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ORIGINAL PAPER

Differentiation of *Myxobolus* spp. (Myxozoa: Myxobolidae) infecting roach (*Rutilus rutilus*) in Hungary

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Abstract During a survey on fishes from Hungarian freshwaters, the occurrence of eight *Myxobolus* species was registered in roach (*Rutilus rutilus* L.). Most species had a specific location within the fish host. Of the known species infecting roach, the intramuscularly developing species *Myxobolus pseudodispar* was found to be the most common. Besides *Myxobolus intimus* and *Myxobolus diversicapsularis* infecting the capillary network of gill lamellae, *Myxobolus feisti* infecting the cartilaginous gill rays were observed. Of the "muelleri-type" *Myxobolus* spp, *Myxobolus rutili* and *Myxobolus sommervillae* sp. n. formed elongated plasmodia in the gill filaments outside and inside the arteria efferens, respectively, while *Myxobolus wootteni* sp. n. developed cysts in fins. Plasmodia and spores of *Myxobolus fundamentalis* sp. n. were detected in the cartilaginous gill arch under the basis of gill filaments. Despite similarities of some species in spore morphology, 18S rDNA sequences and phylogenetic analyses showed clear differences among all species examined. The findings of the study demonstrate that morphologically similar spores could only be correctly identified by considering the location of plasmodia and the genetic characters of the myxozoan species.

Introduction

Roach, *Rutilus rutilus* (L.), is one of the most common fish in Europe. Its parasite fauna, among them *Myxobolus* infections, are also well studied (Donec and Shulman 1984; Landsberg and Lom 1991; Lom and Dyková 1992; Longshaw et al. 2003). Donec and Shulman (1984) recorded more than 20 *Myxobolus* spp. from roach and its subspecies. However, Eiras et al. (2005), in their synopsis on the genus *Myxobolus*, listed only eight species described from roach as a type host (*Myxobolus alievi* Gasimagomedov, 1970; *Myxobolus chernovae* (Chernova, 1970); *Myxobolus diversicapsularis* Slukhai, 1984; *Myxobolus dujardini* Thelohan, 1892; *Myxobolus elegans* Kashkovski, 1966; *Myxobolus marginatus* Kulemina, 1969; *Myxobolus pseudodispar* Gorbunova, 1936 and *Myxobolus rutili* Donec and Tozyyakova, 1984). Besides these species, *Myxobolus buckei* Longshaw et al. (2005) from the vertebral column and *Myxobolus feisti* Molnár et al. (2008) from the gill cartilage were recently described from roach. *M. buckei* was described from chub as original host, but its occurrence in roach was also assumed. Morphological and molecular studies by Molnár et al. (2006) on the *Myxobolus* fauna of chub suggest that *Myxobolus* species are rather host-specific parasites, and seem to infect a single host species or genetically closely related hosts. The above mentioned paper and previous studies (Molnár 1994; Molnár et al. 2002; Eszterbauer 2002, 2004) also suggest that *Myxobolus* species bear strict tissue specificity and moderate organ specificity.

This paper presents data on the spore morphology and phylogeny of five known and three new *Myxobolus* species found in roach. Furthermore, the typical locations of infection and the histological changes in fish caused by these species are also investigated.

Materials and methods

Roach (*Rutilus rutilus*) specimens originated from several Hungarian lakes and rivers. Most specimens were collected

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in the framework of fish parasitological monitoring projects in Lake Balaton (no. of specimens were 339) and the Kis-Balaton water reservoir (217 specimens), but roach were examined also from Danube (12 specimens) and Tisza rivers, and from some small creeks (35 specimens). The majority of roach specimens were 4- to 13-cm long fingerlings of the age group 1+ year, but 14 to 25 cm long, 3- to 6-year-old specimens were also examined (Table 1).

The fishes were subjected to complete parasitological dissection. However, infection of muscle, by compressing pieces between two glass plates, was studied only partially. When mature plasmodia were found, some of the spores were studied in fresh preparations, some of them were collected into Eppendorf tubes and stored at –20°C until further molecular characterization, while the rest of the spores were preserved in glycerine-gelatine as slide preparations. Tissue samples from infected organs containing developing and mature plasmodia were fixed in Bouin's solution, embedded in paraffin wax, cut to 4–5-μm sections, and stained with haematoxylin and eosin. Naïve spores were studied by Nomarski differential interference contrast of an Olympus BH2 microscope. The spores were photographed with an Olympus DP10 digital camera, or recorded on videotapes; and then digitized images were obtained, and measurements were taken. All measurements are given in micrometer.

Molecular methods

For DNA extraction, samples were centrifuged at 5,000×*g* for 5 min. Spore pellets were suspended in 500 μl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, and .4 mg/ml proteinase K) and incubated at 55°C for 3–4 h. DNA was then purified using the Miniprep Express Matrix (BIO 101, USA). Genomic DNA was amplified with the primer pair 18e (5′-CTG GTT GAT TCT GCC AGT-3′) (Hillis and Dixon 1991) and 18r (5′-CTA CGG AAA CCT TGT TAC-3′) (Whipps et al. 2003). The total volume of the PCR reactions was 50 μl, which contained approximately 10 to 50 ng DNA, 1× Taq PCR reaction buffer (MBI Fermentas), 1.5 mM MgCl2, 0.2 mM dNTP mix (Sigma), 25 μM of each primer, and 2 U of Taq DNA polymerase (MBI Fermentas). Amplification conditions were 95°C for 50 s, 58°C for 50 s, and 72°C for 80 s for 35 cycles, with a terminal extension at 72°C for 7 min. When a weak band was detected on 1.0% agarose gel in Tris–acetate–EDTA (TAE) buffer, the amplification was followed as nested PCR assay with inner primer pairs MX5 (5′-CTG CGG ACG GCT CAG TAA ATC AGT-3′) and MX3 (5′-CCA GGA CAT CTT AGG GCA TCA CAG A-3′) (Andree et al. 1999), or SphF (5′-ACT CGT TGG TAA GGT AGT GGC T-3′) and SphR (5′-GTT ACC ATT GTA GCG CGC GT-3′) (Eszterbauer and Székely 2004). Biometra T1 thermocycles were used for amplification. Cycling conditions with primers MX5 and MX3 were 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s for 35 cycles, and were terminated with an extension period at 72°C for 7 min. For PCR using SphF and SphR primers, the same condition was applied for those with the 18e–18r primer pair. The PCR products were electrophoresed in 1.0% agarose gels (Sigma) in TAE buffer, and then purified with QIAquick Gel Extraction Kit (Qiagen).

For samples with insufficient amount of amplified PCR product for direct DNA sequencing, purified DNA was cloned into a pGEM-T Vector System II (Promega) following the manufacturer's manual. Purified PCR products and/or at least two positive clones per sample were sequenced in both directions using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). The following primers were used for sequencing: amplification primers 18e and 18r, MX5 and MX3, SphF and SphR, Myx4r and Act1f by Hallett and Diamant (2001) and MB5, MB3, MC5, and MC3 described by Eszterbauer (2004). For sequence assembling, the STADEN Sequence Analysis Package version 2001.0 (Staden 1996) was used. DNA sequence similarities were calculated with the Sequence Identity Matrix of the software BioEdit (Hall 1999).

Phylogenetic analyses

Nucleotide sequences were aligned with the software Multalin (Corpet 1988) available online. The alignment was corrected manually using the GeneDoc sequence alignment editor program. The dataset for the alignment was chosen on the basis of the results of BLAST searches and morphological findings. Phylogenetic analyses using neighbor-joining (with K2P; NJ) and maximum likelihood (ML) algorithm were conducted in PAUP* version 4.0b10 (Swofford 2001). An optimal evolutionary model (GTR+I+G) for the alignment was determined with AIC in Modeltest 3.06 (Posada and Crandall 1998). Maximum likelihood analysis employed a heuristic search algorithm with random sequence addition (10 replicates) and TBR branch swapping. Bootstrap confidence values were calculated with 1,000 and 100 repetitions for NJ and ML, respectively. Bayesian inference analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). A general time reversible model (GTR) with gamma-shaped rate variations across sites (Invgamma) was chosen for the analysis. Two independent runs were conducted with four chains for 1 million generations. Trees were sampled every 100 generations. The first 25% of the samples were discarded from the cold chain

Table 1 Number of examined roach specimens of two size groups collected in different water basins in the course of the 8-year-long survey (2001–2008)

	Lake Balaton		Kis-Balaton		Danube		Other places		All
	4–13	14–25	4–13	14–25	4–13	14–25	4–13	14–25	
2001	20	18	8	4	–	–	12	–	62
2002	16	8	3	7	–	2	–	–	36
2003	32	14	4	–	–	3	3	–	56
2004	18	18	–	10	–	–	–	4	50
2005	29	13	12	–	–	–	–	2	56
2006	33	24	20	6	4	–	7	5	99
2007	8	6	5	–	1	–	–	–	20
2008	51	31	98	40	–	2	–	2	224
	207	132	150	67	5	7	22	13	603
Total	339		217		12		35		603

Two size groups (4–13 and 14–25) are given in cm

(burninfrac=0.25), and a 50% majority-rule consensus tree was created, which was visualized by MEGA 4. *Myxobolus cerebralis* was chosen as the outgroup.

Results

Five known species (*Myxobolus intimus* Zaika, 1965; *M. diversicapsularis* Slukhai, 1984; *M. rutili* Donec and Tozyyakova, 1984; *M. feisti* Molnár et al. 2008; and *M. pseudodispar* Gorbunova, 1936) and three new species (*Myxobolus fundamentalis* sp. n, *Myxobolus sommervillae* sp. n. and *Myxobolus wootteni* sp. n.) were identified in roach in the course of the 8-year-long survey. Each species had a specific location within the fish host (Table 2). Measurements of *Myxobolus* spp. examined are listed in Table 3, while their molecular characterization is summarized in Table 4.

M. intimus Zaika, 1984

The species was found in 3- to 6-year-old roach specimens from Lake Balaton and Danube River in March and April of each year. Mature plasmodia filled with spores were found in April. In March, plasmodia harbored sporogonic stages and some young spores. In May, only some aged plasmodia were found. No occurrence of the species was recorded in other seasons of the year. Although in a year's relation, only 6% of the examined roach were infected with this species, in spring, the infection in larger roach specimens was over 40%.

Round plasmodia of 60 to 220 μm in diameter were located in the capillary network of gill lamellae. The shape and size of the characteristic spores, with a small extension at the anterior pole (Fig. 1a, Table 3), corresponded to the data given by Rácz et al. (2004); Donec and Shulman (1984) and Lom and Dyková (1992).

Histology Small, round plasmodia developed in the capillary network of gill lamellae. Plasmodia filled most of the capillary network of the lamellae.

Molecular data 18S rDNA sequence of *M. intimus* (AY325285) resembled the best Aurantiactinomyxon "type 1" (AY495708) by Hallett et al. (2006) with 99.9% similarity. Between the two *M. intimus* replicates sequenced, a T/C nucleotide alteration was detected in position no. 52.

M. diversicapsularis Slukhai, 1966

The species was found in 3- to 6-year-old roach specimens from Lake Balaton and Danube River in April and May of each year. Mature plasmodia were first found in April. No occurrence of the species was recorded in any other seasons of the year. Although only 2.6% of the examined roach was found infected in the course of the entire study, in spring 2008, 37% prevalence was detected among roach specimens >3 years of age. Concurrent infections with *M. intimus* were frequently recorded. Ellipsoidal plasmodia of 100–150×175–250 μm in diameter were located in the capillary network of gill lamellae (Fig. 2). Shape and size of the characteristic spores with unequal polar capsules (Figs. 1b and 4a, b) corresponded to the data given by Shulman (1966) and Lom and Dyková (1992).

Type material Voucher spores in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum,

Table 2 Number of roach specimens found to be infected with *Myxobolus* spp. in different habitats in the course of the 8-year-long survey

Name of the species	Location in host	Lake Balaton	Kis-Balaton	Danube	Other localities	In all localities
Myxobolus intimus	Gill lamellae	35/339 (10.3)	0/217 (0)	1/12 (8.3)	1/35 (2.9)	37/603 (6.1)
M. diversicapsularis	Gill lamellae	16/339 (4.7)	0/217 (0)	1/12 (8.3)	0/35 (0)	17/603 (2.8)
M. pseudodispar	Muscles	82/339 (24.2)	130/217 (60)	2/12 (16.7)	30/35 (85.7)	244/603 (40.5)
M. fundamentalis sp. n.	Gill arch	8/339 (2.4)	0/217 (0)	0/12 (0)	0/35 (0)	8/603 (1.3)
M. wootteni sp. n.	Fins	24/339 (7.1)	0/217 (0)	1/12 (8.3)	0/35 (0)	25/603 (4.1)
M. feisti	Gill rays	27/339 (7.9)	52/217 (24)	0/12 (0)	1/35 (2.9)	80/603 (13.3)
Myxobolus rutili[a] and *M. sommervillae* sp. n.	Gill filaments	18/339 (5.3)	6/217 (2.8)	0/12 (0)	2/35 (5.7)	26/603 (4.3)
All *Myxobolus* spp.		210/339	188/217	5/12	34/35	437/603

Prevalence values are in parenthesis (%). Number of fish infected by *Myxobolus* spp/examined fish from a certain water basin

[a] The identification of these species without detailed examination of spores was uncertain

Budapest, Coll. No. HNHM-18215. The 18S rDNA sequence of *M. diversicapsularis* was deposited in Genbank under the accession number GU968199.

Histology Small- to middle-sized ellipsoidal plasmodia (Fig. 2) filled most of the capillary network of the lamellae.

Molecular data 18S rDNA sequences of *M. diversicapsularis* samples resembled the best Triactinomyxon "type 3" (AY495706) with 99.6% similarity (Hallett et al. 2005). Three nucleotide alterations were detected among sample replicates in position no. 1420. (T/C), no. 1429 (T/C), and no. 1508 (T/G).

Table 3 Morphological features and measurements of *Myxobolus* spp. found in roach

	M. intimus	*M. diversicapsularis*	*M. pseudodispar*	*M. fundamentalis* sp. n.	*M. wootteni* sp.n.	*M. rutili*	*M. sommervillae* sp.n.	*M. feisti*
Location of plasmodia	Gill lamellae	Gill lamellae	Muscle cells	Gill arch	Fins	Gill filaments	Gill filaments	Cartilaginous gill rays
Shape of plasmodia	Small, roundish	Small, roundish	Large, elongated	Large, roundish	Small, ellipsoid	Large, elongated or roundish	Large, elongated	Small, round or ellipsoid
Spore shape in FW	Ellipsoidal with extension	Ellipsoidal, often deformed	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
Spore length	13.5±0.96 (12.6–14.7)	10.3±0.51 (9.3–11.0)	12.2±0.79 (11.0–13.6)	15.5±0.81 (14.4–17.0)	13.3±0.96 (11.6–15.0)	13.1±1.26 (11–15.7)	11.8±0.95 (10.5–13.5)	11.7±0.93 (11.5–13.2)
Spore width	9.2±0.4 (8.6–9.7)	8.7±0.41 (8.0–9.0)	7.0±0.7 (5.8–8.3)	11.8±0.58 (11.2–13.2)	11.6±0.73 (10.2–13.0)	9.9±0.77 (8–10.8)	9.7±0.76 (8.4–11.2)	10.0±0.77 (9.4–10.8)
Spore thickness	8.0±0.4 (7.0–8.5)	6.5±0.15 (6.3–6.8)	5.6±0.3 (5.0–6.0)	9.2±0.3 (9.0–9.6)	5.7±0.37 (5.5–6)	6.8±1.17 (6.0–9.0)	7.2±0.39 (6.6–7.5)	6.7±0.19 (6.6–7.0)
Length of (larger) PC	5.5±0.4 (4.8–6.2)	5.4±0.34 (4.8–6.0)	5.9±0.39 (5.6–6.6)	6.7±0.24 (6.5–7.2)	6.2±0.75 (5.0–7.0)	5.9±0.9 (4.0-7.2)	6.0±0.38 (5.4–6.7)	6.2±0.14 (6.0–6.3)
Width of (larger) PC	3.4±0.5 (2.9–4.8)	3.5±0.16 (3.3–3.6)	2.9±0.35 (2.1–3.3)	4.0±0.26 (3.7–4.3)	3.9±0.49 (3.0–4.4)	3.4±0.62 (3.0–3.7)	3.3±0.35 (3.0–4.2)	3.7±0.22 (3.3–4.0)
Length of smaller PC	–	3.4±0.55 (2.5–4.6)	3.8±0.45 (3.5–5.0)	–	–	–	–	–
Width of smaller PC	–	2.6±0.38 (2.2–3.0)	2.8±0.13 (2.7–3.0)	–	–	–	–	–
IP length	n.d.	–	–	1.7±0.1 (1.6–1.8)	2.7±0.76 (2.0–3.5)	1.3±0.31 (0.7–1.5)	1.8±1.85 (1.5–2.7)	2.0±0.32 (1.8–2.4)
Number of PFC	5–6	4–5/3–4	4–6/3–4	6–7	6–7	6	6	6
Reference	Rácz et al. 2004	Present study	Present study	Present study	Present study	Present study	Present study	Molnár et al. 2008

FW frontal view, *PC* polar capsule, *IP* intercapsular process, *PFC* polar filament coils, *n.d.* no data, – absent

Table 4 *Myxobolus* spp. from roach examined at DNA level. The positions of nucleotide alterations are noted in Results

Species	No. of replicates sequenced	Genetic similarities (%)	GenBank accession number (length of 18S rDNA fragment)
M. intimus	2	99.9	AY325285 (1583 nt)
M. diversicapsularis	3	99.7–99.9	GU968199 (1891 nt)
M. fundamentalis sp. n.	2	99.9	GU968200 (1357 nt)
M. wootteni sp. n.	2	99.9	DQ231157 (1599 nt)
M. rutili	2	100	GU968201 (1326 nt)
M. sommervillae sp.n.	2	100	GU968202 (1321 nt)
M. feisti	1	–	EU598804 (1331 nt)

Remarks It is assumed that prior to 2007, immature plasmodia, and less frequently, mature plasmodia of *M. diversicapsularis*, might be erroneously identified in some cases as *M. intimus* occurring concurrently in the same location in fish host.

M. pseudodispar Gorbunova, 1936

Plasmodia or spores of this species were found in each age group and in most of the examined specimens of roach. Plasmodia located in muscle cells showed no seasonality, although developing plasmodia were found mostly in spring, except fingerlings, which got infected in early summer. In autumn and winter, mostly aged plasmodia or disseminated spores in macrophage centers of different organs were detected. The shape and size of the characteristic spores with unequal polar capsules (Fig. 1c, Table 3) corresponded to the data given by Shulman (1966); Lom and Dyková (1992), and Molnár et al. (2002).

M. fundamentalis sp. n.

Plasmodia (Fig. 3), round or ellipsoidal, up to 1.2 mm in length and 0.7 to 0.8 mm in width develop in the cartilaginous gill arch under the gill filaments. Spores develop in disporic pansporoblasts. Spores are relatively large, ellipsoidal in frontal view (Figs. 1d and 4c), and lemon-shaped in sutural view (Figs. 1d (inset) and 4d). The other characteristics of the spore are the following: spore length, 15.5±0.81 (14.4–17.0; n=50), width, 11.8±0.58 (11.2–13.2; n=50), and thickness, 9.2±0.3 (9.0–9.6; n=15). Polar capsules are equal in size, pyriform, relatively short, slightly converging anteriorly, 6.7±0.24 (6.5–7.2) long (n=50) and 4.0±0.26 (3.7–4.3) wide (n=50). Six to seven polar filament coils are arranged perpendicular to the capsule length. Relatively small triangular intercapsular appendix measuring 1.7±0.1 (1.6–1.8; n=16) are located anteriorly between the capsules. Sutural protrusion forms a circular rim around the spore emerging about 0.8 to 1.0 μm over the surface of the spore. The thickness of the rim in sutural view measures about 0.8 μm. Sutural edge markings (Nos. 8 to 9) are poorly seen in fresh spores. Single binucleated sporoplasm with large, round iodinophilous vacuole is present. Mucous envelope is not found.

Type host: roach, *Rutilus rutilus* (L.) (Cyprinidae).

Localities: Lake Balaton and Kis-Balaton water reservoir

Site of tissue development: connective tissue in the gill arch

Type material Syntype spores in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18211. The 18S rDNA sequence of *M. fundamentalis* sp. n. has been deposited in Genbank under the accession number GU968200.

Etymology The species is named after its specific location in the cartilaginous gill rays at the basis of filaments.

Histology of infection Most plasmodia were located in the gill arch close to the basis of gill filaments, but several plasmodia were found inside the gill arch, attached to the wall of arteries (Fig. 3). Semi-mature plasmodia contained mature spores in the central region, and young sporogonic stages at the periphery.

Molecular data The DNA sequences of two samples of this species were 99.9% similar, as only a single nucleotide difference in position no. 350 (A/T alteration) was detected over a 1,357-bp-long fragment. The most closely related species was *Myxobolus gayerae*, with 93.5% similarity in their 1330-bp-long aligned 18S rDNA sequence.

Remarks M. fundamentalis sp. n. resembled in spore morphology *Myxobolus cyprinicola* Reuss 1906 (DQ439805) from the common carp, and *Myxobolus*

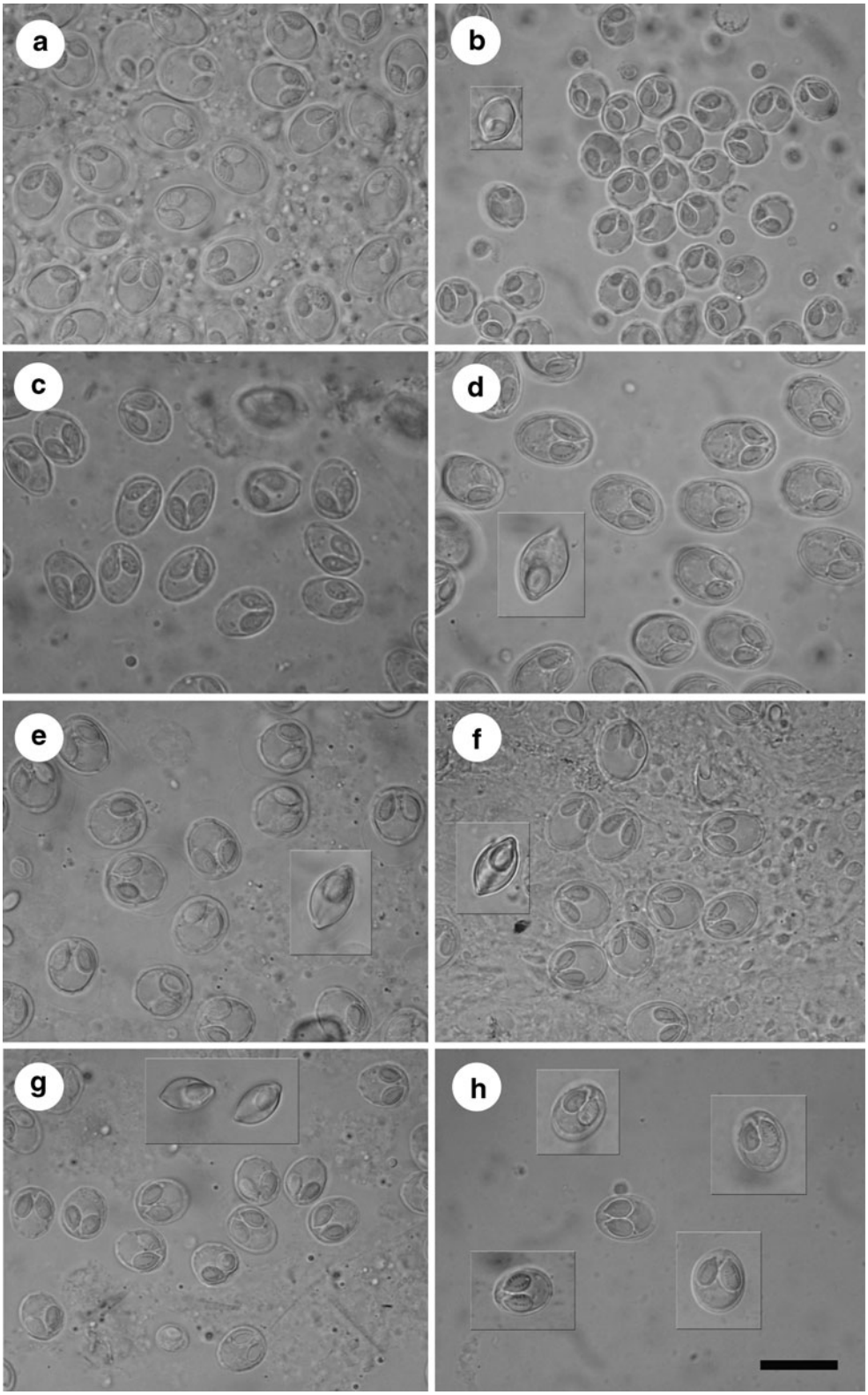
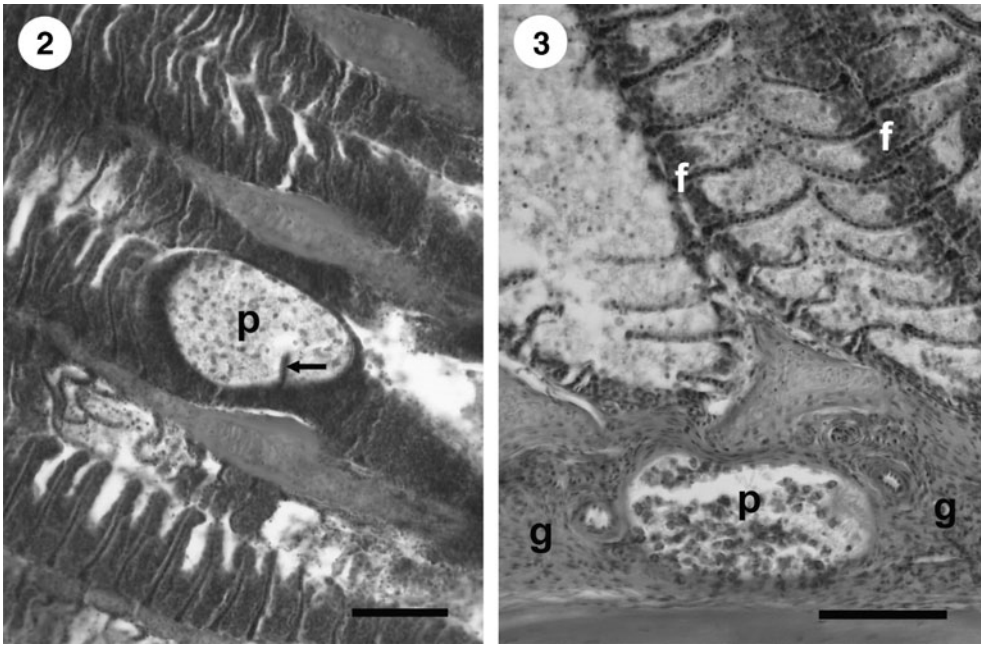


Fig. 1 Spores of *Myxobolus* spp. found in roach. **a** *M. intimus.* **b** *M. diversicapsularis* in frontal view, *inset* in sutural view. **c** *M. pseudodispar.* **d** *M. fundamentalis* sp. n. in frontal view, *inset* in sutural view. **e** *M. wootteni* sp. n. in frontal view, *inset* in sutural view. **f** *M. rutili* in frontal view, *inset* in sutural view. **g** *M. sommervillae* sp. n. in frontal view, *inset* in sutural view and **h** *M. feisti*. Fresh mount. *Bar*=15 μm

cycloides ex *Leuciscus* (DQ439810), a typical parasite of the swimbladder of chub, but it clearly differed from them at the DNA level (86.8% and 93.2% similarity, respectively). By its typical basifilamental location, *M. fundamentalis* sp. n. resembles also *Myxobolus basilamellaris* (AF507971), the parasite of the common carp, but its spores are larger, and their 18S rDNA sequence similarity is only 86.9%.



Figs. 2-3 Plasmodium (*p*) of *M. diversicapsularis* in a gill lamella deforming the neighboring lamellae. The rest of the attacked capillary (*arrow*) can still be seen. Histological section, H&E. *Bar*=200 μm Plasmodium (*p*) of *M. fundamentalis* sp. n. in the lumen of the cartilaginous gill arch (*g*) under the basis of a gill filament (*f*). Histological section, H&E. *Bar*=200 μm

Occurrence of "muelleri-type" spores in different organs of the roach

In the course of the present study, four different "muelleri-type" *Myxobolus* species (*M. wootteni* sp. n., *M. feisti, M. rutili* and *M. sommervillae* sp. n.) were found infecting different organs of roach. Their spores morphologically resembled very much the spores of *Myxobolus muelleri* Buetschli, 1882, a gill-parasite of chub, but differed from it in their site selection and 18S rDNA sequences. The description of these species is as follows:

M. wootteni sp. n.

Large, white, round plasmodia of 300–400×100–180 were found in the fins of roach fingerlings (Figs. 5 and 6). "Muelleri-type" spores were short, ellipsoidal or roundish, somewhat enlarged anteriorly in frontal view (Figs. 1e and 4e), and lemon-shaped in lateral view (Figs. 1e (inset) and 4f). Sutural line is indistinct and the sutural edge is moderately protruding. Valves are thin, symmetrical, and smooth. Spores are 13.3±0.96 (11.6–15) long, 11.6±0.73 (10.2–13) wide, and 5.7±0.37 (5.5–6.0) thick. Two polar capsules pyriform, closely equal in size, 6.2±0.75 (5.0–7.0) long and 3.9±0.49 (3.0–4.4) wide, tapering toward the discharging canals of polar filaments. Polar filaments coiled with six to seven turns in the polar capsule situated perpendicularly to the longitudinal axis of the capsule. The spore has a strong, large, triangular intercapsular appendix of 2.7±0.76 (2.0–3.5) at the anterior end. Sporoplasm nuclei are indiscernible, and a large iodinophilous vacuole is found in the sporoplasm. Four to six sutural edge markings and a thin oval mucous envelope around the posterior end of the spores.

Type host: roach, *Rutilus rutilus* (L.) (Cyprinidae).

Locality: Lake Balaton, Hungary

Site of tissue development: connective tissue between the fin rays

Type material Voucher spores of *M. wootteni* sp. n. in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18212. The 18S rDNA sequence of *M. wootteni* sp. n. has previously been deposited in Genbank by Eszterbauer et al. (2006) under the accession number DQ231157.

Etymology The species is named after the well-known British fish pathologist Dr. Rodney Wootten.

Histology In histological sections, plasmodia were found in the connective tissue under the skin between two cartilaginous fin rays (Fig. 6). The ectoplasm of the plasmodium was bordered by a thin connective tissue layer. No connection was found between plasmodia and cartilaginous elements.

Molecular data DNA sequences of two *M. wootteni* samples from different roach specimens showed 99.9% similarity (G/A nucleotide alteration in position no. 930), and differed from *M. muelleri* ex. chub in 11.2%, from *M. fundamentalis* sp. n. in 10.2%, from *M. gayerae* in 8.9%. Triactinomyxon "type D-1" (DQ231146) characterized by Eszterbauer et al. (2006) showed 99.9% similarity to *M.*

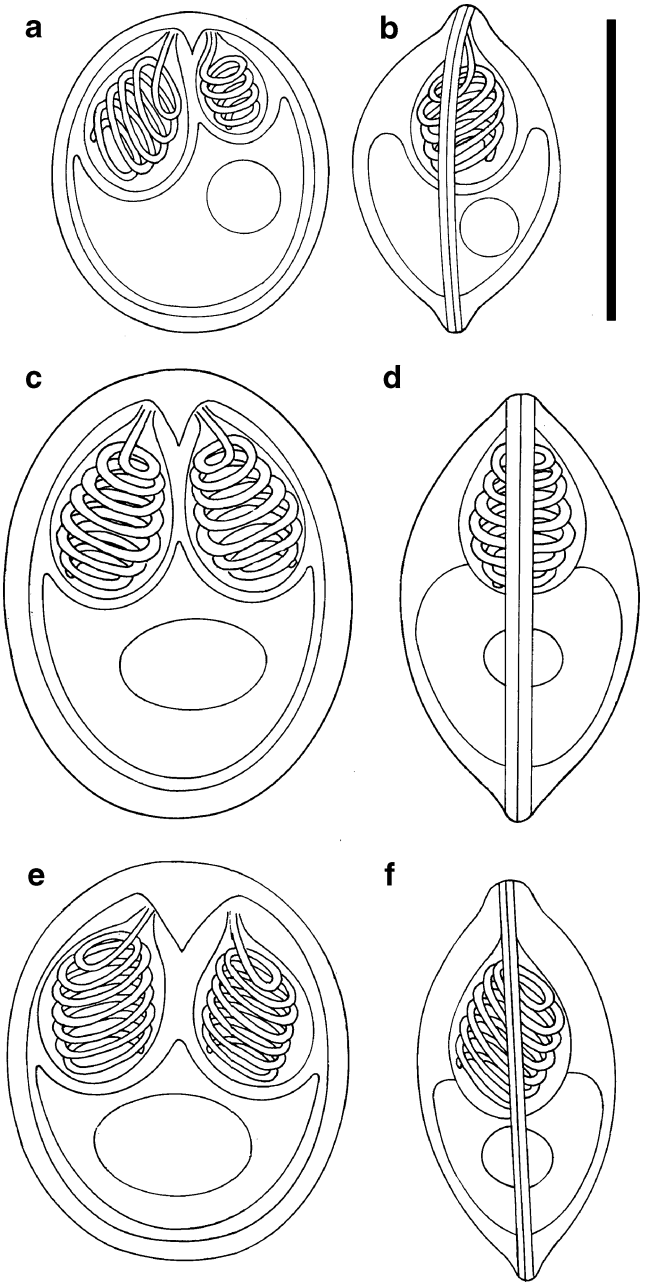


Fig. 4 Schematic drawings of spores of different *Myxobolus* spp. infecting roach. **a** *M. diversicapsularis* in frontal view and **b** in sutural view. **c** *M. fundamentalis* sp. n. in frontal view and **d** in sutural view. **e** *M. wootteni* sp. n. in frontal view and **f** in sutural view. *Bar*=10 μm

wootteni sp. n. (1 nt difference over a 894-bp-long DNA fragment).

Remarks In its spore morphology, *M. wootteni* resembles *Myxobolus bramae* Reuss 1906, *M. muelleri* Buetschli, 1882, and other "muelleri-type" *Myxobolus* spp. (*Myxobolus muellericus, M. rutili, M. sommervillae*, and *M. feisti*), but differs from them in its larger and wider spores and in its well-developed intercapsular appendix. We found *M. wootteni* sp. n. plasmodia that developed exclusively in the fin of roach fingerlings.

M. rutili Donec et Tozyyakova, 1984

Elongated plasmodia of this species, reaching a size of 1–1.5 mm, were located in the central part of gill filaments between the cartilaginous gill ray and the arteria afferens (Fig. 7). Spores were ellipsoidal or short ellipsoidal in frontal view (Figs. 1f and 10a) and lemon-shaped in lateral view (Figs. 1f (inset) and 10b). Spores were 13.1±1.26 (11.0–15.7) long, 9.9±0.77 (8.0–10.8) wide, and 6.8±1.17 (6.0–9.0) thick. Two polar capsules pyriform, closely equal in size, 5.9±0.9 (4.0–7.2) long, and 3.4±0.62 (3.0–3.7) wide, tapering toward discharging canals of polar filaments. Polar filaments are coiled with six turns in polar capsule, situated perpendicularly to the longitudinal axis of the capsule. Spore has a 1.3±0.31 (0.7–1.5) long triangular intercapsular appendix at the anterior end. Sutural line is indistinct. The sutural edge is moderately protruding. Valves are thin, symmetrical, and smooth with indistinct four to six edge markings. Sutural extensions are present, approximately 0.6 μm at the anterior and 1 μm at the posterior end of the spores. Sporoplasm nuclei are indiscernible, and a small iodinophilous vacuole is found in the sporoplasm. Mucous envelope is not found.

Type host: roach, *Rutilus rutilus* (L.) (Cyprinidae).

Locality: Kis-Balaton water reservoir, Hungary

Additional localities: Lake Balaton, small creeks inflowing to Lake Balaton

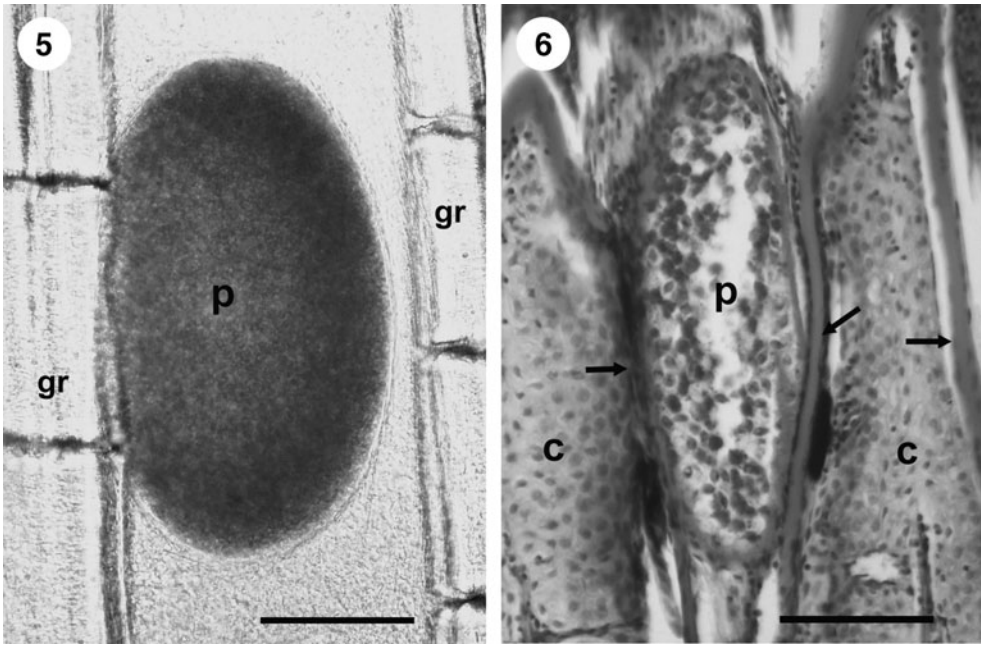
Site of tissue development: central region of the gill filament

Type material Syntype spores in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No.HNHM-18214. The 18S rDNA sequence of *M. rutili* was deposited in Genbank under the accession number GU968201.

Histology Large, elongated plasmodia developed at the central part of gill filaments in the connective tissue attaching externally to the arteria afferens (Fig. 7).

Molecular data 18S rDNA sequences of the two *M. rutili* samples were 100% identical. It resembled the best *Myxobolus dogieli* isolate 1 (EU003977) from the heart of *Abramis brama* (95.8%).

Remarks While studying "muelleri-type" *Myxobolus* spp. of roach, Donec and Tozyyakova (1984) (Donec and Shulman 1984) observed that different organs (cornea,



Figs. 5-6 Plasmodium (***p***) of *M. wootteni* sp. n. in the fin between two gill rays (*gr*) attaching to one of the rays. Fresh mount. *Bar*=100 μm. Inside the cartilaginous wall of the fin ray (*arrows*), loose connective tissue can be seen (*c*). Histological section, H&E. *Bar*=100 μm

gills, gill arch, muscles, and fins) were infected with various sizes and shapes, but morphologically with similar spores and described spores with a size of 14–16.5 μm in length as *M. rutili*. In the same study, Donec (Donec and Shulman 1984) depicted some other spores as well, the shape of which better resembles *M. sommervillae* and *M. feisti*. We assume, therefore, that the original description of *M. rutili* covers more than one *Myxobolus* species. Due to our findings, only spores collected from large cysts locating extravasally in the central region of filaments should be designated as *M. rutili*.

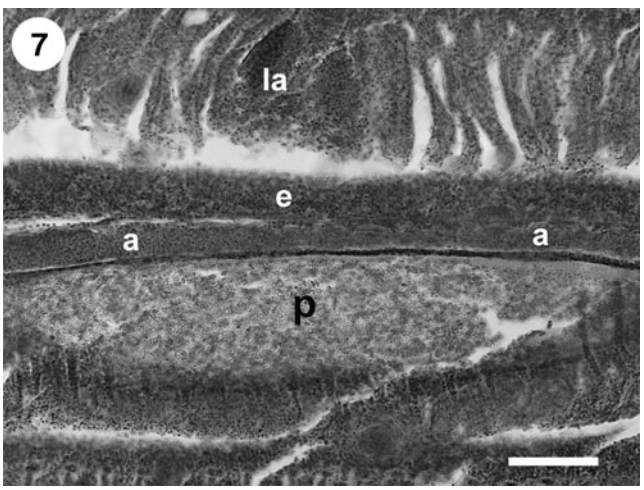


Fig. 7 Plasmodium (*p*) of *M. rutili* lays in the center of a gill filament close to arteria afferens (*a*) covered by multilayered epithelium of the non-lamellated part of the filament. Lamellae (*la*) of the neighboring filament are also seen. Histological section, H&E. *Bar*=50 μm

On the basis of spore morphology, *M. rutili* resembles other "muelleri-like" *Myxobolus* spp., but it differs from them in its 18S rDNA sequence and its specific tissue location. The spores of this species were somewhat more elongated and longer than those of *M. sommervillae* sp. n., but in most cases, no clear morphological difference was observable between the spores of the two species. Both species form elongated plasmodia in the gill filaments, but plasmodia of *M. sommervillae* sp. n. develop inside the lumen of arteria efferens. Plasmodia of *M. rutili*, however, are located outside the lumen of arteries in the central part of gill filaments between the aorta and the cartilaginous gill rays. Two 18S rDNA sequences obtained from samples collected from roach in the Lesence creek entering Lake Balaton and from the Kis-Balaton water reservoir were 100% identical. Their DNA sequences differed from *M. sommervillae* sp. n. in 10.2%. *M. rutili* showed a relatively low similarity to *M. muelleri* (90.5%) and *M. muellericus* (90.4%) as well.

M. sommervillae sp. n.

Elongated plasmodia of the species, reaching a size of 1–1.5 mm, are located in the lumen of the arteria efferens of gill filaments (Fig. 8). Spores are short ellipsoidal in frontal view (Figs. 1g and 10c), and lemon-shaped in lateral view (Figs. 1g (inset) and 10d). Spores are 11.8±0.95 (10.5–13.5) long, 9.7±0.76 (8.4–11.2) wide, and 7.2±0.39 (6.6–7.5) thick. Two polar capsules pyriform, closely equal in size, 6.0±0.38 (5.4–6.7) long, and 3.3±0.35 (3.0–4.2) wide, tapering toward discharging canals of polar filaments. Polar filaments are coiled with six turns in polar capsule, situated perpendicularly to the longitudinal axis of the

capsule. The spore possesses a 1.8±1.85 (1.5–2.7) long triangular intercapsular appendix at the anterior end. Sutural extensions, approximately 0.6 at the anterior and 1 at the posterior end of spores, are seen only in sutural view. Sutural line is indistinct. Sutural edge is moderately protruding. Valves are thin, symmetrical, and smooth with indistinct four to six edge markings. Sporoplasm nuclei are indiscernible, and a small iodinophilous vacuole is found in the sporoplasm. Mucous envelope is not found.

Type host: roach, *Rutilus rutilus* (L.) (Cyprinidae)
Locality: Kis-Balaton water reservoir, Hungary
Additional localities: Tisza River
Site of tissue development: artery of the gill filament

Type material Syntype spores in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18213. The 18S rDNA sequence of *M. sommervillae* was deposited in Genbank under the accession number GU968202

Etymology The species is named after the well-known British fish parasitologist Prof. Christina Sommerville.

Histology Large, elongated plasmodia developed inside the arteria efferens of gill filaments (Fig. 8). Mature spores from ruptured plasmodia were often released into the bloodstream (Fig. 9).

Molecular data 18S rDNA sequences obtained from two roach specimens collected in the Tisza River region and the Kis-Balaton showed 100% identity. The DNA sequence of *M. sommervillae* sp. n. showed high similarity to *M. muelleri* from chub (DQ439806; 98.8% similarity) and *M. bramae* from common bream (AF507968; 98.7%).

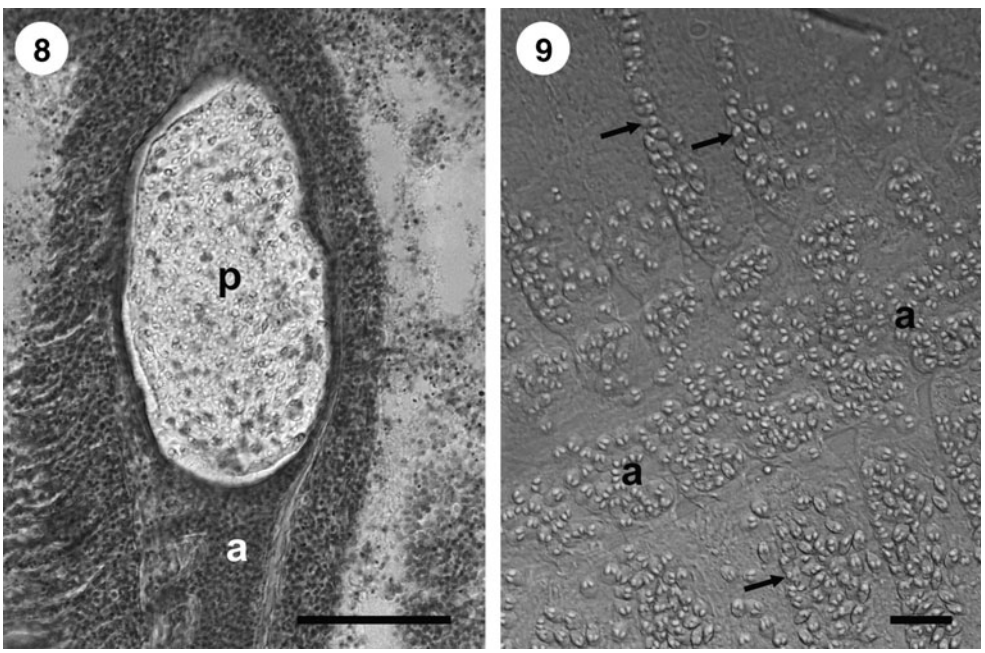
Remarks On the basis of spore morphology, *M. sommervillae* sp. n. (Fig. 10) resembled other "muelleri-like" *Myxobolus* spp., but it differed from them in its site selection and tissue tropism. Except for minor differences in the size of spores (Table 3) and their site selection, this species resembled the best *M. rutili*. However, the two species clearly differed from each other in their 18S rDNA sequences, as they share only 89.8% identical nucleotides over a 1,323-bp-long DNA fragment.

M. feisti Molnár et al. 2008

The occurrence, histology, and spore morphology (Fig. 1h) of this species was recently described by Molnár et al. (2008). The prevalence of this species might be higher than stated in Table 2, since, in case of low intensity of infection, the detection of small plasmodia in the cartilaginous gill rays is rather difficult.

PCR, DNA sequencing, and phylogenetic analyses

The primer pairs 18e–18r, MX5–MX3, and SphF–SphR successfully amplified approximately 2,000, 1,600, and 1,400 bp fragments of the 18S rDNA from samples



Figs. 8-9 Plasmodium (*p*) of *M. sommervillae* sp. n. located inside the artery (*a*) of a gill filament. Histological section, H&E. *Bar*=100 μm *M. sommervillae* sp. n. spores released from a disrupted plasmodium in the filament artery (*a*) and in the lamellae (*arrows*). Fresh mount. *Bar*=30 μm

examined, respectively. The 14 samples sequenced belonged to eight well-distinguishable *Myxobolus* species, three of which have not been studied at the DNA level previously. 18S rDNA sequences obtained were deposited in GenBank under the accession numbers listed in Table 4. Phylogenetic analyses were performed on the basis of a 1,661-bp-long edited alignment that contained 41 DNA sequences.

Maximum likelihood and Bayesian inference analyses confirmed the topology of the phylogenetic tree generated by neighbor-joining analysis (Fig. 11). Of the "muelleri-type" species, only *M. sommervillae* sp. n. was located within the cluster of *M. muelleri*. Despite the similar location in gill lamellae, the location of *M. diversicapsularis* sp. n. samples were distant from *M. intimus*, which formed a sister clade of the muscle parasite *M. pseudodispar*. *M. wootteni* sp. n. clustered with the group of *M. fundamentalis* sp. n. and *M. diversicapsularis*. Furthermore, *M. rutili*, another "muelleri-type" species, was located more closely to *M. muellericus* then to *M. muelleri* itself.

Discussion

Most of the known *Myxobolus* species have been described from European freshwater fishes (Eiras et al. 2005). However, the number of papers describing new myxozoan species from North and South America (Cone et al. 2004; Eiras et al. 2010), from Asia (Baska et al. 2009; Kaur and Singh 2010), from Africa (Abdel-Ghaffar et al. 2008), and from marine fishes (Ali et al. 2007) have been constantly increasing in recent years.

Roach *(Rutilus rutilus)*, a common cyprinid fish in Europe, seems to be infected by several, mostly site-specific *Myxobolus* species. Of them, three new species were found in Hungarian lakes and rivers, and the occurrence of five known species was recorded. Of the species studied in this paper, *M. pseudodispar* was detected most frequently. The occurrence, development, electron microscopic, and molecular aspects of the parasite were previously studied in detail (Baska 1986; Székely et al. 1999, 2001; Alvarez Pellitero et al. 2002; Molnár et al. 2002), although several questions concerning its host specificity and genetic variability still need to be answered. In the course of the present study, the plasmodia of this species could easily be detected in the muscle of fish, and its disseminated spores were found in different seasons in the melano-macrophage centers of inner organs like kidney. Similarly, *M. intimus* infection studied by Rácz et al. (2004) in roach from Lake Balaton proved to be rather common in spring months. However, only the present study revealed that *M. diversicapsularis* often caused a concurrent infection with *M. intimus*, and their plasmodia developed in the same location inside the gill lamellae of roach. Due to the DNA sequence analyses of *M. intimus*, developmental stages (both myxospores and actinospores) obtained and preserved from transmission experiments by Rácz et al. (2004), our findings questioned that triactinomyxon-type actinospores observed by Rácz et al. (2004) are the developmental stage of *M. intimus*. Our molecular findings suggested that the triactinomyxons from the transmission experiments of *M. intimus* are the actinospore of *M. diversicapsularis*, as DNA sequences of *M. diversicapsularis* and actinospores from *M. intimus* experiments were 100% identical (data not shown). Besides, a triactinomyxon-type actinospore found by Hallett et al. (2005) showed 99.6% similarity to *M. diversicapsularis*. The DNA sequence analysis of the present study demonstrated that *M. intimus* was 99.9% similar to aurantiactinomyxon "type 1" found by Hallett et al. (2006) in a naturally infected oligochaete stock. These findings suggest that *M. intimus* most likely possesses an aurantiactinomyxon-type actinospore.

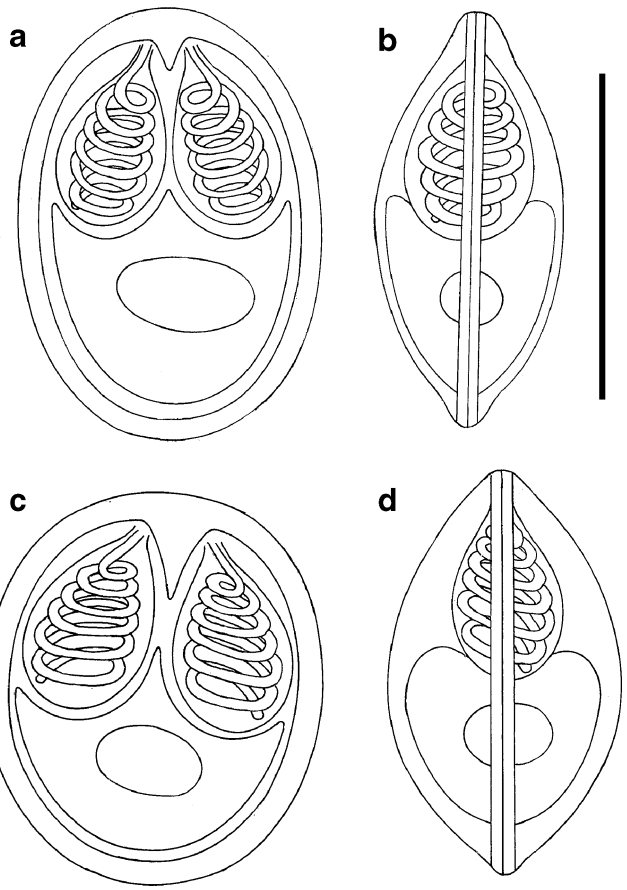


Fig. 10 Schematic drawings of spores of different *Myxobolus* spp. infecting roach. **a** *M. rutili* in frontal view and **b** in sutural view. **c** *M. sommervillae* sp. n. in frontal view and **d** in sutural view. *Bar*=10 μm

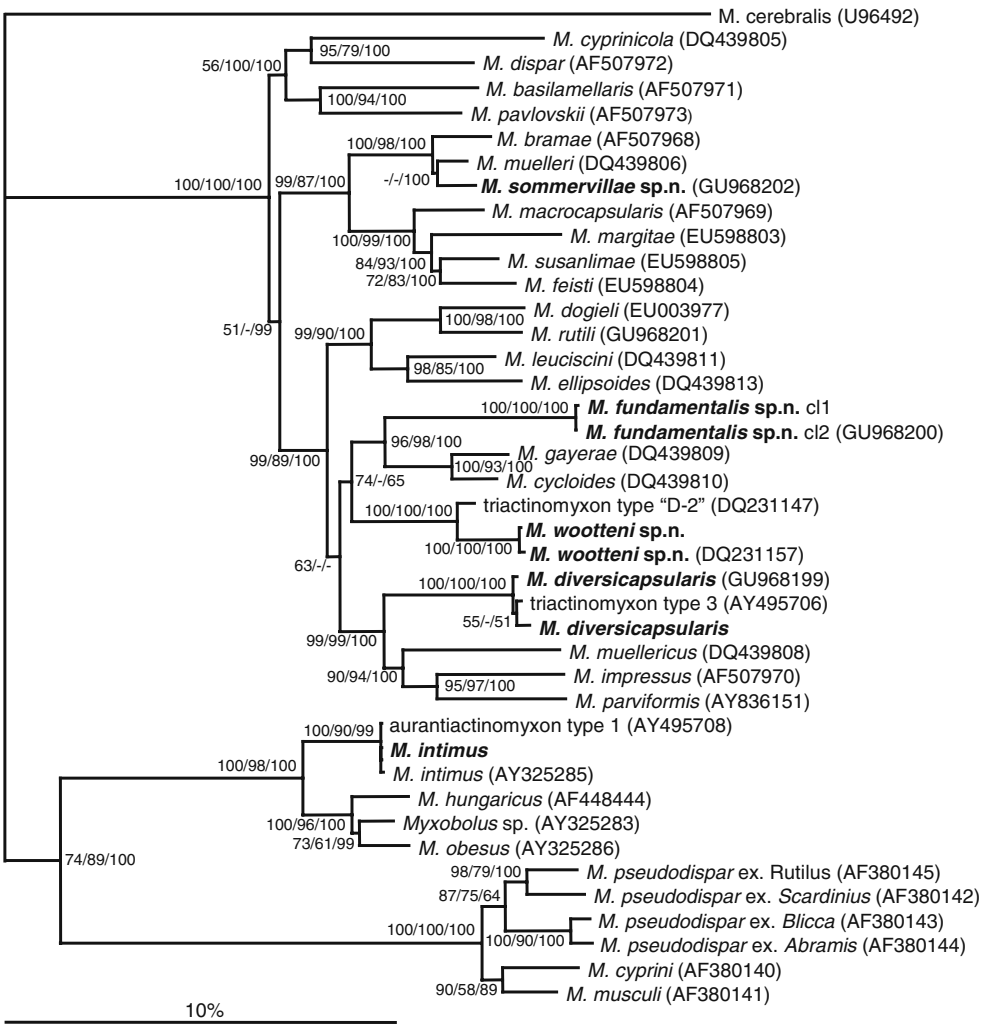


Fig. 11 Phylogenetic tree constructed based on neighbor-joining, maximum likelihood, and Bayesian inference analyses of the 18S rDNA sequences of selected myxozoans. *Numbers at nodes* indicate the respective bootstrap confidence levels in percent. *Myxobolus cerebralis* was used as the outgroup

The above *Myxobolus* spp., as well as *M. fundamentalis* sp. n., have relatively unique spore shapes, and besides their typical locations, they could easily be differentiated even on the basis of morphological features. However, some other *Myxobolus* spp. examined showed rather similar spore morphology, which corresponded to the original description of *M. muelleri* Buetschli, 1882, one of the first described *Myxobolus* species of cyprinid fishes. *M. muelleri* was for a long time thought to be a parasite of several cyprinid fishes (Shulman 1966). However, Reuss (1906) separated "muelleri-type" spores developing in common bream from the above species and described *M. bramae*, while Donec and Tozyyakova (1984) (Donec and Shulman 1984) found slight morphological and size differences in "muelleri-type" spores of roach, and erected *M. rutili* as a new species. Although studies on the fine morphology, site selection, and tissue tropism of morphologically similar spores assist species identification, molecular characterization of spores is required for proper species differentiation. In the course of the present study, four morphologically similar "muelleri-type"species (*M. feisti, M. rutili*, *M. wootteni* sp. n., and *M. sommervillae* sp. n.) were examined. Of them, 18S rDNA sequences of *M. rutili, M. sommervillae* sp. n., and *M. wootteni* sp. n. clearly differed from each other and from the sequences of *M. feisti* described by Molnár et al. (2008). The 89.8% difference between DNA sequences of *M. rutili* and *M. sommervillae* sp. n. was unexpected, as the two species developed in a similar intrafilamental location in roach, and only slight size differences were found in spore measurements. Detailed histological examinations, however, proved that besides differences in 18S rDNA sequences, the two species show different histotropism as well. *M. sommervillae* sp. n. belongs to the filamental vascular group, while *M. rutili* is a filamental but extravascular parasite, which forms plasmodia in the non-lamellar part of the central connective tissue, and located between arteria efferens and the cartilaginous gill ray. Molnár et al. (2007) pointed out that in chub, great differences were observed among the 18S rDNA sequences of the filamental species *M. muelleri* and

the DNA sequence of *M. muellericus*, a species developing in gill lamellae. Both in chub and roach, spores, which had a "muelleri-like" morphological character, but were observed in different locations of gills, possessed different 18S rDNA sequences. Of the filamental species with morphologically similar spores, clear difference (89.8%) was detected between *M. rutili* and *M. sommervillae* sp. n., and the chondrophil species *M. feisti* showed only 92.6% similarity to *M. sommervillae* and 87.4% to *M. rutili*. Spores identified as *M. wootteni* sp. n. seem to differ somewhat from the group of *M. muelleri*-like species. By the widened anterior part of its spores, by its roundish polar capsules, and its large intercapsular appendix, this species can even morphologically be distinguished easily from other species examined.

Spores of *M. fundamentalis* sp. n. differ from spores of other species examined in its large, symmetrical spores and in its specific location in the cartilaginous gill arch under the filaments. Its location resembles *M. basilamellaris* from common carp, but the latter species has smaller spores. In its large spores, *M. fundamentalis* sp. n. also resembles morphologically *M. cycloides* Gurley, 1894; *M. cyprinicola* Reuss 1906 and *M. gayerae* Molnár et al. 2007, but it differs from them in its 18S rDNA sequence, and in locating typically in the cartilaginous gill arch.

Although in the course of the survey a great number of fish was dissected, and in most cases complete parasitological examinations were carried out, the prevalence of *Myxobolus* spp. infection in roach (Table 2) has to be considered with caution. In order to be able to detect a given *Myxobolus* infection, fish of various size groups should be collected in large numbers from several habitats and in different seasons. For *M. pseudodispar*, the plasmodia of which could be found both in fingerlings and aged fish and disseminated spores often occur in inner organs, the rate of infection was easily evaluated. However, for species like *M. intimus* and *M. diversicapsularis*, which develop spores in older fish specimens only in spring, prevalence data could have been exact only for a given season and fish age class. The same concerns the occurrence of *M. wootteni* sp. n., which infected only the fins of fingerlings. The observation of small-sized plasmodia in gill cartilage and hidden plasmodia of *M. fundamentalis* sp. n. in the gill arch is rather time consuming and requires a special technique of dissection. Finding large plasmodia of *M. rutili* and *M. sommervillae* sp. n. seemed to be more simple, but in this case, histology and molecular characterization were also required to correctly identify the species.

Three other species (*M. alievi* Gazimagomedov, 1970, *M. chernovae* (Chernova 1970, syn. *M. lomi* Chernova, 1970), *M. marginatus* Kulemina, 1969) have been described from roach. We were unable to identify these poorly described and inadequately illustrated species with those found in the survey.

The primary purpose of the survey was to enlarge our knowledge on the myxosporean fauna of roach. A more general purpose was, however, to obtain further data on the host specificity and site selection of *Myxobolus* spp. Previous studies on chub (Molnár et al. 2007, 2008), a leuciscinid-cyprinid fish with close relation to roach, showed that chub is regularly infected at least by eight different *Myxobolus* species. Though in the present study eight *Myxobolus* species was also found in roach, DNA sequence analysis revealed that none of the morphologically identical or similar species corresponded to those found in chub.

Results obtained in the course of this survey suggest that the number of host-, site- and tissue-specific species is much higher than hitherto expected. A single cyprinid species might be infected by more than a dozen specific *Myxobolus* spp. In the study, our aim was also to highlight the need for the consideration of the type host in the original species description. Our opinion is that in those cases, when the author of the original species description designated more than one fish as type host, the typical host (i.e., in which the parasite is most frequently found) should be considered valid at re-descriptions.

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