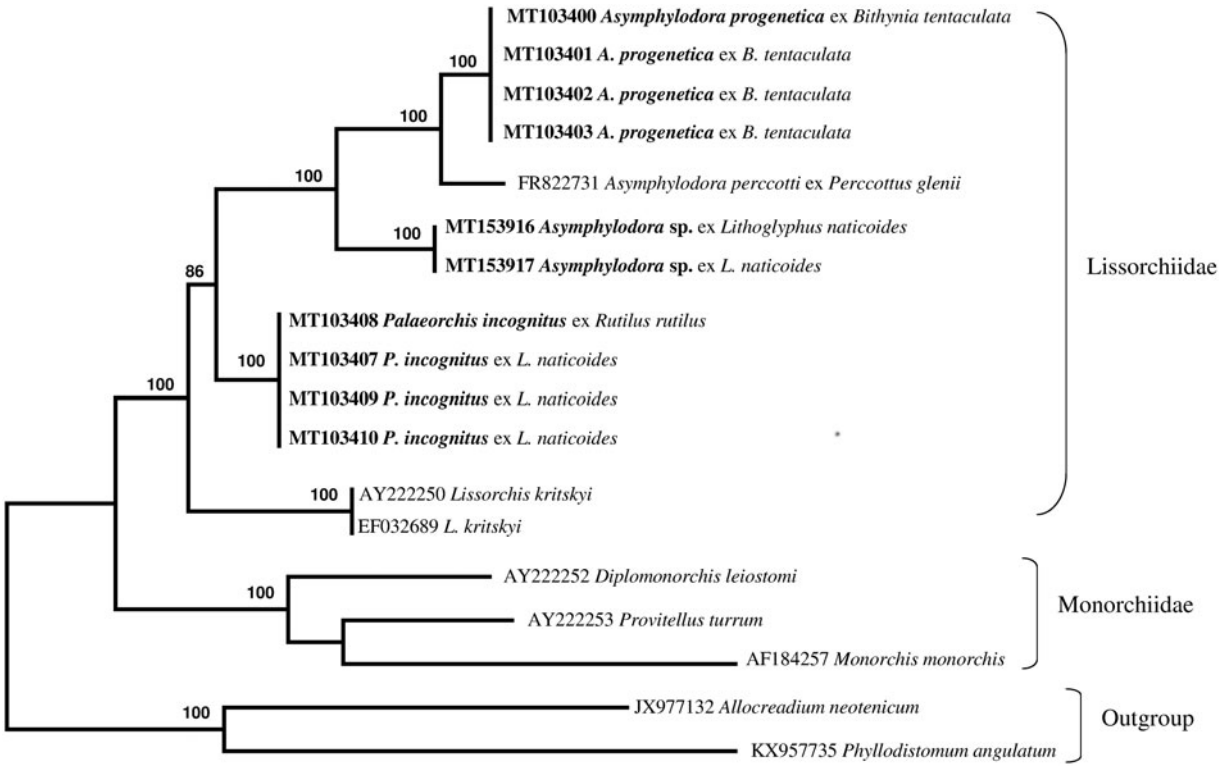

Fig. 4. Phylogenetic tree based on maximum likelihood analysis of partial sequences of the 28S nuclear rDNA gene. Bootstrap support values lower than 70% are not shown. The species sequenced in this study are indicated in bold.

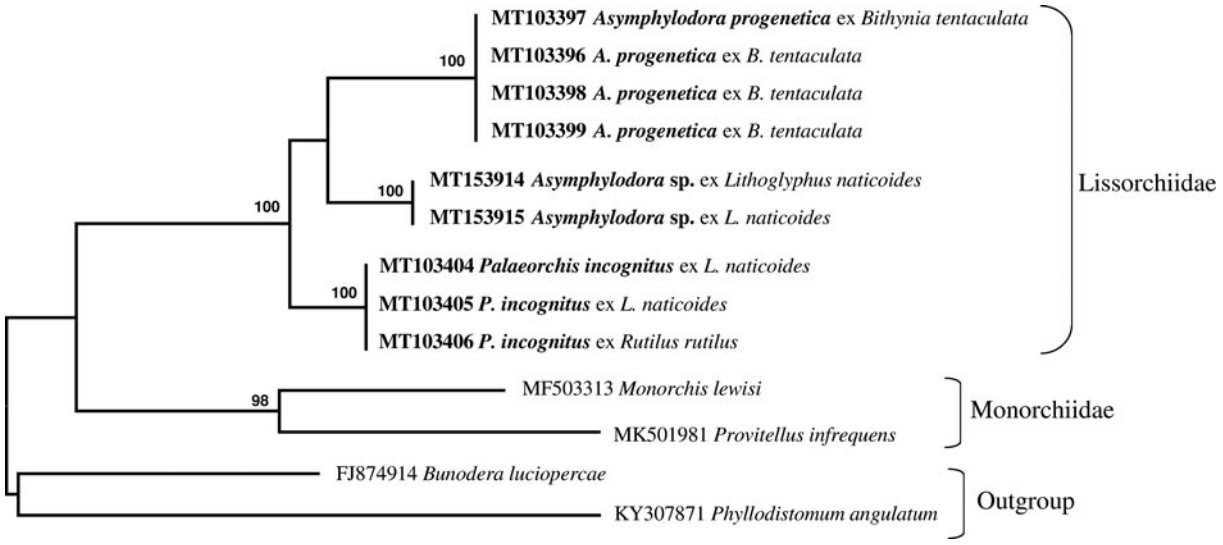

Fig. 5. Phylogenetic tree based on maximum likelihood analysis of the ITS nuclear rDNA region. Bootstrap support values lower than 70% are not shown. The species sequenced in this study are indicated in bold.

tegumental spines that are inconspicuous posteriorly. The prepharynx is conspicuous, the pharynx is almost globular and the oesophagus is long, flexuous, bifurcating in front of the acetabulum. The excretory vesicle is thick-walled, elongate-oval, flexible. Cystogenic gland cells numerous. The genitalia are not clearly visible. Daughter rediae with only a few developing cercariae and several germ balls (in older specimens).

Tyutin & Slynko (2010) found rediae and cercariae morphologically similar to the local species *Parasymphylodora* (=*Asymphylodora*) *markewitschi* (Kulakowskaja, 1947) in 15.5% of examined *L. naticoides*, which usually had a thin-walled shell, as compared to uninfected individuals.

Comparative ITS2 and 28S sequence analysis carried out by us confidently confirmed the link between redial and cercarial isolates

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ex L. naticoides from different populations in Lithuania and Hungary, and the adult stages of *P. incognitus* parasitizing *R. rutilus* from Kaunas water reservoir, supporting their conspecificity. No intraspecific variation was detected between these isolates. In our analysis, representatives of three genera of Lissorchiidae produced strongly supported clades. *Palaeorchis* is represented in the analysis by just one species, which forms a distinct genetic lineage. Cercariaeum obtained from snails collected in the Danube River clusters together with representatives of the genus *Asymphylodora*. The species identification of this cercariaeum is not presently possible, but the collection locality, host record and sequence data will all be valuable reference information for the future collection of adults. The number of DNA sequences deposited in GenBank is rapidly increasing and molecular analysis represents a straightforward method to approach questions related to the species identity of larval stages. Redial and sexually mature stages of *A. progenetica* were originally described from *B. tentaculata* collected near Leningrad, Russia (Serkova & Bychovsky, 1940). The life cycle of this species is truncated to one host, and the mollusc is used as a definitive host as well, or another pathway exists in which trematodes mature in fish (Kulakova, 1982). Despite the altered life cycle, this species shows close affinities with *A. perccotti* from the Russian southern Far East, which lacks the capacity for progenesis (Besprozvannykh *et al.*, 2012).

It should be noted that adult *P. incognitus* was detected only in those Lithuanian water bodies where the *L. naticoides* was also found – that is, the Nemunas River, Curonian lagoon and Kaunas water reservoir (Rauckis, 1988). *Palaeorchis incognitus* was also recorded from fish hosts from the four localities under investigation in the Danube River system in Central Europe (Moravec *et al.*, 1997), where *L. naticoides* is widely distributed. On this basis, it can be inferred that *P. incognitus* is specific to its first intermediate host. A direct consequence of species specificity of parthenitae in many species of trematodes is almost a complete coincidence of their ranges with the ranges of molluscan hosts.

Prior to the present study, sequence data were available for only two lissorchiid species from the Russian southern Far East and from the south-eastern region of the US: *A. perccotti* and *L. kritskyi*, respectively (Olson *et al.*, 2003; Besprozvannykh *et al.*, 2012). This study contributed novel molecular data from three additional lissorchiid species in two genera. Molecular data from *P. incognitus* represent the first such available data for the genus. Here, the generation of ITS2 rDNA sequences for three lissorchiids represents all available data for the family. In the main, the new sequences reported here serve to improve the dataset for future analyses of diversity, life cycles and phylogenetic relations of lissorchiid species.

Karyological features, as well as molecular data, may indicate the evolutionary distance between species, which may not be obvious at the morphological level, and provide helpful markers that can serve as species-specific characteristics (White, 1978; Petkevičiūtė *et al.*,2014, 2018a). In general, comparative data based on different levels of organization provide a more comprehensive understanding of the evolutionary processes of species divergence and phylogenetic relations. Unfortunately, chromosomal analysis is rarely explored in taxonomic and phylogenetic studies of parasitic flatworms.

The chromosome number and morphology of only five lissorchiid species have been analysed. Diploid chromosome sets of three unidentified species of larval *Asymphylodora*, developing in *B. tentaculata*, comprise 20 or 22 elements with two or one pair of large metacentric elements, respectively (Baršienė, 1993). The karyotype with $2n = 20$ and with similar chromosome morphology was described for unidentified larval stages of lissorchiid from the snails *Melanopsis dufouri* (Férussac, 1823) collected in Spain (Baršienė *et al.*, 1995). Apparently, *Asymphylodora* spp. form a group of karyologically closely related species. The karyotype with $2n = 20$ presumably arose through the centromeric fusion of two acrocentric non-homologous chromosomes (Robertsonian translocation). The only karyotype of *Palaeorchis* sp. known so far from *B. tentaculata* includes 14 chromosomes and does not show any similarity to the chromosome sets of *Asymphylodora* species (Baršienė, 1993). The chromosome set revealed for *P. incognitus* in this study, with $2n = 20$, clearly differs from the karyotype of its congener with $2n = 14$. It resembles the chromosome sets of *Asymphylodora* spp. in diploid number and shows very close relative length values compared to *Asymphylodora* sp. 1 described by Baršienė (1993), but differs in centromere position of corresponding chromosome pairs. Such variation in centromere position of corresponding chromosomes of related species is most easily explained by pericentric inversions. Cytogenetic theory leads to the assumption that the more derived species usually evolve a karyotype consisting of biarmed elements, while a 'primitive' karyotype within a given phyletic group consists, typically, of acrocentric chromosomes (White, 1978; Birstein, 1987). In view of this, the karyotype of *P. incognitus*, consisting of predominantly subtelocentric chromosomes, should be regarded as quite 'primitive' in comparison with karyotypes of *Asymphylodora* spp. composed predominantly of meta- and submetacentric elements. Hence, the karyotypic structure of *P. incognitus* is consistent with its position in the 28S-based phylogenetic tree, where it occupies a basal position in relation to *Asymphylodora* spp. It is notable that trematodes are fairly conservative karyologically, and species related from a morphological point of view most often show close karyological affinities. The existence of radically different karyotypes in the congeneric species of digenean trematodes is an unusual and intriguing phenomenon. It is likely that if karyotype features are plotted over a phylogenetic tree based on molecular data, the processes involving chromosome evolution might be clarified.

The results of the present study have shown that the analysis of larval trematodes, including karyological studies and, in particular, molecular sequencing, can provide markers to identify species and to elucidate life cycles and, finally, determine the true biodiversity of the digeneans in the European populations of molluscs.

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Conflicts of interest. None.

Ethical standards. All applicable institutional, national and international guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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