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## Chromosome analysis of *Phyllodistomum folium* (Trematoda, Gorgoderidae) infecting three European populations of zebra mussels

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**Abstract** The mitotic chromosome sets of the larval stages of *Phyllodistomum folium* infecting three European populations of *Dreissena polymorpha* were studied using conventional Giemsa staining and karyometrical analysis. The karyotype, described herein for the first time, consisted of nine chromosome pairs ( $2n = 18$ ). The chromosomes were comparatively small and measured from 1.19  $\mu\text{m}$  to 5.22  $\mu\text{m}$ . The first and second longest chromosome pairs were, respectively, approximately 20% and 18% of the mean total chromosome set length and were clearly differentiated from the other seven pairs, which gradually decreased in size. Most chromosomes were biarmed with medially or submedially located centromeres; only pairs 1 and 4 contained subterminally located centromeres. No significant differences in the relative length values of corresponding chromosomes were observed between the three populations studied. The main interpopulation differences were detected in the centromeric index values of some corresponding chromosome pairs. These data, when analysed in conjunction with those of other Gorgoderidae, indicate that the karyotype of *P. folium* and other species in this family do not show any clear affinities with “typical” plagiorchiate chromosome sets.

### Introduction

Trematodes of the genus *Phyllodistomum* Braun, 1899 are cosmopolitan in distribution and live as adults in the urinary bladders and/or ureters of fish and

amphibians (Pigulevsky 1953; Dawes 1968; Kudinova 1994). Although the genus is very large, containing more than 110 species, life histories have been determined experimentally for only a few species. Species for which life cycles are known utilise bivalves (e.g., Sphaeriidae, Unionidae, and Dreissenidae) as first intermediate hosts and produce cercariae of different types, e.g., rhopalocercous, macrocercous, cystocercous, cercarium, which can be separated primarily by tail structure (Goodchild 1943; Coil 1954; Thomas 1958; Schell 1967; Wanson and Larson 1972; Ivanciv and Kurandina 1985; Zhokhov 1987). Cercarial *Phyllodistomum folium*, a cercarium, was described by Sinitsin (1905) as having a short, stumpy tail which becomes vestigial at the time of the development of gut bifurcation. However, only one species with this type of cercaria has been described among phyllodistomes, and it seems that this is the only known *Phyllodistomum* species infecting *Dreissena*. Recently, Zdun et al. (1994) provided a more detailed description of the developmental stages of *P. folium* from *Dreissena polymorpha*.

The taxonomic status of the sexually mature phyllodistomes is still problematic due to the close morphological similarity and remarkable variability of many diagnostic characters, some of which may change with development, and/or be insufficiently characterised in the original descriptions. The type species, *P. folium*, was described by Olfers (1817) (cited by from Sinitsin 1905) based on specimens from pike (*Esox lucius* L.). Later disagreement on species synonymy created a chaotic taxonomic status and provided considerable discussion for well over a century. For example, Pigulevsky (1953) regarded *P. folium* sensu Sinitsin 1905, nec Olfers, 1816 as synonymous with *Phyllodistomum dogieli* Pigulevsky, 1953. Dawes (1968) concluded that many European species of *Phyllodistomum* were identical with *P. folium* and that *P. folium* sensu Sinitsin, 1905 was a synonym of *Phyllodistomum macrocotyle* (Lühe, 1909). Recent investigation of the morphological variability of *Phyllodistomum* spp. has indicated that both *P. macrocotyle* and *P. dogieli* can be regarded

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as ecophenotypes of *P. folium* and that the polymorphic state of many morphological features depends on peculiarities of the excretory system of the fish host (Kudinova 1994). *P. folium* has been reported from cyprinid, esoxid, percid, salmonid, silurid, and other fish (Bykhovskaya-Pavlovskaya and Kulakova 1987). Thus, it is necessary for more criteria to be used in systematics, which can provide additional information for the taxonomic characterisation of the species.

Chromosome analysis is an essential part of the systematic studies of any group of organisms, and can play an important role, especially in taxonomically complicated genera. The present work represents the first description of the chromosomes of *P. folium*. A karyological analysis was carried out on three disjunct populations of parthenitas infecting *D. polymorpha*. The data should be considered as a partial contribution towards a much broader study of the chromosomal variation and evolution of Gorgoderidae.

## Materials and methods

Mitotic metaphase preparations were made from cells of the parthenitae of *P. folium* obtained from 25 specimens of naturally infected *D. polymorpha*. Mollusks were collected from the following three European localities: a water reservoir at the Lithuanian Power Station (near Elektrėnai), Lukomskoe Lake (120 km northeast of Minsk, Belarus), and the Moselle River (Metz, France) (6, 17 and 2 specimens were collected from each locality, respectively). The species was identified according to the morphological features of the intramolluskan stages. The morphology of the sporocysts and cercariae corresponded to the description of *P. folium* provided by Zdun et al. (1994).

Infected mollusks were treated with 0.005% colchicine in well water for 3–5 h. Gill tissue containing the sporocysts was dissected and immersed in distilled water for 30–40 min at room temperature for hypotony. This material was fixed in three changes of a freshly prepared solution of ethanol and glacial acetic acid (3:1) for 20 min and stored at 4°C. Fixed tissue was smeared on clean microscope slides, air-dried for at least 1 day, treated with 1 N HCl for 10–15 min, and rinsed three times in distilled water. Slides were stained with 4% Giemsa solution for 40 min, rinsed in tap water and allowed to dry. Suitable mitotic metaphases were photographed with a 100× objective using Mikrat-300 film. For karyotyping, chromosomes were cut out of the photographs and paired on the basis of size and centromere position. Within each population, measurements on the chromosomes of karyotypes from different animals were made. Nine metaphase plates from the Lithuanian, ten from the Belarussian and six from the French population were measured. Means ± SD are given. Relative chromosome length is expressed as a percentage of the total length of the haploid complement. The centromeric index (Ci) was calculated by dividing 100× the length of the short arm by the total chromosome length. The terminology used to describe the centromere position follows that of Levan et al. (1964). A chromosome was metacentric (m) if the Ci fell in the range 37.5–50.0, submetacentric (sm) in the range 25.0–37.5, subtelocentric (st) in the range 12.5–25.0, and acrocentric (a) if < 12.5. When a centromere position was on the borderline between two categories, two chromosome categories were listed. Student's *t*-tests were used for statistical comparison between populations for measurements of the centromeric index and relative length of comparable chromosomes. Results were considered significant when  $P < 0.05$ .

## Results

Analyses of more than 400 metaphase plates of *P. folium* demonstrated that the mode diploid number of chromosomes for this species was 18. The percentage of aneuploid cells ( $2n = 16, 17, 19$ ) was 37.6% (from 266 metaphase plates analysed), 30.5% (from 118 plates), and 31.6% (from 29 plates), respectively, in the Lithuanian, Belarussian, and French populations. The great majority of aneuploid cells were considered to be the result of errors inherent in preparation. Overall, the karyotypes of all populations were very similar. The chromosomes were comparatively small; the largest and the smallest chromosomes measured 5.22 µm and 1.19 µm, respectively. Table 1 gives the means ± SD of absolute length, relative length and centromeric indexes, with the chromosome classification of the three populations studied. Representative karyotypes for *P. folium* are shown in Fig. 1. Most chromosomes are biarmed with medially or submedially located centromeres. Pairs 1 and 4 contain subterminally located centromeres with the centromere index not exceeding 25.0%. No chromosomes possessing terminal centromeres were observed in the karyotypes. The longest and second longest chromosome pairs were, respectively, approximately 20% and 18% of the mean total chromosome set length, and were clearly differentiated from the other seven pairs, which gradually decreased in size. (Table 1, Fig. 1).

No significant difference in the relative length values of corresponding chromosomes was observed between the three populations studied. Measurements of the absolute length showed that the metaphase chromosomes from the Belarussian population were somewhat larger than in the Lithuanian and French populations. The absolute length of a chromosome may vary due to fixation or the degree of advance of metaphase. Thus, it is better to compare relative rather than absolute magnitudes. Comparing the centromeric indexes of individual chromosome pairs, some chromosomes showed significant ( $P < 0.05$ ), but not very striking differences. Part of the variability of centromeric index values could be a result of the difficulty in accurately locating the centromeres. Differences in pairs 2, 5 and 8 were the most outstanding. Chromosome pair 2 in the French population possessed a more medially located centromere and was clearly metacentric, but in the Lithuanian and Belarussian populations this pair had a submeta-metacentric structure. Chromosome pair 5 was metacentric in the Lithuanian population, but submetacentric in the other two, while pair 8 had a subterminally located centromere in chromosome sets from the Lithuanian population and submedially located centromeres in the Belarussian and French populations. In order to better visualise the existing interpopulation differences, ideograms of the chromosome sets of the three populations were constructed based on relative

**Table 1** Measurements (means  $\pm$  SD) and classification of chromosomes of *Phyllodistomum folium*. L Lithuanian, F French, B Belarusian, m metacentric, sm submetacentric, st subtelocentric chromosomes

Chromosome number		Absolute length ( $\mu\text{m}$ )	Relative length (%)	Centromeric index	Classification
1	L	4.25 $\pm$ 1.09	20.16 $\pm$ 1.48	23.27 $\pm$ 2.56	st
	F	4.11 $\pm$ 0.58	20.40 $\pm$ 1.11	25.22 $\pm$ 2.19	st-sm
	B	5.22 $\pm$ 1.22	20.54 $\pm$ 1.37	21.91 $\pm$ 3.84	st
2	L	3.77 $\pm$ 0.78	18.03 $\pm$ 1.03	35.02 $\pm$ 2.53	sm
	F	3.78 $\pm$ 0.62	18.74 $\pm$ 1.36	43.71 $\pm$ 2.93	m
	B	4.77 $\pm$ 1.24	18.70 $\pm$ 1.49	37.36 $\pm$ 3.81	sm-m
3	L	2.83 $\pm$ 0.42	13.63 $\pm$ 0.97	32.78 $\pm$ 3.19	sm
	F	2.62 $\pm$ 0.32	13.00 $\pm$ 0.61	29.78 $\pm$ 2.31	sm
	B	3.57 $\pm$ 0.82	14.11 $\pm$ 0.78	30.94 $\pm$ 2.90	sm
4	L	2.44 $\pm$ 0.50	11.72 $\pm$ 0.90	13.19 $\pm$ 3.43	st
	F	2.23 $\pm$ 0.46	10.97 $\pm$ 1.23	16.48 $\pm$ 3.86	st
	B	3.03 $\pm$ 0.85	11.88 $\pm$ 0.82	17.63 $\pm$ 4.38	st
5	L	1.86 $\pm$ 0.30	8.98 $\pm$ 0.67	42.14 $\pm$ 3.88	m
	F	1.86 $\pm$ 0.31	9.21 $\pm$ 0.86	35.26 $\pm$ 3.37	sm
	B	2.18 $\pm$ 0.48	8.62 $\pm$ 0.57	31.34 $\pm$ 4.52	sm
6	L	1.59 $\pm$ 0.24	7.66 $\pm$ 0.42	34.34 $\pm$ 3.31	sm
	F	1.57 $\pm$ 0.19	7.82 $\pm$ 0.20	34.78 $\pm$ 4.08	sm
	B	1.99 $\pm$ 0.42	7.90 $\pm$ 0.46	32.78 $\pm$ 5.20	sm
7	L	1.51 $\pm$ 0.27	7.25 $\pm$ 0.67	31.08 $\pm$ 4.84	sm
	F	1.47 $\pm$ 0.16	7.30 $\pm$ 0.55	28.98 $\pm$ 3.35	sm
	B	1.71 $\pm$ 0.32	6.83 $\pm$ 0.90	28.49 $\pm$ 3.27	sm
8	L	1.40 $\pm$ 0.27	6.70 $\pm$ 0.60	20.79 $\pm$ 5.02	st
	F	1.32 $\pm$ 0.08	6.62 $\pm$ 0.74	27.47 $\pm$ 4.69	sm
	B	1.53 $\pm$ 0.37	6.08 $\pm$ 0.69	29.12 $\pm$ 5.25	sm
9	L	1.22 $\pm$ 0.21	5.87 $\pm$ 0.67	42.78 $\pm$ 4.35	m
	F	1.19 $\pm$ 0.13	5.95 $\pm$ 0.85	45.61 $\pm$ 1.27	m
	B	1.35 $\pm$ 0.30	5.34 $\pm$ 0.56	46.12 $\pm$ 1.25	m

chromosome length and centromeric index values (Fig. 2).

## Discussion

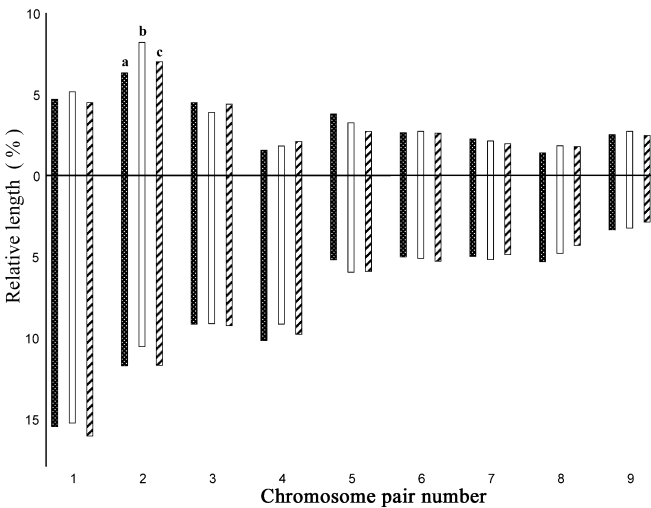
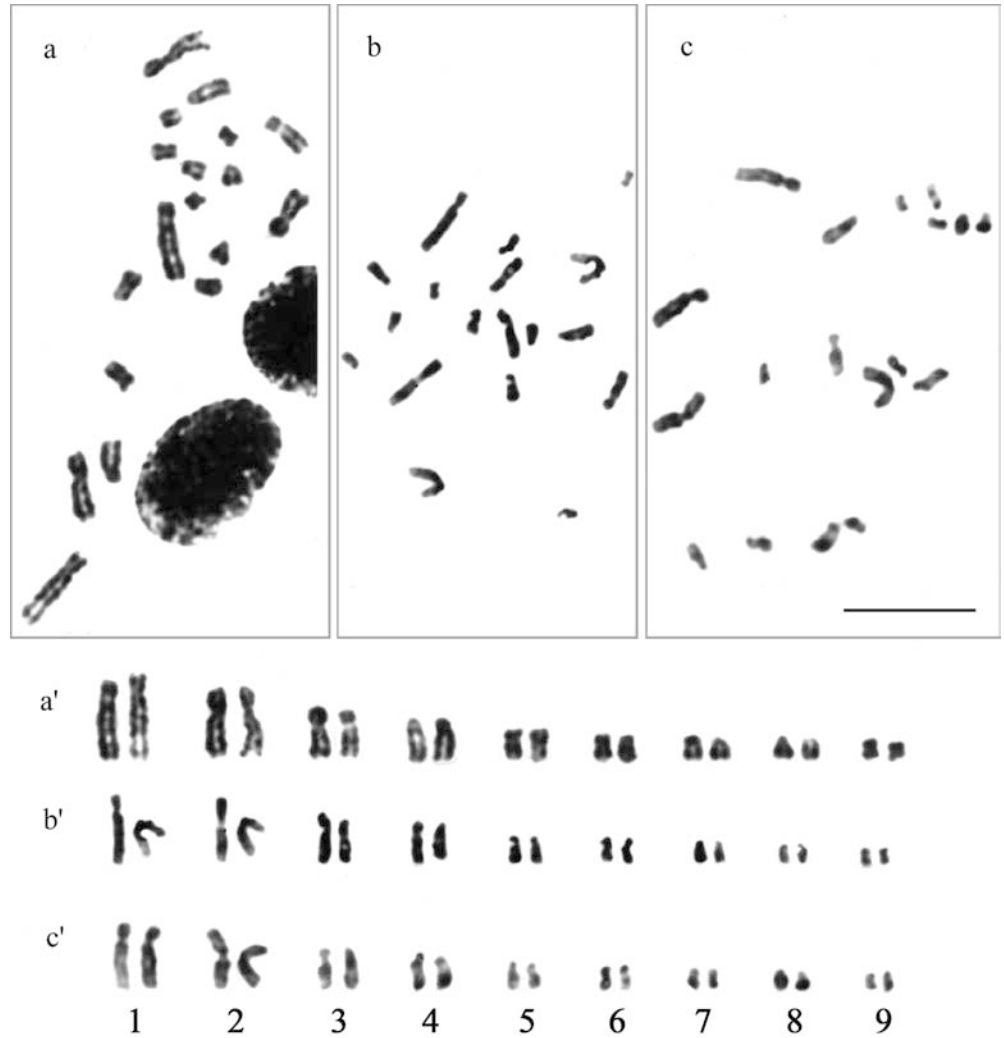
Despite the great advances made in the karyology of trematodes (see Baršienė 1993), only a small part of existent species have undergone karyological analysis. Chromosomal data for gorgoderids are very limited, with information for only seven species available. Early reports provided only the number of chromosomes and lacked morphological detail. Cytogenetic studies of this group began with Markell (1943), who showed the existence of  $n=6$ ,  $2n=12$  chromosomes in *Probolitrema californiense* by means of analysis of serial sections of generative tissues. Britt (1947) revealed the chromosome number  $n=7$ ,  $2n=14$  for *Gorgoderina attenuata* and  $n=8$ ,  $2n=16$  for *Gorgoderina amplicava*, both based on sectioned material. Dhingra (1954) found eight chromosomes in haploid complements of *Phyllodistomum spatula*. However, it is possible that in sectioned material some of the chromosomes (especially the smallest) could have been overlooked. Recent studies on colchicine-treated, air-dried material has provided more detailed and more accurate karyological information on three species: *Gorgoderina pagenstecheri*,  $2n=18$  (Baršienė 1991), *Phyllodistomum conostomum*,  $2n=16$ , and *Phyllodistomum pungitii*,  $2n=18$ , (Baršienė 1993; Orlovskaya et al. 1995). The karyotype of *P. folium*, described therein, resembles that

of *P. pungitii* and *G. pagenstecheri* in having  $2n=18$  and close values of the relative lengths of corresponding chromosomes. However, both *P. pungitii* and *G. pagenstecheri* possess less symmetrical karyotypes, with un-armed chromosomes of the acrocentric type and chromosomes with subterminally localised centromeres prevailing. Similarity of chromosome relative length among the three taxa showed that this parameter is too stable and cannot discriminate between species or even genera. The variation in the centromere position of the corresponding chromosomes among species under consideration is most easily explained by pericentric inversions of a different entity.

The chromosome complement of *P. conostomum* with  $2n=16$  and one pair of large metacentric elements constituting 34.6% of the total length of the haploid set (Orlovskaya et al. 1995) presumably arose from a karyotype with  $2n=18$ . The appearance of the large metacentrics, clearly distinguished from the remaining elements, could be the result of the centromeric fusion of two acrocentric non-homologous chromosomes (Robertsonian translocation). The reduction of chromosome numbers by means of centromeric fusion rather than their elimination is also characteristic of the karyotypic evolution of other trematodes (Grossman et al. 1981; Petkevičiūtė et al. 1990; Baršienė 1993).

The Gorgoderidae Looss, 1899 comprises a group of plagiorchiate trematodes. Their closest relatives among the other plagiorchiates are not known with any degree of confidence (Brooks and Macdonald 1986). Cable

**Fig. 1** Mitotic chromosomes of three populations of *Phyllodistomum folium*. *a, a'* Mitotic metaphase and karyotype from the Lithuanian population, *b, b'* the Belarussian population, *c, c'* the French population. Bar = 10  $\mu$ m



**Fig. 2** Ideograms of chromosomes of three populations of *P. folium*. *a* Lithuanian, *b* French, *c* Belarussian

(1974) and Odening (1974) have linked the gorgoderids with zoogonids, opecoelids, lissorchiids, and some monorchids. However, cytogenetic parameters, such as

chromosome number and morphology, can also be used to investigate phylogenetic patterns. Unfortunately, karyological observations are too few to verify the taxonomic affinity between the families noted. So far, chromosome numbers are reported for only one species of Zoogonidae; the diploid number of chromosomes in *Zoogonus mirus* was reported by early workers to be 10 or 12 (reviewed by Walton 1959). Nothing is known of the structure of the chromosome complement of any member of the family Lissorchiidae. *Sphaerostomum bramae*, the only karyologically studied representative of the family Opecoelidae, resembles that of the many other pliorchiate species in the families Troglotrematidae, Pliorchiidae, Prosthogonimidae, Lecithodendriidae, and Telorchidae (reviewed by Baršienė 1993), in having  $2n=22$  and the first large pair of chromosomes being of the metacentric type (Petkevičiūtė et al. 1995). Trematodes are fairly conservative karyotypically, with related species on generic and even family levels differing by a few chromosome rearrangements and possessing 'typical' karyotypes. The 'typical' pliorchiate chromosome complex (i.e.,  $2n=22$  and with the first pair as large metacentric elements) does not

show any affinities with the gorgoderid chromosome sets that have been described so far. Recent molecular data also suggest placement of the family Gorgoderidae outside of the Plagiorchiata (Tkach et al. 2001).

The only plagiorchiates with karyotypes of  $2n=18$  are three species in the family Microphallidae. Chromosome sets of *Microphallus piriformes*, *Microphallus pygmaeus* and *Microphallus triangulatus* consist of nine pairs of biarmed chromosomes: two pairs of comparatively large and seven pairs of smaller elements (Birstein and Mikhailova 1989). Thus, it seems that the diploid numbers and comparatively close sizes of chromosomes of the microphallids and gorgoderids support a taxonomic affinity between these families. Furthermore, there is one striking biological peculiarity common to some species in both families. The above mentioned species of *Microphallus* form the 'pygmaeus' group, characterised by an aberrant life-cycle without free-living cercariae (Belopolskaya 1949). Cercariae of *P. folium* develop in sporocysts and encyst in them as metacercariae (Sinitsin 1905). However, in the phylogeny based on molecular data, the representatives of the Microphallidae were clustered within one of the two main clades of Plagiorchiata (Tkach et al. 2001). Karyological data find little support for such a relationship. It seems that a much more comprehensive sampling of taxa would be needed before these relationships between the taxa under consideration could be justified.

Despite the comparatively conservative nature of the karyotype structure of trematodes, intraspecific differences have been documented in several species. For example, the overall karyotypes of seven strains of *Schistosoma mansoni* were similar, with  $2n=16$ , but small differences were revealed which could be accounted for by relatively small pericentric inversions and/or translocations (Short et al. 1989). Comparative karyological analysis of two populations of *Diplo-discus subclavatus* revealed small, but statistically significant ( $P < 0.05$ ) differences in the centromeric index values of some chromosome pairs (Petkevičiūtė et al. 1989). Only slight differences in the chromosome morphology of some corresponding chromosome pairs, which occurred as a result of small pericentric inversions, were found in a comparison of *Opisthioglyphe ranae* karyotypes obtained from three populations (Baršienė et al. 1995). The data obtained in the analysis of *Notocotylus ephemera* showed an interpopulation polymorphism, mainly in the location of the centromeres in several chromosomes (Mutafova et al. 1995). Many authors have indicated that some statistically significant differences in measurements might be artefacts resulting from the incorrect homologue matching of certain morphologically similar members, or difficulty in accurately locating centromeres. These problems are also inherent in the methods of analysis employed in this investigation. The comparative results obtained for the three populations of *P. folium* are in accordance with the earlier findings in other trematode species. The relative length values were very stable and no statistically significant differences

were revealed. The main interpopulation differences were detected in the centromeric index values of some chromosomes, and these could be most easily explained by small pericentric inversions.

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