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Comparison of the developmental stages of some European allocreadiid trematode species and a clarification of their life-cycles based on ITS2 and 28S sequences

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Abstract Genetic data were used to examine the diversity in some allocreadiid trematodes. Nuclear ribosomal DNA (ITS2 and partial sequences of 5.8S and 28S) was sampled from sexual adult and 'larval' stages. From these and previous reference datasets phylogenetic trees were constructed. The results uncovered genetically distinct lineages within *Bunodera luciopercae* (Müller, 1776), suggesting that the two Palaearctic subspecies, *B. l. luciopercae* and *B. l. acerinae* Roitman & Sokolov, 1999, and Nearctic *B. luciopercae* from *Perca flavescens* may represent distinct species with a restricted host-specificity. Identical rDNA was revealed for the sexual adult of *B. l. acerinae* and 'larval' *B. luciopercae* described by Wiśniewski (1958). An unexpected match between the rDNA sequences of adult *B. l. luciopercae* and 'larval' *Allocreadium isoporum* (*sensu* Wiśniewski, 1958) was also detected. The adult *A. isoporum* (Looss, 1894) differs significantly from the 'larval' *A. isoporum*, the level of rDNA sequence divergence between them (8.6 % for 5.8S-ITS2-28S sequences and 6.26% for 28S) being consistent with the level expected for intergeneric variation. These results revealed the possible existence of a cryptic species complex within the nominal species *B. luciopercae* and a clear need for reconsideration of some of the accepted, but largely untested, tenets regarding allocreadiid life-cycles.

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Introduction

The trematodes of the family Allocreadiidae Looss, 1902 are among the most common and widely distributed freshwater parasites in the intestines of teleosts (Yamaguti, 1971; Caira, 1989; Caira & Bogea, 2005). In Europe the Allocreadiidae includes the genera *Allocreadium* Looss, 1900, *Bunodera* Railliet, 1896 and *Crepidostomum* Braun, 1900 (see Niewiadomska & Valtonen, 2007). Only one species of *Bunodera* has been described in European waters, i.e. *B. luciopercae* (Müller, 1776). The definitive hosts reported for this species include members of several piscine families, but its main hosts are considered to be *Perca* spp. (Sokolov, 2004; Choudhury & Leon Regagnon, 2005; Sokolov et al., 2006). In North America, *B. luciopercae* appears to be primarily a parasite of the yellow perch *Perca flavescens* (Mitchill) (see Hoffman 1999).

The existence of a host-associated species complex within *B. luciopercae* has been suggested on the basis of some morphological differences of the

'adult[1]' stage (Roitman & Sokolov, 1999). Recently two subspecies, *B. luciopercae luciopercae* (Müller, 1776) and *B. l. acerinae* Roitman & Sokolov, 1999, have been recognised based on morphology, RAPD profiles and host-specificity (Sokolov et al., 2006). The latter authors pointed out the need for life-cycle studies in order to confirm the rank of these taxa.

In general, allocreadiids follow a three-host strategy, involving redial, cercarial and metacercarial stages. The cercariae develop within rediae in sphaeriid bivalves rather than in gastropods and are of a typical ophthalmoxiphidiocercaria type. They are characterised by several morphological characters, including eye-spots and the presence of a stylet in the oral sucker. Free-swimming cercariae encyst in the second intermediate host, which is generally an aquatic arthropod. Classical life-cycle determination by experimental infections is difficult, time consuming and not always feasible. The life-cycle of *B. luciopercae* was established by Wiśniewski (1958) in Poland and later by Cannon (1971) in Canada. However, differences exist in the Palaearctic and Nearctic *B. luciopercae* that cannot be attributed to interpopulational variation. Cercariae of *B. luciopercae* (*sensu* Cannon, 1971) possess only two rather than three penetration glands on each side and have less developed adhesive ventral sucker papillae. The second intermediate hosts are rather different (cladocerans in the Palaearctic and amphipods in the Nearctic). The information on chromosome set structure also is controversial, and different authors have given different results for karyological studies on *B. luciopercae* (see Petkevičiūtė & Stanevičiūtė, 2008). These facts have lead to the presumption that *B. luciopercae* sensu Cannon (1971) could represent a cryptic species within the nominal species *B. luciopercae*.

As an alternative to classical methods, molecular tools have been used in recent years to identify various stages involved in trematode life-cycles (Jousson & Bartoli, 2000; Bartoli et al., 2000; Nolan & Cribb, 2004; Petkevičiūtė et al., 2004; Pina et al., 2007). In this study *B. l. luciopercae* and *B. l. acerinae* were obtained from their definitive hosts and two regions of their rDNR (ITS2 and partial 28S) were sequenced and alignments compared with those of allocreadiid 'larval' stages collected from naturally infected sphaeriid clams in Europe in order to obtain markers to distinguish closely related taxa. 'Adult' and 'larval' stages of *Allocreadium isoporum* (Looss, 1894), the type-species of the genus, as well as other relevant GenBank data on allocreadiids were also included in the analyses.

[1] In this work 'adult' refers to the sexual adult or marita, and 'larva' refers to the other life-history stages, although the parthenitae are not larval stages.

Materials and methods

Developmental stages of allocreadiid species used in this study, their hosts, the geographical origin of the material and the GenBank accession numbers for the corresponding sequences are presented in the Table 1.

Adult specimens of *Bunodera luciopercae* were recovered from the intestines of the European perch *Perca fluviatilis* (L.) and ruff *Gymnocephalus cernuus* (L.) caught in the River Tvertsa (upper Volga Basin, Russia) and Lake Segozero (Karelia). Adult *Allocreadium isoporum* were obtained from intestine of bleak *Alburnus alburnus* (L.) caught in Lake Oster (Karelia).

Sphaeriid clams were collected from different freshwater bodies in Lithuania, Russia and the Ukraine using a hand-net and dissected to obtain parthenitae from the host tissues. 'Larval' allocreadiids were identified using morphological features of the cercariae and rediae. Identifications were based on the descriptions given by Wiśniewski (1958) and Caira (1989). 'Larval' *Crepidostomum* material from *Sphaerium corneum* (L.) possibly belongs to the species *C. farionis* (Müller, 1780); however, it is referred to here as *Crepidostomum* sp., because, as the cercariae were obtained during the dissection of the molluscs, some uncertainties arose due to differences in their maturity.

Voucher specimens from the same collecting event and extracted total DNA are deposited in the P. B. Šivickis Laboratory of Parasitology, Institute of Ecology of Nature Research Centre, Vilnius.

'Adult' and 'larval' allocreadiid stages were fixed in ethanol and stored at -20°C before genomic nucleic acid extraction. The 'adult' worms or parthenitae were placed in TBE buffer for 5 min to wash out the ethanol. Single 'adult' specimen or several parthenitae from a single mollusc were ground between two

Table 1 Allocreadiid material for which the DNA was sequenced

Parasite species and stage	Host species	Geographical origin	GenBank Accession No.	
			5.8S-ITS2-28S	28S
Bunodera luciopercae luciopercae 'adult'	*Perca fluviatilis* (L.)	River Tvertsa (upper Volga River basin, Russia);	FJ874917	GU462123
			GU647218	GU462124
		Lake Segozero (Karelia)	FJ874918	GU462115
B. luciopercae acerinae 'adult'	*Gymnocephalus cernuus* (L.)	River Tvertsa (upper Volga River basin, Russia)	FJ874913	GU462122
		Lake Segozero (Karelia)	FJ874914	GU462114
Allocreadium isoporum 'adult'	*Alburnus alburnus* (L.)	Lake Oster (Karelia)	FJ874921	GU462125
				GU462126
B. luciopercae (*sensu* Wiśniewski, 1958) 'larva'	*Pisidium amnicum* (Müller)	Dammed up River Nemunas near Kaunas (Lithuania)	FJ874910	GU462112
			FJ874911	GU647219
		River Tvertsa (upper Volga River basin, Russia)	FJ874912	GU462113
A. isoporum (*sensu* Wiśniewski, 1958) 'larva'	*Sphaerium rivicola* (Lamarck)	Dammed up River Nemunas near Kaunas (Lithuania)	FJ874916	GU462116
		River Teterev (Dnieper River basin, Ukraine)	FJ874915	GU462111
Crepidostomum sp. 'larva'	*Sphaerium corneum* (L.)	River Belka (Dnieper River basin, Ukraine)	FJ874919	GU462121

microscope slides with TBE buffer. The homogenate was collected and incubated at 95°C for 10 min and placed on ice for 3 min, and then centrifuged at 12,000 g for 5 min using an Eppendorph 5415R. Subsequently, the supernatant with total DNA was transferred to a fresh tube and stored at −20°C.

An entire nuclear 5.8S-ITS2-28S DNA sequence of ribosomal RNA (~460bps: 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence) was amplified using primers: 3S (5′-CGG TGG ATC ACT CGG CTC GTG -3′), forward direction; 28S (5′- CCT GGT TAG TTT CTT TTC CTC CGC -3′), reverse direction (Bowles et al., 1995). The 5′ end of the 28S rRNA gene sequence (~1,230 bps), not overlapping with the previous sequence, was amplified using two primers: Digl2 (5′- AAG CAT ATC ACT AAG CGG -3′) forward direction; L0 (5′- GCT ATC CTG AG(AG) GAA ACT TCG-3′) reverse (Tkach et al., 1999). Additionally, two newly designed primers were used to check and cover flaws in the 28S rRNA gene sequences: Alloc28F (5′- ATG CGC GTT GCT CTT ATC AA -3′), forward direction; Alloc28R (5′- GTT TCA AGA CGG GTC AGG TG -3′), reverse direction. DNA fragments were amplified via a standard Polymerase Chain Reaction (PCR) using Taq DNA polymerase (Fermentas Inc.) in an Eppendorf Master Gradient thermal cycler under the following conditions: 36 cycles of 95°C, 30 sec; 53°C, 1 min; 72°C, 2 min. All PCR reactions included negative controls to detect possible contamination. The samples were sequenced in both directions at Macrogen (Seoul, Korea). Sequence confirmation was accomplished by comparing complimentary DNA strands. Editing of the DNA sequences, contig assembly and the alignment of the consensus sequences were carried out using the software program Sequencher 4.7 (Gene Codes Corporation). Sequences have been deposited to GenBank (Table 1). Sequences of the following 12 allocreadiid species were downloaded from GenBank and included in the phylogenetic analysis: *Bunodera luciopercae* (DQ029331), *B. sacculata* Van Cleave & Mueller, 1932 (DQ029333), *B. inconstans* (Lasee, Font & Sutherland, 1988) (DQ029330), *B. mediovitellata* Tsimbaluk & Roitman, 1966 (EF202573, DQ029332), *Megalogonia ictaluri* Surber, 1928 (EF032694), *Wallinia chavarriae* Choudhury, Daverdin & Brooks, 2002 (DQ991918), *Crepidostomum cornutum* (Osborne, 1903) (EF032695), *C. cooperi*

Hopkins, 1931 (DQ029328) and *Allocreadium lobatum* Wallin, 1909 (EF032693, DQ029327). *Phyllodistomum folium* (Olfers, 1816) (AF533015, AY288828), *Polylekithum catahoulensis* Curran, Tkach & Overstrett, 2006 (EF032698) and *P. ictaluri* (Pearse, 1924) (EF032697) were included as outgroups in this study. Gorgoderid trematodes exhibit a close association with allocreadiids in molecular phylogenies and also use sphaeriid bivalves as intermediate hosts. *Polylekithum* spp. have been classified within the Allocreadiidae, but recent molecular analyses have showed that they are not allocreadiids (Curran et al., 2006).

The ribosomal RNA sequence datasets were analysed phylogenetically using MEGA version 4 (Tamura et al., 2007). The genetic distances were calculated using the Maximum Composite Likelihood method (Tamura et al., 2004), and the phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987).

Results

Sequences from two different regions of rDNA were used for phylogenetic analyses: the 5.8S-ITS2-28S and the 5′ end of the 28S gene, which does not overlap with the previous sequence. PCR amplification of 5.8S-ITS2-28S rDNA and subsequent sequencing of amplified fragments gives a single product, which varies in size from 446 to 452 bp, depending on the isolate (GenBank accession numbers are presented in Table 1). Alignment of these sequences revealed that complete sequences of ITS2 are more variable and have different lengths: 287 bp for *Bunodera l. acerinae* (parasitic in *Gymnocephalus cernuus*) and 'larval' *B. luciopercae* (obtained from *Pisidium amnicum* (Müller)); 286 bp for *B. l. luciopercae* (parasitic in *Perca fluviatilis* from the River Tvertsa and Lake Segozero); 285 bp for a second isolate of *B. l. luciopercae* (parasitic in *P. fluviatilis* from the River Tvertsa) and 'larval' *Allocreadium isoporum* (*sensu* Wiśniewski, 1958); and 283 bp for *A. isoporum* (parasitic in *Alburnus alburnus* from Lake Oster) and *Crepidostomum* sp. cercariae. The ITS2 sequence alignment comprises 51 variable (17.77% of 287 sites) and 42 (14.63%) parsimony informative sites. In contrast, 5.8S gene sequences (128 bps) are homogeneous for all isolates. A high level of variation occurs in the short sequence (37 bps) at the beginning of the 28S gene: 8 variable (21.62%) and 3 parsimony informative sites (8.11%) were detected. Blast searches performed on these sequences demonstrated the highest matches with other digenean trematode sequences. Neighbour-joining analyses of these sequences gave a tree with three major clades within the ingroup taxa (Fig. 1). The first clade is formed by identical 5.8S-ITS2-28S rDNA sequences of 'adult' *B. l. acerinae* and 'larval' *B. luciopercae*. The sequences of 'adult' specimens isolated from *G. cernuus*, originating from geographically distant populations (Table 1), differ in just a single base transversion. The second clade comprises two branches: one formed by identical 5.8S-ITS2-28S rDNA sequences of 'adult' *B. l. luciopercae* (from Lake Segozero and the River Tvertsa); and the other by identical sequences of 'adult' *B. l. luciopercae* (from the same locality of the River Tvertsa) and 'larval' *A. isoporum* (*sensu* Wiśniewski, 1958). Pairwise comparison of these *B. l. luciopercae* sequences revealed that there are four base pair differences (0.83% of 480 sites). Sequence analysis of the 5.8S-ITS2-28S rDNA clearly distinguished *B. l. luciopercae* and *B. l. acerinae*, where a difference as great as 3.75 % at this rDNA locus (453 bps) was revealed.

Surprisingly, 'adult' and 'larval' *A. isoporum* are located in different clades. The level of 5.8S-ITS2-28S rDNA sequence divergence between them is high, involving 39 variable sites (8.57% of 455).

The rRNA gene sequences ($\sim$1,230 bps) localised at the 5′ end of the 28S rDNA gene are included in the phylogenetic analysis together with those sequences obtained from GenBank which were not shorter than 812 bps. An alignment of the 28S rDNA dataset comprised 830 characters (including spaces), of which 114 bps (excluding the outgroup) and 308 bps (including the outgroup) are parsimony informative. A phylogenetic tree, constructed using 28S rDNA gene sequences (Fig. 2), has an identical topology to the 5.8S-ITS2-28S rDNA tree described above (Fig. 1).

In an alignment of 10 new sequences of *Bunodera* spp. and *Allocreadium* spp., comprising 1,214 characters (including one space), all 82 variable sites were parsimony informative (6.76%). The 28S rDNA gene sequences (1,213 bps) of 'adult' *B. l. acerinae*, isolated from *G. cernuus*, differ in just a single base

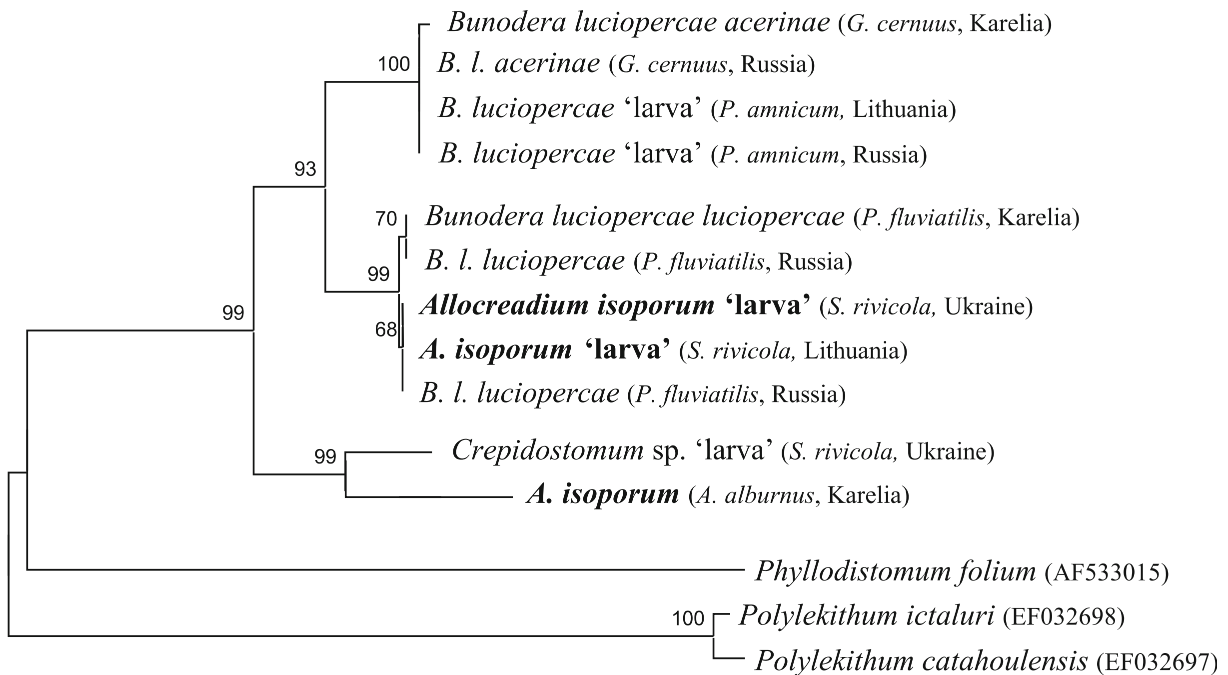

Fig. 1 Phylogenetic tree based on the analysis of 454 sites of ribosomal DNA sequences (5.8S and 28S partial sequences and ITS2 complete sequence). The tree was constructed using the neighbour-joining method (bootstrap reps = 10,000, pairwise deletion, distance method: Maximum Composite Likelihood). Host name and sampling locality are indicated in parentheses following the species name. Accession numbers are indicated for sequences obtained from GenBank. Note the position of 'adult' and 'larval' *Allocredium isoporum* (bold) located in different clades

transversion. Among the *B. l. luciopercae* isolates studied, two different sequences were detected that differ in 5 bps (0.41% of 1,213 bps); one sequence was identical with that of 'larval' *A. isoporum* (*sensu* Wiśniewski, 1958). More differences were detected between the *B. l. acerinae* and *B. l. luciopercae* 28S rDNA sequences (1,213 bps), i.e. 7 (0.58%) or 10 (0.82 %) variable sites, depending on the isolates compared. The alignment of the 28S rDNA sequence (812 bps) of Nearctic *B. luciopercae* (GenBank No. DQ029331) with those of the Palaearctic subspecies revealed more conspicuous differences: eight different sites (0.99%) with *B. l. acerinae* and 12 (1.48%) or 9 (1.11%) with *B. l. luciopercae*, depending on the isolates compared.

The 'adult' *A. isoporum* occurs in a single clade with *A. lobatum* (Fig. 2). The level of 28S rDNA sequence divergence between 'adult' *A. isoporum* and 'larval' *A. isoporum* is high, occurring at as many as 76 sites (6.26% of 1,214 sites, including one space).

Discussion

Species of parasites known to exploit several host species may in reality represent a complex of morphologically similar species with a restricted host-specificity (for examples, see Combes, 2001). *Bunodera luciopercae* (*sensu lato*) follows the typical pattern of a wide definitive host range (Bykhovskaya & Kulakova, 1987; Hoffman, 1999). In the present study, sequence analyses were undertaken in order to provide molecular markers for the discrimination of two host-specific subspecies, *B. l. luciopercae* from *Perca fluviatilis* and *B. l. acerinae* from *Gymnocephalus cernuus*, and to try and link allocreadiid 'adults' with 'larval' stages developing in European sphaeriid bivalves. Previous studies on helminths have shown the ITS to be a sensitive marker for species boundaries, even in cryptic species that are morphologically similar (see examples in Blair, 2006). Nucleotide sequencing of the ITS region is at present the most reliable molecular marker for monogenean species

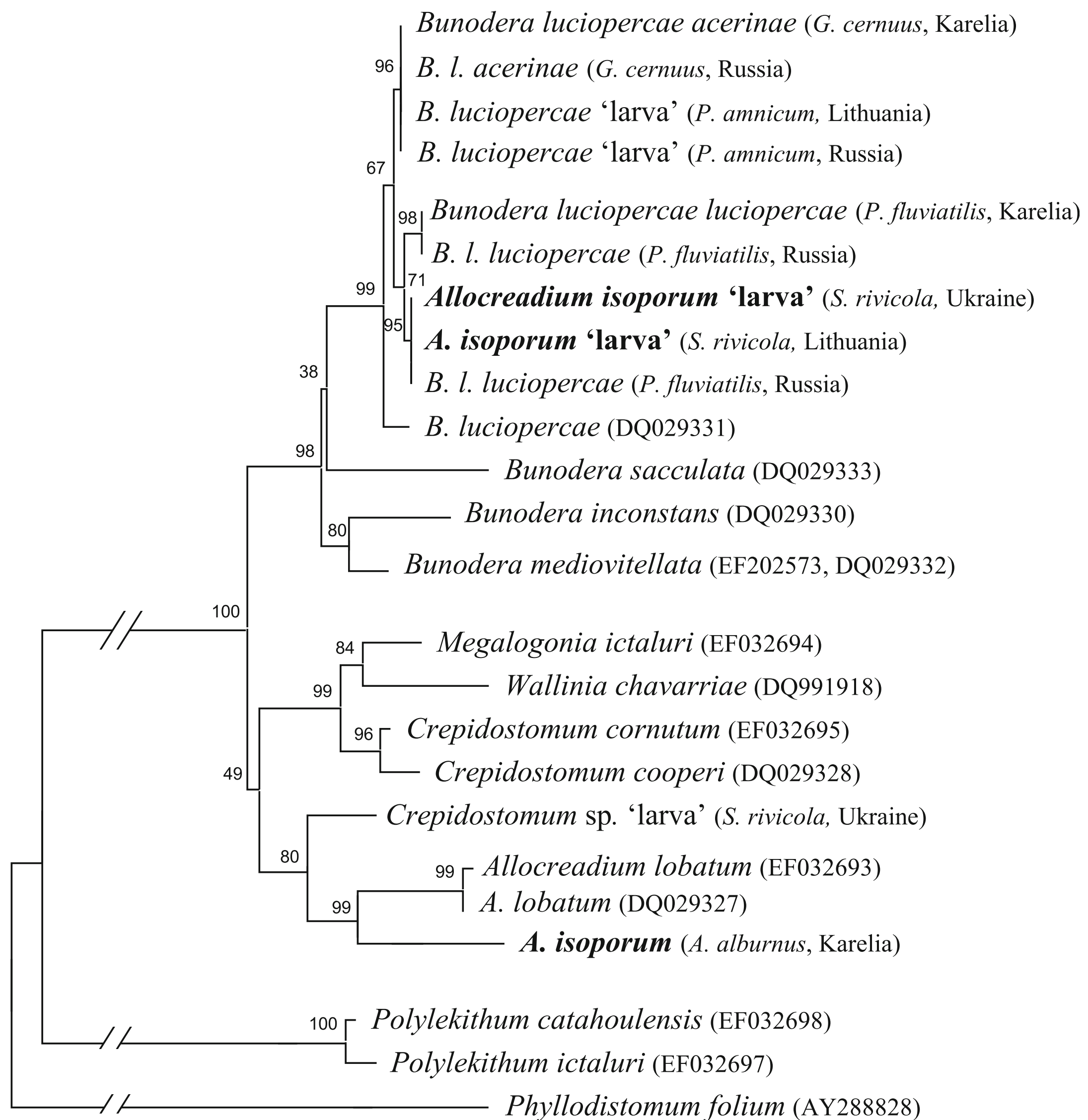

Fig. 2 Phylogenetic tree based on analysis of 796 sites of the 5′ end of the 28S rRNA gene sequence. The tree was constructed using the neighbour-joining method (bootstrap reps = 10,000, pairwise deletion, distance method: Maximum Composite Likelihood). Host name and sampling locality are indicated in parentheses following the species name. Accession numbers are indicated for sequences obtained from GenBank. Note the position of 'adult' and 'larval' *Allocredium isoporum* (bold) located in different clades

(Cunningham & Matejusova, 2006). Nevertheless, the utility of ITS2 sequences for the discrimination of *Ichthyocotylurus* spp. (Digenea: Strigeidae) was found to be limited by extremely high levels of intrageneric conservation within this region, levels which were markedly higher than those previously recorded for other digenean genera (Bell et al., 2001). On the other hand, the identical ITS2 sequences provided strong evidence for the conspecificity of nominal species of *Echinostoma* Rudolphi, 1808 and *Apatemon* Szidat, 1928 (Morgan & Blair, 1995; Bell & Sommerville, 2002). Data of Curran et al. (2006) showed that ITS2 rDNA is the most practical target for a quick differentiation between species of *Polylekithum* Arnold, 1934 (a genus of uncertain systematic position, previously classified within the Allocreadiidae) due to its high variability and short length. Our results are consistent with those who

suggest that the ITS2 may be sufficiently variable to permit discrimination at the species level (Allard et al., 1993; Anderson & Barker 1998; Galazzo et al., 2002). The 28S gene has mainly been studied to infer the phylogeny of various parasitic groups, and for this purpose partial sequences of this gene are sufficient. The 28S region has been more widely employed in allocreadiid studies and now provides the most diverse reference database of molecular characters. Intraspecific variation is slight or non-existent in the 28S genes of parasitic flatworms (Blair, 2006).

The results of this study have shed new light on the diversity of well-known fish parasites. The phylogenetic trees produced by neighbour-joining analysis have given statistical support for the existence of distinct genetic species within the nominal species *B. lucioperca*e.

Sequence analyses of the ITS2 and 28S clearly distinguish 'adult' *B. l. lucioperca*e and *B. l. acerinae*, differences as large as 3.75 % and 1.11 %, respectively, suggesting a fundamental separation of these two taxa. The divergence showed between them greatly exceeds that suggested by Jousson et al. (1999) as maximal (1.5%) for intraspecific variation in the ITS sequences of trematodes. It is also worth noting that Ziętara & Lumme (2002) proposed a guide of 1% variation in the ITS sequences for the separation of *Gyrodactylus* spp. (Monogenea). Several intraspecific point-mutations were observed for *B. l. lucioperca*e samples isolated from *P. fluviatalis* from geographically distant populations, suggesting that intraspecific variation exists in the ITS2 rDNA region of allocreadiids. Single base substitution between geographically distant isolates was also recorded for *B. l. acerinae* specimens obtained from *G. cernuus*. The 28S was comparatively less variable, although an intraspecific difference at one site was revealed in the isolates of *B. l. lucioperca*e coming from the same locality.

Nearctic *B. lucioperca*e (DQ029331) is closely related to the Palaearctic subspecies, but formed a separate branch in the 28S tree (Fig. 2). The sequence difference reached 1.48% of 812 bps. Hence, the statement of Cannon (1971) that *B. lucioperca*e is a single Holarctic species cannot be supported. Considering the molecular results, differences in the life-cycle (e.g. cercarial morphology; second intermediate hosts range) and discrepancies in karyological results (see Petkevičiūtė & Stanevičiūtė, 2008), *B. lucioperca*e from *Perca flavescens* may represent a cryptic species.

The most obvious diagnostic application of gene sequences is in the identification of 'larval' forms and the elucidation of complex life-cycles. Our knowledge of the life-history data for allocreadiid species is quite limited. The affiliation between the 'larval' and 'adult' stages living in different organisms may be difficult to establish, so it is hardly surprising that, to date, the life-cycles of only two European allocreadiids are known from experimental infections. The development of *B. lucioperca*e was elucidated by Wiśniewski (1958) and that of *Crepidostomum farionis* by Brown (1927). It is notable, that Wiśniewski (1958) used *Gymnocephalus cernuus* (as *Acerina cernua*) for his experimental infections. So, it is not surprising that identical ITS2 and 28S sequences were revealed for 'adult' *B. l. acerinae* from *G. cernuus* and 'larval' *B. lucioperca*e identified using Wiśniewski's description. Such a result provides strong evidence that these are life-cycle stages of one and the same species.

'Larval' stages of *A. isoporum* have been known for more than a century. Looss (1894) and Dollfus (1949), basing their conclusions on morphological similarities and ecological evidence, identified an ophthalmoxiphidiocercaria developing in sphaeriid bivalves as a larval stage of *A. isoporum*. Although Wiśniewski (1958, p. 304) indicated that the "development cycle has not been determined yet", he supported Looss' supposition on the basis of ecological evidence. Subsequently, other authors presented their own descriptions of the cercaria of *A. isoporum* (see Frolova 1975; Chernogorenko 1983). They noted slight differences in cercarial morphology and pointed out the possibility that the cercariae of *A. isoporum* described by different authors might represent different species, but by common assent the name *A. isoporum* was used for all of these cercariae with eye-spots, stylet and the tail surrounded by a broad tegumental inflation and developing within rediae in *Sphaerium* or *Pisidium* spp. It should be emphasised that it is not possible to link the 'larval' stages to an 'adult' fluke based solely on morphological characters and ecological indications. Moreover, the small body size, lack of hard parts and overall body plasticity make the identification of systematically useful characteristics in cercariae a difficult task. Specifically, the number of characteristics visible via the standard light microscopy of live animals is limited and measurements are extremely variable in both living and preserved specimens.

However, difficulties in our understanding of trematode life-cycles may be overcome through the use of molecular techniques. The most surprising result of our molecular analyses was the finding that the ITS2 and 28S sequences of 'larval' *A. isoporum* (*sensu* Wiśniewski, 1958), occurring in *Sphaerium rivicola*, were identical to those of 'adult' *B. l. luciopercae* from *P. fluviatilis*. This exact match and the positions in the phylogenetic trees (Figs. 1, 2) strongly suggests that the parthenitae identified as *A. isoporum,* following descriptions of Wiśniewski (1958), and 'adult' *B. l. luciopercae* represent one and the same species. 'Adult' *A. isoporum* obtained from *Alburnus alburnus* differ significantly from 'larval' specimens supposed to represent the same species, the level of 5.8S-ITS2-28S sequence divergence (8.5%) and 28S divergence (6.26%) between them being consistent with the expected level for intergeneric variation.

It is notable that the chromosome sets of 'larval' allocreadiids involved in the present study have previously been described (Petkevičiūtė & Stanevičiūtė, 2008). Comparative study revealed a close karyological affinity between 'larval' *B. luciopercae* and *A. isoporum* (*sensu* Wiśniewski, 1958). Both karyotypes contains the same chromosome number, $2n = 14$, and differ mainly in the centromere position of only one chromosome pair (No. 3). A high degree of similarity between the karyotypes suggests a close phylogenetic relationship, and the differences revealed can be regarded as intrageneric. So, both karyological and molecular analyses confirm the possible placement of both 'larvae' within the genus *Bunodera*. Morphologically, cercariae of *A. isoporum* and *B. lucioperae* are closely related. Wiśniewski (1958) pointed out that the glands of the ventral sucker of his *A. isoporum* cercariae were less developed and less visible than in *B. luciopercae* cercariae; however, the main difference is in the structure of the tail. In the case of *A. isoporum,* the cercarial tail has a broad, dorsoventral tegumentary inflation. However, cercarial tails as phylogenetic indicators can be very misleading (Cable 1965); in the Allocreadiidae there can be a pronounced variation in the structure of the cercarial tail in members of the same genus (Madhavi, 1980).

'Larval' *Crepidostomum* sp. differ more distinctly in their chromosome set structure, i.e. $2n = 12$ (Petkevičiūtė & Stanevičiūtė, 2008), and form a separate branch in the molecular trees for allocreadiids (Figs. 1, 2). There was no match for this 'larva' with any adult allocreadiid, but, hopefully, with the accumulation of sequence data for 'adult' forms, identification of this 'larva' will be possible.

Our molecular data, together with previously determined differences in morphology, RAPD profiles and host-specificity (Sokolov et al., 2006), represent strong evidence that the subspecies of *B. luciopercae* represent distinct species.

Based on our results, it is evident that both ITS2 and 28S sequences can serve both for the construction of phylogenetic trees and for the clarification of species relationships between allocreadiids, as well as for analyses of their life-cycles. However, the ITS2 is shorter and more variable and, hence, more practical for species identification. The results of the present study also revealed the need for reconsidering some of the accepted but largely untested tenets regarding allocreadiid life-cycles, and, specifically, have shown that the life-cycle of *A. isoporum* has yet to be elucidated.

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