Cytogenetic and sequence comparison of adult *Phyllodistomum* (Digenea: Gorgoderidae) from the three-spined stickleback with larvae from two bivalves

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SUMMARY

Due to the low informative value of available morphological characters, cytogenetic and molecular methods, based on rDNA sequencing, were used to characterize adult and larval stages of *Phyllodistomum* spp. Species studied have 18 chromosomes with comparable absolute and relative lengths. Conventional Giemsa staining and karyometric analysis revealed clear differences in chromosome morphology of larval *Phyllodistomum* spp. infecting two bivalve host species, *Sphaerium corneum* and *Pisidium amnicum*. However, karyotypes of adult *P. folium* from three-spined sticklebacks and larval stages from *S. corneum* appear almost identical both with respect to the relative lengths and centromeric indices of the corresponding chromosome pairs. The entire internal transcribed spacer (ITS) region (ITS-1, 5.8S and ITS-2) and the D1-D3 region of 28S gene were sequenced and compared. Again, sufficient differences were observed between larval *Phyllodistomum* spp., while adult *P. folium* and larvae from *S. corneum* showed a high level of similarity. So, both cytogenetic and molecular data support the suggestion that they represent developmental stages of the same species. The results were compared with published data obtained by cytogenetic and molecular studies on the other *Phyllodistomum* species. Differences revealed in karyotype and rDNA sequences leads to the conclusion that the cercariaeum of *P. folium* sensu Sinitsin, 1905 could not be regarded as the larva of adult *P. folium* from three-spined stickleback.

Key words: *Phyllodistomum*, Trematoda, karyotype, chromosomes, ribosomal DNA, life-cycles.

INTRODUCTION

There is considerable taxonomic confusion within the genus *Phyllodistomum* Braun, 1899. The type species, *Phyllodistomum folium*, was described by Olfers (1817) (cit. from Sinitsin, 1905) based on specimens recovered from pike (*Esox lucius* L.) collected in Europe. Many of the phyllodistomes found by subsequent workers in the bladders and urinary ducts of various fishes have been referred to this species. This fact has created the extremely difficult taxonomic problem of attempting, with merged data, the separation and identification of valid species. Sexually mature phyllodistomes have few distinctive qualitative differences and exhibit a considerable intraspecific variation in most morphological characters, including those previously used for species differentiation (Koval, 1978; Kudinova, 1994). A study of the figures presented as *P. folium* in the literature (see Lewis, 1935; Dawes, 1946; Pigulewsky, 1953; Kozicka, 1959; Koval, 1978; Bykhovskaya-Pavlovskaya & Kulakova, 1987) clearly indicate, that either the species is extremely variable (statement of Kudinova, 1994 and Dugarov, 2000) or, more plausibly, the older taxa consist of assemblages of numerous species.

A wide range of host fishes is recorded for *P. folium*, and obvious disagreement exists regarding the degree of specificity of this species. According to Bykhovskaya-Pavlovskaya & Kulakova (1987) this parasite may infect cyprinid, esoxid, percid, salmonid, silurid and other fish. Pigulewsky (1953), on the other hand, stated that pike is the only host of *P. folium*. According to Chappell (1969), Kennedy (1974) and Nie & Kennedy (1992), *P. folium* is mainly a parasite of three-spined sticklebacks, *Gasterosteus aculeatus* L.

The life-cycles so far reported for *Phyllodistomum* spp., have varied greatly both in larval structures and typical sequences. Species for which life-cycles are known utilize bivalves (e.g. Sphaeriidae, Unionidae, and Dreissenidae) as first intermediate hosts. Cercaria *P. folium*, a cercariaeum, was described by Sinitsin (1905) from *Dreissena polymorpha*. Zdun *et al*. (1994) have provided a more detailed description of developmental stages of *P. folium*.

Following the discovery of *P. folium* in the urinary bladder of three-spined sticklebacks in Vilnelė River in Vilnius, we investigated the molluscan fauna at this station. The only bivalve molluscs, known as potential hosts of phyllodistomes, were *Sphaerium corneum* (Linnaeus, 1758); *Dreissena* spp. were

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completely absent. Dissection of several hundreds of specimens revealed that *S. corneum* was harbouring cercariae of the gorgoderid kind. The cercariae were of a macrocercous, cystocercous type and closely resembled those of *P. elongatum* described by Zhokhov (1987). The other gorgoderid cercaria, used in a comparative study, was found to infect *Pisidium amnicum* (O. F. Müller, 1774) from a Lithuanian power station reservoir in Elektrėnai. This cercaria with a filiform, notatory tail was described by Ginetzinskaya (1959) as *Phyllodistomum* sp. from the same intermediate host collected in the Rybinsk water reservoir (Russia).

Generally, in order to identify phyllodistomes, a description of the entire life-cycle, with developmental stages needs to be obtained. This is extremely difficult in practice and contributes to the taxonomic confusion of the group. Because morphological characterization of *Phyllodistomum* spp. provides a limited number of criteria for species determination, better comparative markers are needed to distinguish species and to identify developmental stages. This study aims to characterize *Phyllodistomum* species using various genetic methods, including cytogenetics and rDNA analysis and to compare their distinctive capacity. Cytogenetic parameters such as chromosome number and morphology are of undoubted taxonomic importance and provide investigative tools for definition and differentiation of biological species. To distinguish among morphologically similar species a variety of molecular markers, including nucleotide sequences, are increasingly being used (Blair, Bray & Barker, 1998; Morgan & Blair, 1998; Dvorak *et al*. 2002). Ribosomal DNA genes and ITS sequences, which are localized between ribosomal genes are widely used on all trematode taxonomic levels because they consist of regions displaying different degrees of variability. This study is the first attempt to characterize genetically the developmental stages of *Phyllodistomum* in order to establish the affiliation between larvae ant their respective adults.

MATERIALS AND METHODS

Collection of specimens

Specimens of *P. folium* were recovered from the urinary bladders of three-spined sticklebacks caught in the Vilnelė River in Vilnius in June 2002. After capture, fishes were transported alive to the laboratory and dissected within the following 48 h. Living trematodes were recovered from the urinary bladder and placed into a saline (0·65% NaCl). Specimens were identified *in vivo* according to the key of Bykhovskaya-Pavlovskaya & Kulakova (1987). Seven specimens of *Sphaerium corneum* naturally infected with *Phyllodistomum* sp. were collected in the Vilnelė River (in the same place where the infected fish were caught); 5 specimens of naturally infected *Pisidium amnicum* were collected in the water reservoir of the Lithuanian Power station in Elektrėnai in June–August, 2002. Larval phyllodistomes were identified using morphological features of the cercariae.

Chromosome preparations

For cytogenetic studies, a total of 16 live, complete adult specimens of different size and maturity were placed into 0·01% colchicine (Aldrich Chemical Co.) solution for 3–4 h at room temperature, then transferred to distilled water for 30–40 min for hypotony, fixed in freshly prepared ethanol-acetic acid (3 : 1) and stored at 4 °C. For slide preparations, fixed worms were stained with aceto-orsein for 12–14 h, briefly soaked in 45% acetic acid and squashed under a cover-slip. Squashes were sealed with Canada balsam and examined for cell divisions. The best mitotic plates were photographed under an oil-immersion system.

Chromosome preparations of larval stages of *Phyllodistomum* spp. were made from cells of parthenitae obtained from naturally infected specimens of sphaeriid molluscs. Before dissection, sphaeriids were treated with 0·01% colchicine in well water for 12–14 h. They were dissected and the tissues containing trematode larvae removed and treated with distilled water for 30 min for hypotony. The material was fixed in ethanol–acetic acid (3 : 1). Microscope preparations were made using the air-dried method (Petkevičiūtė & Stanevičiūtė, 1999), and stained with 4% Giemsa solution in Sörensen's buffer (pH 6·8) for 30–40 min.

Measurements (absolute length in micrometers, relative length in percent, and centromeric indices) are given as mean values and standard deviations (S.D.). Measurements are based on all chromosomes from 10 metaphase spreads for each species. The classification of chromosomes followed that of Levan, Fredga & Sandberg (1964). Data were analysed by the Student's *t*-test. Results were considered significant when $P < 0{\cdot}05$.

DNA isolation, PCR amplification and sequencing

Adult and larval stages (sporocysts and sporocyst-infected tissues) of *Phyllodistomum* spp. were fixed in 70% ethanol and stored at −20 °C before genomic nucleic acid extraction. Genomic DNA was extracted using either DNA/RNA Isolation Kits (Amersham Life Sciences, Inc., Cleveland, OH) or FastDNA extractions kits (Qbiogene Inc., Carlsbad, CA) and was stored at −80 °C in purified water.

Oligonucleotide primers were used to amplify DNA fragments localized at the 5′ end of 28S gene and the ITS regions (Fig. 1). Main parts of DNA sequences were amplified with primers, which are

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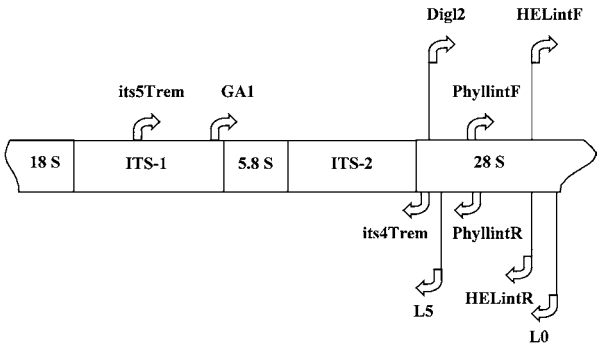

Fig. 1. Localization and direction of rDNA primers.

universal for Digenea. To amplify the 5′ end of the 28S gene the following were used: Digl2 (AAGCATATCACTAAGCGG), forward direction (Tkach, Pawlowski & Mariaux, 2000); L0 (GCTATCCTGAG(AG)GAAACTTCG), reverse (Tkach *et al*. 2000). To amplify the ITS1, 5.8S and ITS2 regions: L5 (TTCACTCGCCATTACT), reverse direction (Jousson, Bartoli & Pawlowski, 1999); GA1 (AGAACATCGACATCTTGAAC), forward (Cribb *et al*. 1998); its5Trem (GGAAGTAAAAGTCGTAACAAGG), forward (Dvorak *et al*. 2002); its4Trem (TCCTCCGCTTATTGATATGC), reverse (Dvorak *et al*. 2002). Additionally newly designed primers (Stunžėnas, Cryan & Molloy, 2004), together with universal primers, were used to check and cover flaws in DNA sequences, which were obtained with universal primers. To amplify ribosomal DNA fragments localized at the 5′ end of 28S gene: PhyllintF (TGCGCCTCGGTTGTTTAT), forward direction; PhyllintR (ATAAACAACCGAGGCGCA), reverse; HELintF (AGTAACATGTGCGCGAGT), forward; HELintR (ACTCGCGCACATGTTACT), reverse.

DNA fragments were amplified *via a* standard Polymerase Chain Reaction (PCR) using *Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA) under the following conditions: 36 cycles of 94 °C, 30 s; 53 °C, 1 min; 72 °C, 2 min. All PCR reactions included negative controls to detect possible contamination. Double-stranded PCR products were visualized on 1–2% agarose gels, purified using Geneclean III DNA Purification Kits (Bio101, Vista, CA), directly sequenced with Big Dye Version 3 Cycle Sequencing Ready Reaction with AmpliTaqFS DNA Polymerase (PE Applied Biosystems, Foster City, CA), and fractionated by polymer capillary electrophoresis on either a Prism 3700 DNA Analyzer or a Prism 3100 Genetic Analyzer (PD Applied Bio systems, Foster City, CA).

Sequence confirmation was accomplished by comparing complimentary DNA strands. Editing of nucleotide sequences, contig assembly, and manual alignment of consensus sequences were performed using the software program Sequencher 4.0.5 (Gene Codes Corp., Ann Arbor, MI) for PC. Complete nucleotide sequences are available in GenBank under accession numbers AY281126, AY277703, AY277704, AY277705, AY277706 and AY277707. Phyllodistome sequence identity matrixes, were calculated with BioEdit software version 5.0.6.

RESULTS

A diploid complement of 2n = 18 was found in 32 dividing cells (out of 32 studied) of adult *P. folium*. Mitotic metaphases were mainly observed in early stages of developing embryos in eggs of maturing trematodes (body length > 0·8 mm, colourless eggs). The chromosomes were medium sized and ranged in length from 1·72 to 5·92 μm (Table 1). The mean total length of the haploid complement was 29·57 μm. The two first pairs of homologues were larger than the remaining chromosome pairs, which gradually decrease in size (Table 1, Fig. 2). They contribute approximately 20% and 18% to the mean total chromosome length. Chromosomes with terminally and subterminally located centromeres prevailed in the karyotype. According to the centromere position, chromosome pairs 1 and 2 were acrocentric, pairs 3, 4, 5, 6, 7 and 8 were subtelocentric and pair 9 was metacentric. It is notable that the individual identification of the homologues of pairs 6 and 7 was difficult due to the similarities in size and shape.

Examination of 79 mitotic metaphase plates of larval intramolluscan stages of *Phyllodistomum* sp. from *S. corneum* revealed the same modal diploid number, 2n = 18 (Fig. 2). Eight (9%) aneuploid spreads displaying a chromosome number lower than the mode (2n = 16, 17) were also encountered. Part of these might be related to technical shortcomings. Two (3%) tetraploid cells (4n = 36) were found. Measurements of absolute length gave values from 2·03 to 6·32 μm (Table 1). The total length of the haploid genome was 33·04 μm. Comparative studies revealed no significant ($P < 0{\cdot}05$) differences between the karyotypes of these parthenithas and adult *P. folium*, described above. They were identical both with respect to the relative chromosome length and centromeric indices of the corresponding chromosome pairs. It is notable that even absolute chromosome length, the most variable characteristic of chromosome set, depending also on method of analysis applied, revealed no significant differences.

The chromosomes of 58 cells of larvae from *P. amnicum* were counted. Most cells (94%) had 2n = 18 (Fig. 3). The remainder of the metaphase plates (6%) were aneuploid, with chromosome numbers lower than the modal. The absolute length of chromosomes ranged from 1·57 to 6·02 μm (Table 2). The mean total length of the haploid genome was 29·57 μm. Comparison of the relative length of corresponding chromosome pairs revealed no statistically significant differences with the karyotypes described above. The most distinct interspecific differences were observed

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Table 1. Measurements (means ± S.D.) and classification of chromosomes of *Phyllodistomum* spp.

(Abbreviations: A, adult *P. folium*; L, larvae from *S. corneum*; m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric chromosomes.)

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Classification
1 A	5·92 ± 1·96	20·91 ± 1·51	10·36 ± 3·04	a
L	6·32 ± 0·99	19·08 ± 1·18	10·82 ± 1·70	a
2 A	4·98 ± 1·72	17·61 ± 1·47	10·36 ± 3·37	a
L	5·62 ± 0·78	16·99 ± 0·75	10·30 ± 1·78	a
3 A	3·56 ± 1·06	12·67 ± 0·59	11·62 ± 3·50	a-st
L	4·28 ± 0·55	12·95 ± 0·57	14·58 ± 2·86	st
4 A	3·12 ± 0·88	11·11 ± 0·50	15·43 ± 4·37	st
L	3·78 ± 0·57	11·43 ± 0·79	18·93 ± 3·69	st
5 A	2·64 ± 0·60	9·54 ± 0·47	17·31 ± 3·75	st
L	3·32 ± 0·56	10·01 ± 0·66	19·26 ± 3·57	st
6 A	2·20 ± 0·50	7·95 ± 1·09	23·86 ± 2·37	st-sm
L	2·90 ± 0·37	8·78 ± 0·42	22·96 ± 1·65	st
7 A	2·05 ± 0·50	7·44 ± 0·91	21·06 ± 3·23	st
L	2·57 ± 0·21	7·81 ± 0·64	21·76 ± 1·77	st
8 A	1·77 ± 0·37	6·42 ± 0·60	19·41 ± 4·98	st
L	2·22 ± 0·19	6·75 ± 0·59	21·01 ± 1·68	st
9 A	1·72 ± 0·27	6·32 ± 0·98	40·59 ± 1·27	m
L	2·03 ± 0·15	6·18 ± 0·61	42·69 ± 2·04	m

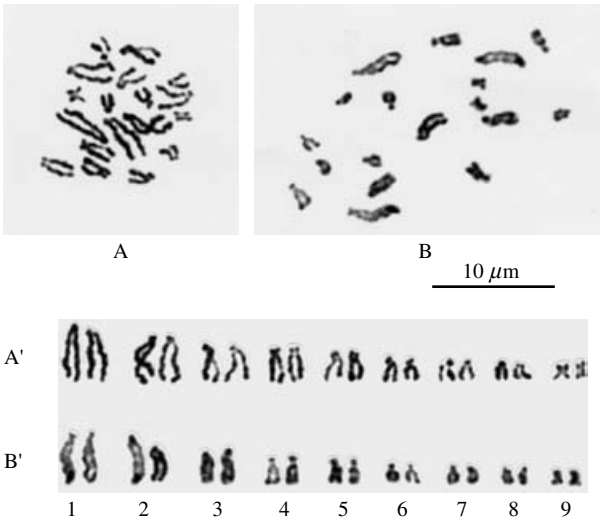

Fig. 2. Mitotic chromosomes of *Phyllodistomum* spp. (A, A′) Mitotic metaphase and karyotype of intramolluscan larval stages of *Phyllodistomum* sp. from its host, *Sphaerium corneum*; (B, B′) mitotic metaphase and karyotype of adults of *P. folium* from its host, *Gasterosteus aculeatus*.

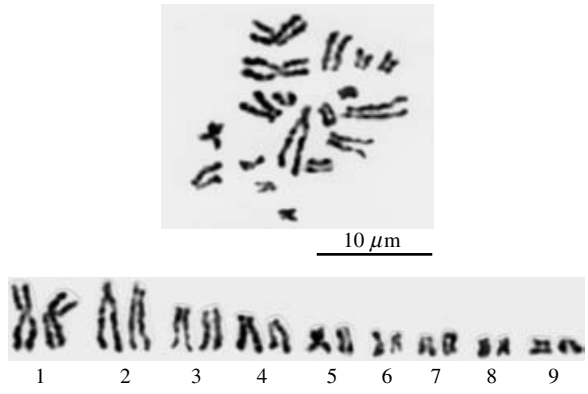

Fig. 3. Mitotic metaphase and karyotype of *Phyllodistomum* sp. from its host, *Pisidium amnicum*.

in the centromeric index values of chromosomes pairs 1, 5, 6,7 and 8. These chromosome pairs are all biarmed, metacentric or submetacentric, in the karyotype of *Phyllodistomum* sp. larvae from *P. amnicum* but are acrocentric or subtelocentric in the other two karyotypes, described above. The best cytogenetic marker appears to be the first pair of large chromosomes, which are clearly metacentric in the karyotype of larvae from *P. amnicum*, but possess a terminally located centromere in the cells of adult *P. folium* and parthenithas from *S. corneum*.

In order to visualize the existing interspecific differences better, idiograms of chromosome sets of studied *Phyllodistomum* spp. were constructed based on the values of relative lengths and centromeric indices (Fig. 4).

To develop the additional argument in support of the hypothesis that cystocercous cercaria from *S. corneum* correspond with adult *P. folium*, we sequenced internal transcribed spacer ITS1-ITS2 and 28S ribosomal RNA (rRNA) genes of adult and larval stages of phyllodistomes. The internal transcribed spacer partial ITS1, 5.8S gene, partial ITS2 and beginning of 28S ribosomal RNA (rRNA) gene of adult *P. folium* from *G. aculeatus* 827 bps (GenBank number AY277705) and 1250 bps (AY277707), respectively; larval stages of phyllodistomes from *P. amnicum* – 718 bps (AY277703) and 1295 bps (AY281126), *S. corneum* 727 bps (AY277704) and 1217 bps (AY277706) were sequenced.

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Table 2. Measurements (means ± S.D.) and classification of chromosomes of *Phyllodistomum* sp. from *Pisidium amnicum*

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric indices	Classification*
1	6·02 ± 1·37	20·25 ± 1·48	43·24 ± 2·20	m
2	5·87 ± 1·03	19·94 ± 1·12	10·09 ± 1·87	a
3	4·03 ± 0·75	13·67 ± 0·76	12·87 ± 5·62	st
4	3·45 ± 0·79	11·61 ± 0·77	13·21 ± 4·72	st
5	2·66 ± 0·48	9·05 ± 0·80	41·21 ± 2·41	m
6	2·26 ± 0·48	7·29 ± 0·83	37·45 ± 4·14	sm-m
7	1·95 ± 0·36	6·61 ± 0·45	32·71 ± 4·40	sm
8	1·76 ± 0·35	5·95 ± 0·42	38·22 ± 4·39	m
9	1·57 ± 0·36	5·29 ± 0·38	29·06 ± 4·71	sm

* See Table 1 for abbreviations.

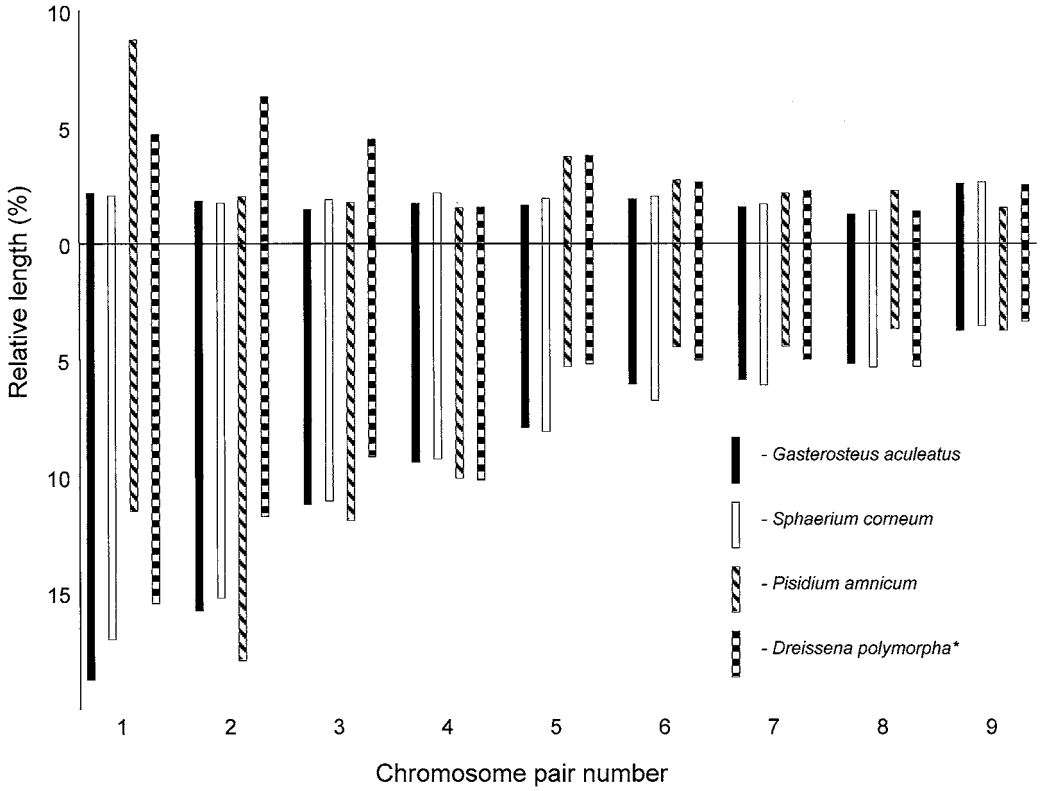

Fig. 4. Idiograms representing the haploid sets of *Phyllodistomum* spp. from different host species. * Previously published data of Petkevičiūtė *et al.* (2003).

The sequences of *Phyllodistomum* spp. were determined and compared with each other. For ITS1-ITS2 sequences, comparisons of 714 bps of sequences of *Phyllodistomum* spp. from *S. corneum* and *G. aculeatus*, and 710 bps of sequence of phyllodistome from *P. amnicum* were utilized. No differences were found between phyllodistomes from *S. corneum* and *G. aculeatus*, however, these sequences differ from sequences of phyllodistome from *P. amnicum* at 154 positions (21·2%).

For the D1-D3 region of 28S rDNA gene, 1173 bps from *Phyllodistomum* spp. from *S. corneum* and *G. aculeatus*, and 1176 bps from *P. amnicum* phyllodistome were compared. Again, no differences were noted between the sequences derived from specimens from *S. corneum* and *G. aculeatus*. These sequences differed from that from the *P. amnicum* phyllodistome at 127 positions (10·8%).

The same ribosomal gene regions were compared with rDNA sequence (Stunžėnas *et al.* 2004) of *P. folium sensu* Sinitsin, 1905 (AF533015) from *Dreissena polymorpha*. This sequence has 127 bps different from D1-D3 region sequence of *Phyllodistomum* sp. from *S. corneum* and *G. aculeatus*, 36 bps different from D1-D3 region sequence of *Phyllodistomum* sp. from *P. amnicum*, as well as 160 bps different from ITS1-ITS2 sequence of *Phyllodistomum* sp. from *S. corneum* and *G. aculeatus*, and 48 bps different

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Table 3. Phyllodistome sequence identity matrixes

ITS1-ITS2 sequence (727 bp)	*P. amnicum* (17*)	*D. polymorpha* (16*)
G. aculeatus (13*)	0·768	0·757
P. amnicum (17*)	1·000	0·932
28S sequence (1178 bp)	*P. amnicum* (2*)	*D. polymorpha* (1*)
G. aculeatus (5*)	0·887	0·891
P. amnicum (2*)	1·000	0·969

* Number of gaps of the sequence in the alignment.

from ITS1-ITS2 sequence of *Phyllodistomum* sp. from *P. amnicum*.

DISCUSSION

Comparison of the karyotypes of closely related morphologically similar species, in the context of a wider study, might throw light on their relationships, as well as on their systematic position in the genus. We also were interested in detecting differences, which might serve to distinguish the species and provide a basis for further analysis of *Phyllodistomum* life-cycles and for evaluation of validity of species.

Although the genus *Phyllodistomum* is large, containing more that 110 species (Kudinova, 1994), chromosomal data are limited, with information available for only 4 species. In an early study, Dhingra (1954) found 8 chromosomes in haploid complements of *Phyllodistomum spatula*. No data on chromosome morphology were presented. Studies on colchicine-treated, air-dried material have provided more detailed and more accurate karyological information on *P. conostomum*, 2n = 16, and *P. pungitii*, 2n = 18 (Orlovskaya, Atrashkevich & Barshene, 1995). The chromosome set of *P. conostomum* with 2n = 16 and one pair of large metacentric elements presumably arose from a karyotype with 2n = 18 by means of centromeric fusion of two acrocentric non-homologous chromosomes (Robertsonian translocation). Trematodes, in general, are karyotypically conservative, and their karyotypes tend to have the same number and closely related gross chromosome morphology on the generic and even family taxonomic level. A high degree of similarity was noted between the karyotypes of adult *P. folium*, described therein, and *P. pungitii*, i.e. obvious differences in the gross morphology of chromosomes are not apparent, and only comparison of karyometric data revealed some statistically significant differences in centromeric indices.

Clear karyotypic differences exist in the centromeric position of the corresponding chromosomes among the larval phyllodistomes from *S. corneum* and *P. amnicum*. Thus we can assume that the main cytogenetic mechanisms, which produce interspecific differences in chromosome sets, are pericentric inversions.

As the karyotype of the larval stages of *P. folium* sensu Sinitsin 1905, nec Olfers, 1817 was recently described (Petkevičiūtė, Stanevičiūtė & Molloy, 2003), a reliable comparison with the adult *P. folium*, described therein, was possible. Surprisingly, the comparison revealed clear differences in karyotype structure. Despite the same chromosome numbers, 2n = 18, and very close values of relative length of chromosomes, measurements showed that the centromeric index values of many chromosomes of larval *P. folium* are larger (most chromosomes are biarmed with medially or submedially located centromeres). The variation in the centromere position of corresponding chromosomes is most easily explained by pericentric inversions. The differences observed are undoubtedly interspecific, and the cercariaeum of *P. folium* sensu Sinitsin, 1905 from *D. polymorpha* could not be regarded as the larva of adult *P. folium* from three-spined stickleback. It is notable that Odhner (1911) stated that the cercaria of Sinitsin (1905) is the larva of *P. macrocotyle* (Lühe, 1909) and Dawes (1946) accepted this opinion. Nybelin (1926) claimed that this cercaria might represent the larva of *P. elongatum*.

On the other hand, the karyotypes of adult *P. folium* and macrocercous cystocercous cercariae from *S. corneum* appear identical both with respect to the relative length and centromeric indices of chromosomes.

Exact taxonomic identification, based on morphological characters, without knowing the age of the worm, the physical conditions of its host and the limits of intraspecific variability of the parasite, is extremely difficult. Even the results of life-cycle studies based upon controlled experimentation do not always contribute to the clarification of the chaotic taxonomic status of sexually mature phyllodistomes. For example, experimental infection of *Tinca tinca* and *Carassius auratus* by Orecchia *et al.* (1975) demonstrated that *Cercaria duplicata* von Baer, 1827 from *Anodonta cygnea* is the larval form of *Phyllodistomum elongatum*; Zhokhov (1987), however, identified the cercaria of *P. elongatum* as stylet-bearing cystocercous cercaria developing in *Pisidium amnicum*!

Recently, some studies have demonstrated the potential of molecular data as an alternative to the classical approaches (i.e. experimental infections) for

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the elucidation of digenean life-cycles (Cribb *et al*. 1998; Jousson *et al*. 1998, 1999). Sequence identity matrixes (Table 3), calculated with BioEdit, show the proportion of identical residues between all of the sequences in the alignment: ITS1-ITS2 and D1-D3 region of 28S sequences of *P. folium* from *G. aculeatus* and *S. corneum* are almost equally different from the phyllodistomes from *P. amnicum* and *D. polymorpha*. The results of the sequence comparisons in our study clearly supported the hypothesis that cystocercous cercaria from *S. corneum* correspond to adult trematoda from three-spined stickleback, thereby *S. corneum* and *G. aculeatus* are utilized by one species of phyllodistome. On the other hand, larvae from *P. amnicum* is the other species of genus *Phyllodistomum*, because it has a lot of gaps and different base pairs throughout all studied ITS and rDNA genes.

It should be emphasized that, on the basis of karyotypic characters, as well as with molecular data, larval phyllodistomes from *P. amnicum* and *D. polymorpha* can be recognized as more closely related to each other than to adult *P. folium* and its respective larvae from *S. corneum*. The karyotype of the latter is less symmetrical, composed of chromosomes with terminally and subterminally localized centromeres (only the 9th chromosome pair is metacentric), while in the karyotypes of phyllodistomes from *P. amnicum* and *D. polymorpha* biarmed, meta- and submetacentric, chromosomes prevail. Hence, we may conclude that karyological research could establish clear discriminative characteristics for *Phyllodistomum* species.

This study reveals a clear need of revision of the genus *Phyllodistomum* and opens a new perspectives in establishing species-specific characters for confident identification of both larvae and their respective adults.

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