Journal of Fish Diseases 2009, **32,** 219–231 doi:10.1111/j.1365-2761.2008.00976.x

Myxobolus erythrophthalmi sp. n. and *Myxobolus shaharomae* sp. n. (Myxozoa: Myxobolidae) from the internal organs of rudd, *Scardinius erythrophthalmus* (L.), and bleak, *Alburnus alburnus* (L.)

K Molnár, E Eszterbauer, Sz Marton, G Cech and Cs Székely

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Abstract

During a survey of myxosporean parasites of cyprinid fish in Hungary, infections caused by unknown *Myxobolus* spp. were found in the internal organs of rudd, *Scardinius erythrophthalmus,* and bleak, *Alburnus alburnus*. Small plasmodia developed in blood vessels of the kidney, liver, testes and intestinal wall. The parasites were studied on the basis of spore morphology and by histological and molecular methods. In most cases, plasmodia were surrounded by host tissue without a host reaction; however, in advanced cases, a connective tissue capsule was seen around plasmodia. Spores collected from the two fish species differed from each other and from the known *Myxobolus* spp. both in their morphology and 18S rDNA sequences. The two species, described as *M. erythrophthalmi* sp. n. from rudd and *M. shaharomae* sp. n. from bleak, are characterized by a specific histotropism to blood vessels, while the organ specificity involves the kidney and for the latter species, most internal organs.

Keywords: 18S rDNA, histology, molecular phylogeny, *Myxobolus*, Myxozoa, site selection.

Introduction

In the course of surveys on the parasite fauna of cyprinids from Hungarian natural waters, several *Myxobolus* species were recorded from cyprinid fish (Lom 1969; Molnár 1971, 1997; Ergens, Gussev, Izyumova & Molnár 1975; Molnár & Baska 1999; Molnár & Székely 1999; Eszterbauer 2004; Molnár, Marton, Eszterbauer & Székely 2006). While several *Myxobolus* species have been described from bleak, *Alburnus alburnus* (L.), little is known about the *Myxobolus* infections of rudd, *Scardinius erythrophthalmus* (L.). In their synopsis on members of the genus *Myxobolus*, Eiras, Molnár & Lu (2005) recorded 751 known species, while Lom & Dyková (2006) reported 792 valid species. Of these, however, only three species, *M. scardinii* Reuss, 1906, a parasite of the gill, *M. physophilus* Reuss, 1906, a parasite from the swim bladder wall and *M. destruens* Schurmans-Stekhoven, 1920, a parasite of the muscles were described from rudd as the type host, while *M. alburni* Donec, 1984, *M. ergensi* Lom, 1969; *M. margitae* Molnár, 2000; *M. obesus* Gurley, 1893 and *M. saidovi* Gazimagomedov, 1970 were identified in bleak. In Hungary only a single species, *M. pseudodispar* has been described from rudd (Molnár, Eszterbauer, Székely, Dán & Harrach 2002) and four species (*M. alburni, M. margitae, M. obesus* and *M. pseudodispar*) have been reported from bleak (Molnár 2000). In the Russian literature, 16 and 20 *Myxobolus* species described from other cyprinid type hosts were recorded as potential parasites of rudd and bleak, respectively (Donec & Shulman 1984).

In this study, two novel *Myxobolus* species infecting the inner organs of rudd and bleak are described, and data are presented on their spore morphology, histotropism and molecular characteristics.

Correspondence *K Molnár, Veterinary Medical Research Institute, Hungarian Academy of Sciences, 21 Hungária krt, H-1143 Budapest, Hungary (e-mail: molnar@vmri.hu)*

Materials and methods

During a survey of the myxosporean infections of several cyprinid species in Hungary, rudd and bleak were collected from March 2000 to September 2007. Rudd were collected from Lake Balaton and from the Kis-Balaton water reservoir. Sixty-nine rudd fingerlings 2–4 cm in length, 42 rudd 4–8 cm in length (1- to 3-year old) and 55 rudd 8–17 cm in length (4- to 7-year old) were seine netted. For bleak, 142 specimens from Lake Balaton and 63 specimens from the River Danube were examined. The total length of 1- to 4-year-old bleak varied from 4–11 cm. The fish were seined, transported to the laboratory alive in oxygenated plastic bags, and kept in aquaria. Fish killed by a cervical cut were examined within 4 days after capture. From 2006, fish were sedated prior to euthanasia by addition of clove oil into water. In the course of a complete parasitological examination the hemibranchs of the gills, and the fins, were examined under a dissecting microscope for the presence of *Myxobolus* plasmodia. Pieces of the kidneys, liver, spleen and testes were compressed between two glass plates and examined first under a stereomicroscope and then at 200–400× magnification with a Zeiss Jenaval compound microscope (Carl Zeiss, Jena, GDR). The gut was first examined whole under a dissecting microscope, then cut open and compressed between two glass plates. Plasmodia were mechanically isolated with preparation needles under a dissecting microscope. *Myxobolus* spores from the isolated and opened cysts were first studied in a wet mount, and then some spores were placed in glycerine-jelly (7 g gelatine in 42 mL water, 50 mL glycerine and 1 g carbolic acid) onto a slide under a cover slip and preserved as a reference slide. Other spores (preferably from the same plasmodium) were collected into Eppendorf tubes and stored at −20 °C for subsequent molecular examination. Tissue samples from infected organs containing developing and mature plasmodia were fixed in Bouin's solution, embedded in paraffin wax, sectioned at 4–5 μm and stained with haematoxylin and eosin (H & E). The maturity of spores was checked by placing spores into a 0.4% urea solution. Spores of a given plasmodium were regarded as mature when at least 90% of them extruded polar filaments in this solution. Unfixed spores were studied by Nomarski differential interference contrast with an Olympus BH2 microscope (Olympus, Tokyo, Japan) Fresh spores were photographed with an Olympus DP10 digital camera (Olympus, Tokyo, Japan) or recorded on videotapes, digitized images were obtained, and measurements were taken with IMAGO® softwares (Demeter Ltd., Budapest, Hungary). All measurements are given in micrometres.

Molecular methods

For DNA extraction, samples were centrifuged at 5000 ***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 0.4 mg mL^{-1} proteinase K) and incubated at 55 °C for 3–4 h. DNA was then purified using the Miniprep Express Matrix (BIO 101). A nested PCR system was used for amplification. DNA was amplified with the 18e–18r universal primer pair. This was followed by a second round PCR with the MX5–MX3 primer pair or in some cases with SphF–SphR (Table 1). In both steps of the nested PCR, the total volume of the PCR reactions was 50 μL, which contained approximately 10–150 ng DNA, 1× *Taq* PCR reaction buffer (MBI Fermentas), 1.25 mM $MgCl_2$, 0.2 mM dNTP mix (Sigma), 1 μM of each primer and two units of *Taq* DNA polymerase (MBI Fermentas). MJ Research PTC-200 (Mf Research Inc., Watertown, MA, USA) and Biometra T1 thermocyclers (Biometra Biomedizinische Analytik GmbH, Goettingen, Germany) were used for amplification. Amplification conditions in the first round were: 95 °C for 50 s, 56 °C for 50 s and 72 °C for 80 s for 35 cycles, with a terminal extension at 72 °C for 7 min. This was followed in the second round with primers MX5 and MX3: 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s for 35 cycles and was terminated with an extension period at 72 °C for 7 min. For PCR using SphF and SphR primers, the same conditions were applied as with the 18e–18r primer pair. The PCR products were electrophoresed in 1.0% agarose gels (Sigma) in TBE buffer and then purified with a QIAquick Gel Extraction Kit (Qiagen).

For samples with an insufficient amount of amplified PCR product for direct DNA sequencing, purified DNA was cloned into a pGEM-T Vector System I (Promega) following the manufacturer's manual. Positive clones were screened by digestion with restriction enzyme *Msp*I and then by sequencing with the universal pUC/M13 primer (Promega). Purified PCR products and/or at least three positive clones per sample were sequenced in both directions with primers listed in Table 1 using the ABI BigDye Terminator version 3.1 Cycle

Table 1 Primers used for PCR and/or sequencing

Name	Sequence (5′ → 3′)	Source
18e	CTG GTT GAT TCT GCC AGT	Hillis & Dixon (1991)
18r	CTA CGG AAA CCT TGT TAC G	Whipps *et al.* (2003)
MX5	CTG CGG ACG GCT CAG TAA ATC AGT	Andree *et al.* (1999)
MX3	CCA GGA CAT CTT AGG GCA TCA CAG A	Andree *et al.* (1999)
MB5r	ACC GCT CCT GTT AAT CAT CAC C	Eszterbauer (2004)
MB3f	GAT GAT TAA CAG GAG CGG TTG G	Eszterbauer (2004)
MC5	CCT GAG AAA CGG CTA CCA CAT CCA	Molnár *et al.* (2002)
MC3	GAT TAG CCT GAC AGA TCA CTC CAC GA	Molnár *et al.* (2002)
SphF	ACT CGT TGG TAA GGT AGT GGC T	Eszterbauer & Székely (2004)
SphR	GTT ACC ATT GTA GCG CGC GT	Eszterbauer & Székely (2004)

Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). 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 BIOEDIT software (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. Phylogenetic calculations were performed with PHYLIP version 3.6a (Felsenstein 1997). The data were analysed with maximum likelihood (DNAML with transversion/transition ratio 1:2, empirical base frequencies, one rate class for nucleotide substitution and global rearrangements) and distance matrix analysis (DNADIST using Kimura-2 parameter followed by FITCH with global rearrangements). Clade support was assessed with bootstrapping (100 replicates for maximum likelihood and 1000 replicates for the distance matrix method). *Ceratomyxa shasta* was chosen as an outgroup.

Results

In the course of the survey, plasmodia containing morphologically similar spores were found in the internal organs of rudd and bleak. However, spores from the two fish species differed from each other in size, structure of the suture and 18S rDNA sequences.

Myxobolus infection in rudd

In squash preparations of the internal organs of rudd, plasmodia of a hitherto unknown *Myxobolus* sp. were found in the kidney. Less frequently, plasmodia with similar spores were found in the liver and the intestinal wall, but these rare cases were not studied in detail. Small plasmodia of this species were found in 13 (19%) of 69 fingerlings, in 28 of 1- to 3-year-old rudd (66% of 42 examined fish) and in 32 (58%) of 55 specimens of 4- to 7-year-old rudd. Globular plasmodia (Fig. 1) containing mature spores of a *Myxobolus* species measuring 200–350 in diameter were located in the interstitium of the kidney, but not affecting the renal tubules. In some cases, young, elongate plasmodia were located in the blood vessels. These contained only plasmodial developmental stages. In addition to the new species, individual spores engulfed by macrophages (Fig. 2) or small groups of spores were also found inside the melano-macrophage centres in the kidney (Fig. 9). They were identified as spores of *M. pseudo-dispar* Gorbunova, 1936, a common parasite of muscle cells. In muscle pieces compressed between two glass plates, elongated intracellular plasmodia of *M. pseudo dispar* were often found in muscle cells. The survey revealed high prevalence in rudd: 48% in

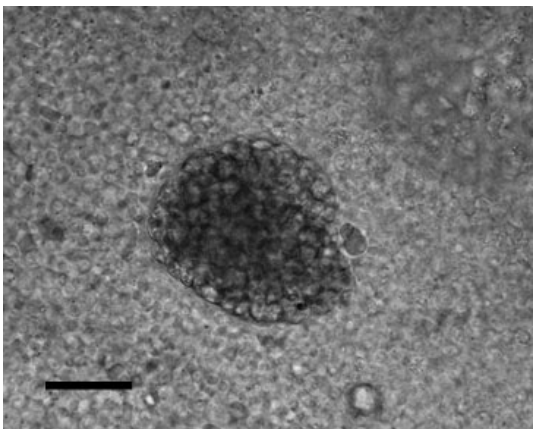

Figure 1 *Myxobolus erythrophthalmi* sp. n. plasmodium filled by mature spores in the renal interstitium of a rudd (fresh mount, bar = 100 μm).

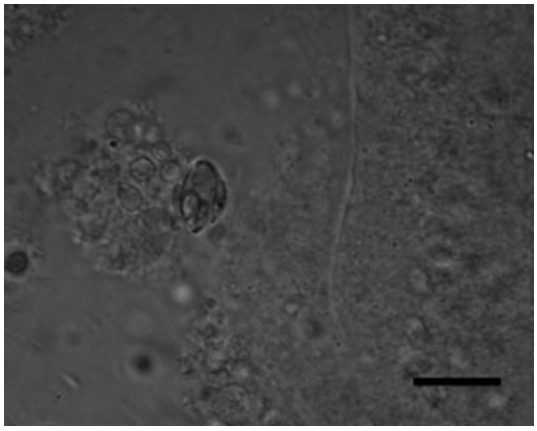

Figure 2 A scattered spore of *Myxobolus pseudodispar* arrow engulfed by a macrophage arrow-head in the renal interstitium of a rudd (fresh mount, bar = 20 μm).

fingerlings, 42% in 1- to 3-year-old fish and 64% in older fish. Intensive infection with *M. pseudodispar* was mostly observed in fingerlings. In addition, cysts of an unknown *Myxobolus* spp. were found in the artery of the gill filaments of 5 and in the fins of 3 rudd. The spores of the above *Myxobolus* sp. differed significantly from spores found in the internal organs. Despite the dissection of approximately the same number of cyprinids other than rudd, no infection caused by this new species was found in other cyprinids living in the same biotope [i.e. *Rutilus rutilus* (L.), *Blicca bjoerkna* (L.) and *Abramis brama* (L.)]. The species was named as *M. erythrophthalmi* sp. n. and is described as follows. Measurements are given in micrometres.

Myxobolus erythrophthalmi sp. n.

Mature plasmodia were round or ellipsoidal up to 350 in length. Young plasmodia containing only early sporogonic stages were elongate. Spores (Figs 3a & 4a) were ellipsoidal in frontal view and lemon-shaped in sutural view (Figs 3b & 4b). Spore measurements are given in Table 2. Polar capsules were pyriform, equal in size, slightly converging anteriorly. Five filament coils arranged obliquely to the capsule length wound loosely in the polar capsule. In most spores, no intercapsular appendix was seen; however, for some spores a very small knob-like thickening between capsules could be observed. A sutural protrusion formed a circular rim around the spore, emerging about 0.7–1.0 over the surface of the spore (Fig. 3c). In sutural view, this rim protruded over the surface of the spore 1–1.2 at the anterior pole, and 0.7–0.9 at the posterior pole. The thickness of the rim measured about 0.7 in sutural view. Seven to nine sutural edge markings were easily observed in fresh spores. There was a single binucleated sporoplasm with round iodinophilous vacuole. Mucous envelope was not found.

Type host

Rudd, *Scardinius erythrophthalmus* (L.) (Cyprinidae).

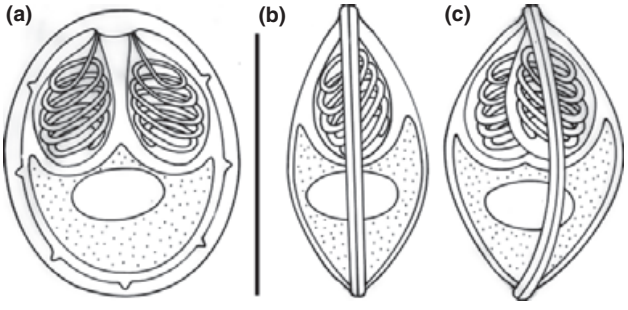

Figure 3 Schematic drawings of *Myxobolus erythrophthalmi* sp. n. spores. (a) Frontal view, (b) sutural view, (c) semi-lateral view (bar = 10 μm).

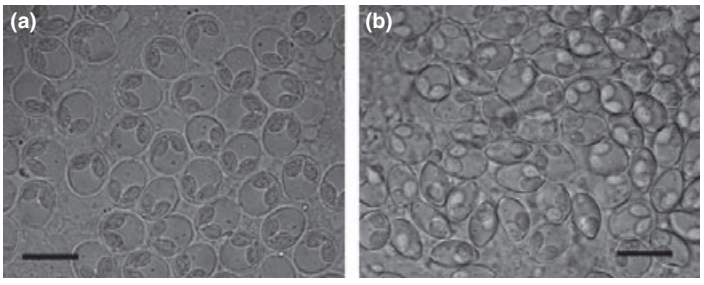

Figure 4 Spores of *Myxobolus erythrophthalmi* sp. n. (a) In frontal view, (b) in sutural and semi-lateral view (bar = 10 μm).

Table 2 Comparison of *Myxobolus erythrophthalmi* sp. n. and *M. shaharomae* sp. n. with related species on the basis of spore morphology

	M. erythrophthalmi	*M. shaharomae*	*M. alburni*	*M. ellipsoides*	*M. destruens*	*M. pseudodispar*
Host	*Scardinius erythrophthalmus*	*Alburnus alburnus*	*Alburnus alburnus*	*Leuciscus cephalus*	*Scardinius erythrophthalmus*	*Rutilus rutilus*
Spore length	11.0 ± 0.52 (10.4–12.0)	13 ± 0.89 (12.1–14.5)	13.7 (13.0–14.0)	12.0 ± 0.28 (11.6–12.3)	10.4 ± 0.82 (9.1–12.0)	(10.0–12.0)
Spore width	9.5 ± 0.44 (8.3–10.2)	10.4 ± 0.80 (9.0–11.3)	11.0 (9.5–12.0)	9.7 ± 0.31 (9.3–10.0)	5.8 ± 1.0 (4.2–7.2)	(7.0–9.5)
Spore thickness	7.0 ± 0.48 (6.3–7.5)	7.3 ± 0.25 (7.0–7.6)	8.0	5.8 ± 0.31 (5.5–6)	4.8	(5.3–6.0)
PC length	5.7 ± 0.46 (5.0–6.3)	6.1 ± 0.31 (5.6–7.0)	5.3 (5.0–5.6)	5.4 ± 0 .31 (5–5.8)	L: 5.8 S: 4.0	L: (4.5–6.2) S: (3.9–5.0)
PC width	3.4 ± 0.26 (3.1–3.7)	3.8 ± 0.18 (3.5–4.1)	3.3 (3.0–3.5)	3.1 ± 0.12 (3.0–3.3)	(1.5–2.0)	L: (3.0–3.7) S: (2.7–3.0)
Thickness of sutural rim	0.7	0.7	n.d.	0.8 ± 0.24 (0.6–1.3)	n.d.	n.d.
No. coils in PC	5	5	4	6	n.d.	3–4
Anterior extension of spore wall	1.0–1.2	0.9	n.d.	n.d.	n.d.	n.d.
Caudal extension of spore wall	0.7–0.9	0.7	n.d.	n.d.	n.d.	n.d.
Reference	Present study	Present study	Molnár 2000	Molnár *et al.* 2006	Schuurmans-Stekhoven 1920	Donec & Shulman 1984

Mean ± SD and range in parentheses are expressed in micrometres (μm).
PC, polar capsule; L, large PC; S, small PC; n.d., no data.

Type locality

Lake Balaton, Hungary.

Other locality

Kis-Balaton water reservoir.

Site of tissue development

Blood vessels in the renal interstitium.

Type material

Syntype spores in glycerine–gelatine and a photo series were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-17828**.** The 18S rDNA sequence of *M. erythrophthalmi* sp. n. was deposited in GenBank under the accession number EU567311.

Etymology

The species is named after the generic name of the host fish.

Histology of infection

Histological studies were made only on plasmodia developing in the kidney. The youngest developmental stages observed were elongated plasmodia inside the blood vessels of the renal interstitium. It was readily observed that plasmodia were located inside a blood vessel and contained late plasmodial developmental stages and some spores (Fig. 5). A part of the eosinophilic wall of the plasmodium attached directly to the endothelium of the blood vessel, the other part, however, was surrounded by erythrocytes flowing freely in the blood. In some histological sections, different parts of the same elongated but curving plasmodia were observed close to each other in the section (Fig. 6). In the late stage of development, plasmodia lost their elongated shape, became round or oval and contained varying numbers of mature spores. The structure of the blood vessel in these plasmodia was no longer observable. These plasmodia were located in the renal interstitium surrounded by a thin connective tissue wall (Fig. 7). In more advanced cases, a capsule composed by multilayered fibrous connective tissue developed around the plasmodium (Fig. 8). As host reaction, melano-macrophages appeared among the spores, infiltrated them,

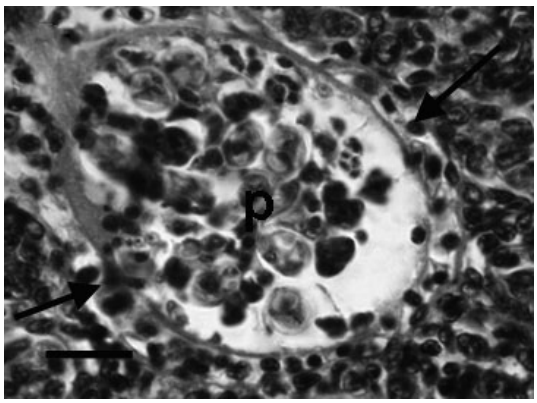

Figure 5 Cross-section of a young *Myxobolus erythrophthalmi* sp. n. plasmodium (p) containing young spores and pansporoblasts in the renal interstitium of a rudd. The plasmodium is surrounded by the capillary (arrows) in which the plasmodium developed (H & E, bar = 10 μm).

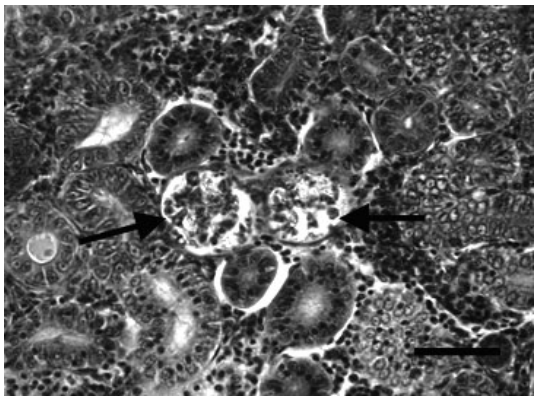

Figure 6 Cross-section of parts of a single elongated plasmodium of *Myxobolus erythrophthalmi* sp. n. in a curving blood vessel of the kidney (arrows) (H & E, bar = 20 μm).

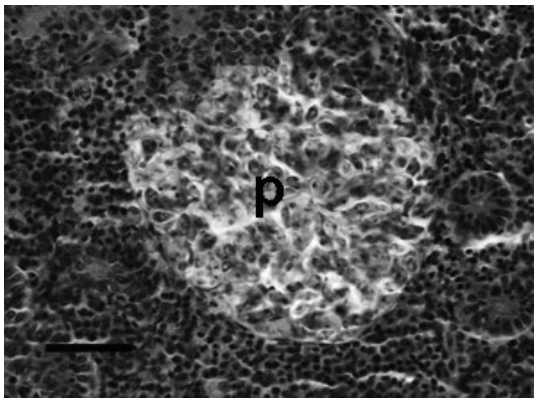

Figure 7 Large plasmodium (p) of *Myxobolus erythrophthalmi* sp. n. filled by spores in the renal interstitium of a rudd (H & E, bar = 20 μm).

and as a final stage, some degenerated spores were observed inside the melano-macrophage centres (Fig. 9). In this case, however, spores of *M. erythophthalmi* sp. n. damaged by macrophages were hardly distinguishable from spores of *M. pseudodispar* commonly occurring at the same site, after having been carried there from ruptured plasmodia in muscle cells (Fig. 10).

Molecular data

18S rDNA sequences of four replicates of *M. erythrophthalmi* sp. n. collected from four rudd specimens showed 99.8–100% identity over a 1303 bp long alignment. Nucleotide alterations were detected at positions 785 (A/T) and 1008

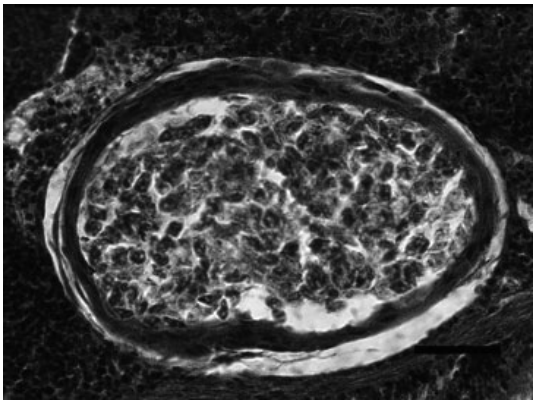

Figure 8 Plasmodium of *Myxobolus erythrophthalmi* sp. n. in the renal parenchyma, encapsulated by thick fibrous connective tissue (H & E, bar = 20 μm).

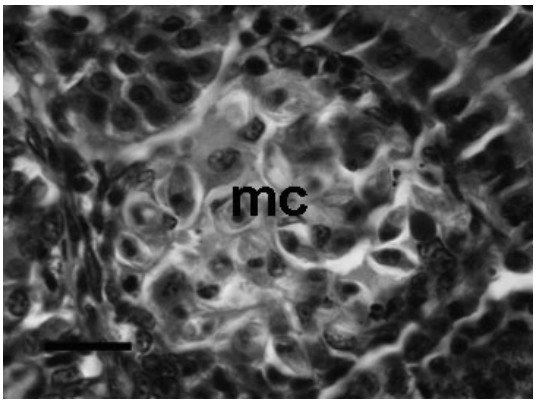

Figure 9 A melano-macrophage centre harbouring spores (mc) of *Myxobolus pseudodispar* and presumed spores of *M. erythrophthalmi* sp. n. in the interstitium of the kidney of a rudd. The spores are located among pale-staining and melanin-containing macrophages (H & E, bar = 20 μm).

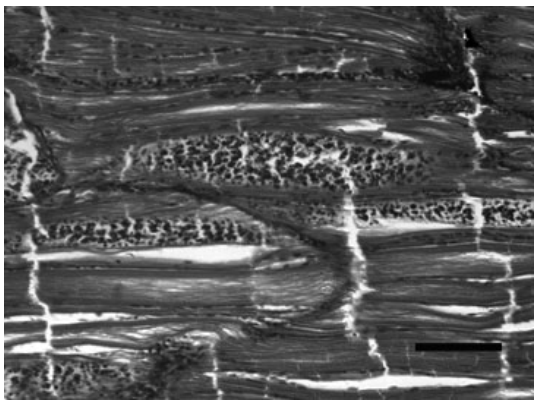

Figure 10 Intensive infection with intracellular plasmodia of *Myxobolus pseudodispar* in muscle cells of a rudd fingerling (H & E, bar = 100 μm).

(A/G) (relative to 18S rDNA accession no. EU567311). The greatest genetic similarity (97.8–97.9%) was with *M. ellipsoides*. The 18S rDNA sequence of *M. alburni,* a fin parasite of bleak, showed 96.0–96.2% similarity, while *M. shaharomae* sp. n. was found to be 95.8–96.1% similar to *M. erythrophthalmi* sp. n. A BLAST search did not reveal any identical actinosporean sequence available in GenBank.

Remarks

Myxobolus scardinii Reuss, 1906, a parasite of the gill of rudd, differs from *M. erythrophthalmi* sp. n. in having a well-developed intercapsular appendix, while the other specific parasite of rudd, *M. physophilus* Reuss, 1906, developing in the swimbladder, differs from this new species by its large polar capsules. The spore morphology of *M. erythrophthalmi* sp. n. resembles *M. ellipsoides* Thelohan, 1892, but the spores of the former species are smaller. The spore also resembles *M. alburni* and *M. leuciscini* Gonzales-Lanza & Alvarez-Pellitero, 1985, however, the latter species have six filament turns in the polar capsule. *Myxobolus erythrophthalmi* sp. n. is similar to *M. pseudodispar* as both species have approximately the same-sized spores without an intercapsular appendix, and loosely and obliquely arranged filaments in the polar capsule. The spores of the kidney-infecting *M. erythrophthalmi* sp. n., however, differ from the muscle-dwelling *M. pseudodispar* by their equal polar capsules, and by their symmetrical shape. *Myxobolus erythrophthalmi* sp. n. also differs from the above *Myxobolus* spp. in the 18S rDNA sequence.

Myxobolus infection in bleak

Myxobolus plasmodia of the new species were found in 15 fish from the River Danube and 37 fish from Lake Balaton (52/205 examined fish, 25.3% prevalence). Infection was found both in small- and large-sized specimens. In infected fish, cysts were most frequently found in the liver (85%), in the kidney (54%), in the testes (33%) and in the gut (21%). Cases occurred in all seasons of the year. No infection by this parasite was found in other organs. However, infection with *M. pseudodispar* was frequently found in the muscles. In spring and summer, *M. alburni* in the fins of bleak was found in the River Danube and *M. obesus* and *M. margitae* in the gills of bleak were also recorded in both biotopes.

Examination of the small cysts was difficult under microscope preparations, but in the pinhead-sized, mostly round plasmodia, spores were easily observable when pieces of the organs were examined between two glass plates. Round plasmodia 100–300 in diameter contained only a few hundred spores. Young plasmodia containing developmental stages were less round and in one case, elongated young cysts were also found in blood vessels of the kidney and the intestinal wall. The species was named as *M. erythrophthalmi* sp. n. and is described as follows. Measurements are given in micrometres.

Myxobolus shaharomae sp. n.

Mature plasmodia were round or ellipsoidal up to 350 in size. Young plasmodia contained only early sporogonic stages and were elongated. Spores (Figs 11a & 12a) were short-ellipsoidal in frontal view. In sutural view, they had a lemon shape (Fig. 11b). Spore measurements are listed in Table 2. Polar capsules were pyriform, equal in size, with slight anterior convergence. Five polar filament coils arranged obliquely to the capsule length wound loosely in the polar capsule. Generally, no intercapsular appendix was seen; however, for some spores, a very small knob-like thickening between capsules could be observed. Sutural protrusion formed a circular rim around the spore, emerging about 0.7–0.9 over the surface of the spore (Fig. 11c). In sutural view, this rim

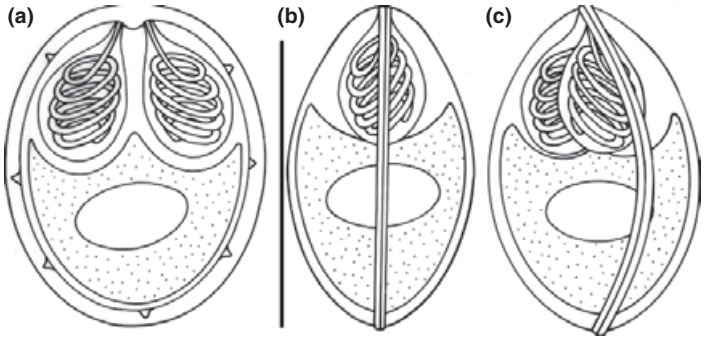

Figure 11 Schematic drawings of *Myxobolus shaharomae* sp. n. spores. (a) Frontal view, (b) sutural view, (c) semi-lateral view (bar = 10 μm).

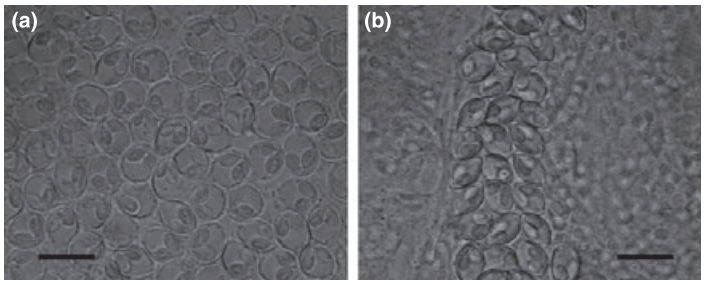

Figure 12 Spores of *Myxobolus shaharomae* sp. n. (a) In frontal view, (b) evacuating spores in a collecting duct of the kidney in sutural and semi-lateral view (bar = 10 μm).

protruded only slightly, or not at all, over the surface of the spore both at the anterior and posterior poles. The thickness of the rim measured about 0.7 in sutural view. Sutural edge markings (no. 5–7) were rarely seen in the spores. There was a single binucleated sporoplasm with ellipsoidal iodinophilous vacuole. Mucous envelope was not found.

Type host

Bleak, *Alburnus alburnus* (L.) (Cyprinidae).

Type locality

Lake Balaton, Hungary.

Other locality

River Danube.

Site of tissue development

Blood vessels in the renal interstitium, liver, testes and lamina propria of the intestinal folds.

Type material

Syntype spores in glycerine–gelatine and a photo series were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-17829**.** The 18S rDNA sequence of *M. shaharomae* sp. n. was deposited in GenBank under the accession number EU567312.

Etymology

The species is named after Faizah Shaharom, the well-known Malaysian fish parasitologist.

Histology of infection

Plasmodia were found in the kidney, liver, testes and the lamina propria of the intestinal folds. The youngest developmental stages were elongated plasmodia inside the blood vessels of the infected internal organs. Plasmodia containing developmental stages were slightly elongated (Fig. 13), while plasmodia with young spores became ellipsoidal or round (Fig. 14). These plasmodia were distinguishable from the neighbouring host cells by a distinct eosinophilic wall of parasite origin. Old plasmodia containing mature spores were bordered only by a thin layer of the endothelium from the hepatic cells (Fig. 15), the basal membrane of the intestine or the parenchymal tissues of the kidney and testes (Fig. 16). No capsule formation was observed in the histological sections studied.

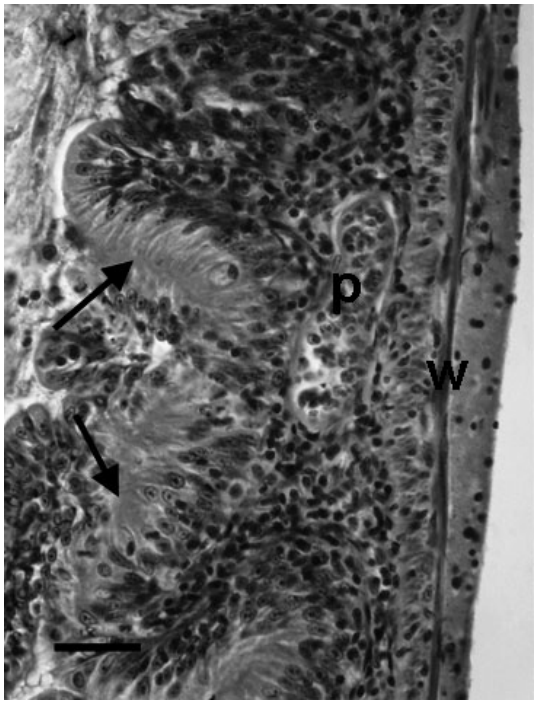

Figure 13 *Myxobolus shaharomae* sp. n. plasmodium (p) containing early sporogonic stages in the lamina propria of the gut. The elongated plasmodium bordered by an eosinophilic ectoplasm and containing mainly pansporoblasts is located between the intestinal wall (w) and the intestinal folds (arrows) (H & E, bar = 20 μm).

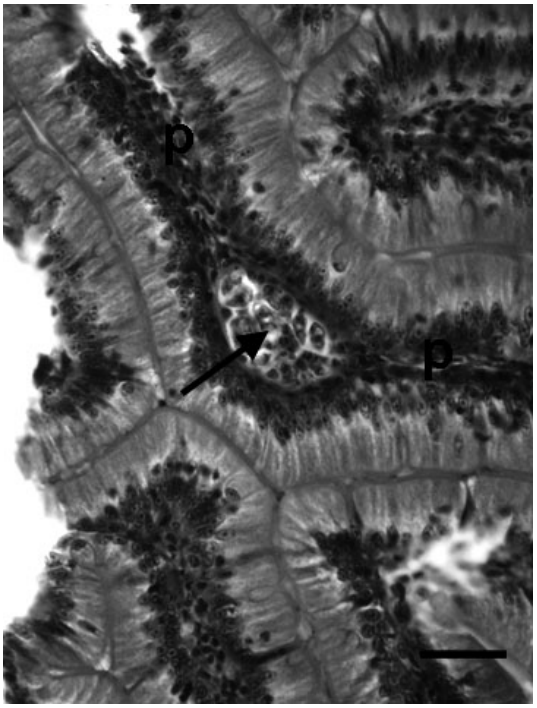

Figure 14 *Myxobolus shaharomae* sp. n. plasmodium (arrow) containing young spores and sporogonic stages in the lamina propria (p) inside an intestinal fold (f) (H & E, bar = 20 μm).

Molecular data

18S rDNA sequences of five replicate samples of *M. shaharomae* sp. n. collected from the liver, testis and kidney of three different bleak specimens showed 99.8–100% identity on the basis of a 1162 bp long overlapping DNA fragment. Nucleotide alteration was detected at position 569 (G/C), besides an 'A' nucleotide insertion at position 61 (relative to 18S rDNA accession no. EU567312). The highest genetic similarity was obtained with *M. alburni* (EU567313), showing 96.9–97.2% similarity in their 18S rDNA sequences. No identity was found to actinosporean sequences available in the GenBank.

Remarks

The spore morphology of *Myxobolus shaharomae* sp. n. resembles *M. erythrophthalmi* sp. n. but its spores are somewhat larger, and sutural protrusions are less observable at the anterior and posterior side of the spores. It also resembles *M. alburni*, but the latter species has only four filament turns in the polar capsule. *Myxobolus shaharomae* sp. n. is also similar to *M. pseudodispar* as both species have about the same-sized spores without an intercapsular appendix, and loosely and obliquely arranged filaments in the polar capsule. The muscle-dwelling *M. pseudodispar*, however, differs from *M. shaharomae* sp. n. by its unequal polar capsules, by the polar filaments having only four turns and by the asymmetrical shape of its spores. *Myxobolus shaharomae* sp. n. also differs from the above-mentioned *Myxobolus* spp. in its 18S rDNA.

Phylogenetic analyses

Phylogenetic analyses were performed on the basis of a 1592 bp long, edited alignment that contained 33 18S rDNA sequences. The distance matrix analysis confirmed the clustering pattern of maximum likelihood analysis, with slight differences in the bootstrap values. On the phylogenetic tree, *M. erythrophthalmi* sp. n. grouped with its closest relative, the fin parasite *M. ellipsoides* from chub, while *M. shaharomae* sp. n. shared a branch with

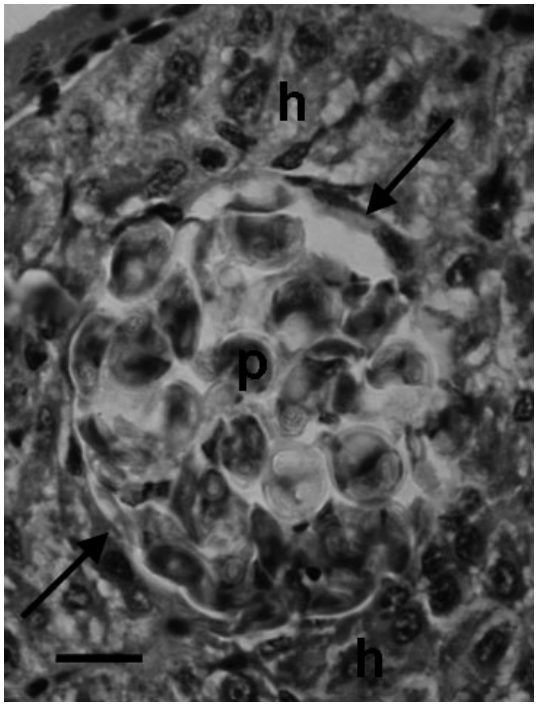

Figure 15 *Myxobolus shaharomae* sp. n. plasmodium (p) in the liver among hepatocytes (h) surrounded only by a thin layer of the rest of the blood vessel (arrow) (H & E, bar = 10 μm).

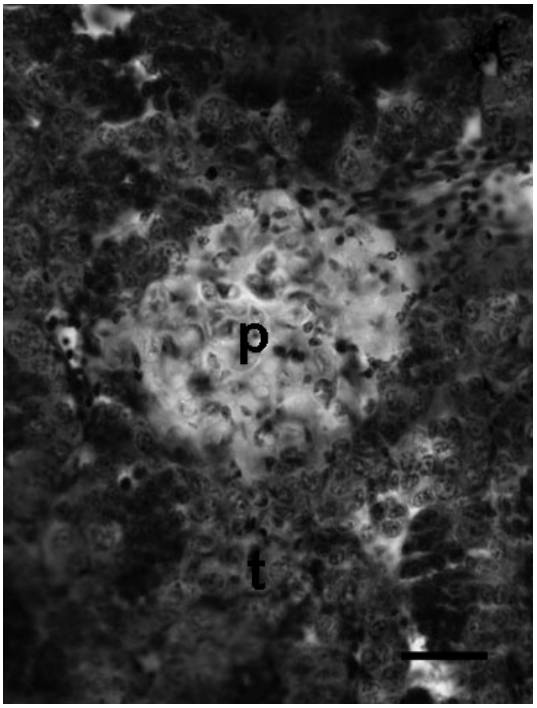

Figure 16 *Myxobolus shaharomae* sp. n. plasmodium (p) in the testis (t) (H & E, bar = 20 μm).

M. alburni parasitising the fin of bleak (Fig. 17). The location of the studied species within the large group of *Myxobolus* spp. with rounded spore shape was supported by high bootstrap values (100 and 97).

Discussion

The present study of the myxosporean fauna of rudd and bleak revealed that these fish are frequently infected by two different unknown *Myxobolus* spp. in their internal organs. The species described as *M. erythrophthalmi* sp. n. from rudd and the other species recorded as *M. shaharomae* sp. n. from bleak had similar spore morphology and tissue tropism, while a clear difference was observed in their 18S rDNA sequence. In the kidney of rudd infected by *M. erythrophthalmi* sp. n., individual spores of *M. pseudodispar* were also common in the melano-macrophage centres, but the two infections were easily distinguishable by the shape of the spores and the characteristic location of *M. erythrophthalmi* in the renal parenchyma. *Myxobolus erythrophthalmi* sp. n. formed elongated and, in cross-section, round plasmodia in the blood vessels of the renal parenchyma. Young plasmodia of *M. erythrophthalmi* developing typically in blood vessels, however, also differed remarkably from the location of individual spores of *M. pseudodispar*. In addition to differences in spore morphology and tissue tropism, there was a clear difference at the DNA level. The 18S rDNA of the examined species also differed from that of *M. ellipsoides, M. leuciscini* and *M. alburni*.

The site selection of *M. shaharomae* sp. n. was similar to that of *M. erythrophthalmi* sp. n., but this species more frequently infected other organs of bleak and, besides renal infections, was commonly found in the liver, testes and intestine. Site specificity of *Myxobolus* spp. is mostly connected to tissues rather than organs, thus *M. erythrophthalmi* sp. n. affecting several organs can be still considered tissue specific, as the species develops plasmodia in blood vessels. In addition, differences in the 18S rDNA sequence clearly showed that *M. shaharomae* sp. n. differs at the DNA level both from the morphologically similar *M. alburni* species and from *M. erythrophthalmi* sp. n.

The two new species, probably together with *M. alburni*, belong to those species which start their development in the capillaries. Histological sections indicate that young plasmodia are located inside the blood vessels, and the early-stage developing

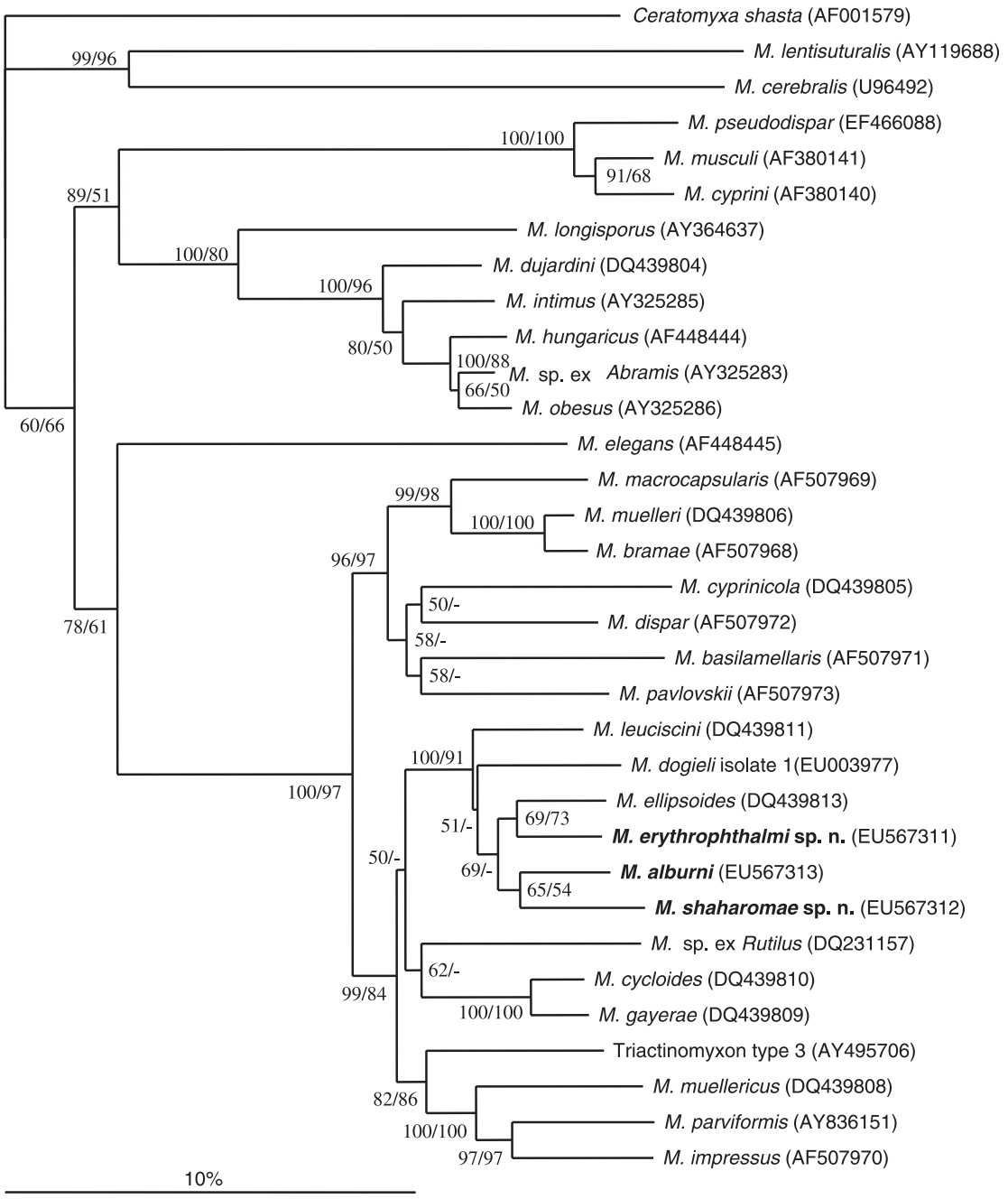

Figure 17 Phylogenetic tree generated by distance matrix and maximum likelihood analyses of the 18S rDNA sequences of myxosporeans, rooted at *Ceratomyxa shasta*. Numbers at nodes indicate bootstrap confidence levels in per cent (distance matrix method: 1000 repetitions; maximum likelihood: 100 repetitions, transversion/transition 1:2). GenBank accession numbers are given in parentheses. The distance scale is shown beside the tree. Myxosporeans examined in the present study are given in bold.

plasmodia with their elongated shape follow the route of the capillary. In morphological aspects, these *Myxobolus* spp. resemble also *M. pseudodispar, M. cyprini* and *M. musculi,* the representatives of the intracellular–intramuscular group; however, at the DNA level the two novel species are distinguishable from the muscle-dwelling species.

Myxobolus pseudodispar, described by Gorbunova (1936) from *R. rutilus* as type host, is a rather common species in leuciscine fish, and commonly

infects the fingerlings of rudd (Molnár, Székely, Csaba, Láng & Majoros 2001). Molnár *et al.* (2002) have shown that the typical asymmetrically shaped spores collected from plasmodia in the muscle cells of *R. rutilus, S. erythrophthalmus, A. brama* and *B. bjoerkna* possess similar, although in most cases not identical 18S rDNA sequences, and they belong to the species *M. pseudodispar*. On the other hand, they concluded that spores with similar morphology infecting common carp, *Cyprinus carpio* L., and barbel, *Barbus barbus* (L.), belong to *M. cyprini* and *M. musculi*, respectively.

Studying the original description of *M. destruens* recorded from the muscles of rudd by Schuurmans-Stekhoven (1920), we assumed that this species might be a senior synonym of *M. pseudodispar* Gorbunova, 1936. Schuurmans-Stekhoven (1920) examined several spores and presented a series of line drawings of the developmental stages of *M. destruens*, but he failed to summarize the data required for an exact identification, and the line drawings were also of poor quality. Therefore, the identity of the two species cannot be proved with certainty. Thus, the species infecting the muscles of rudd is suggested to be the well-characterized species, *M. pseudodispar*. We recommend *M. destruens* is regarded as an invalid species.

The phylogenetic positions of *M. erythrophthalmi* sp. n. and *M. shaharomae* sp. n. show that the two species, having similar spore morphology and site preference, fit in the *M. macrocapsularis* – *M. impressus* group with a rounded to ellipsoid spore shape. The highest genetic similarity was found to *M. ellipsoides* and *M. alburni*, which do not possess an intercapsular appendix and develop in the loose connective tissue of the fins. These species are easily distinguishable from the species of the other main cluster including the range of myxozoans from *M. longisporus* to *M. obesus*, as they are characterized by elongated spores with a pointed anterior end. If only spore morphology was considered, it would be surprising that *M. erythrophthalmi* sp. n. and *M. shaharomae* sp. n. are located far from the morphologically similar group of intramuscular species including *M. pseudodispar*, *M. cyprini* and *M. musculi* on the phylogenetic tree. However, members of the *M. pseudodispar* group form plasmodia in the muscle tissue, while the species described here are found developing in the blood vessels of different internal organs. Considering the close correlation between the histotropism and the genetic relationships of *Myxobolus* spp. (Eszterbauer 2004; Molnár *et al.* 2006), the genetic distance between the two groups is explained by their distinct site preference.

Acknowledgements

The authors thank Györgyi Ostoros for her help with the histological work. This study was granted by the Hungarian Scientific Research Fund (projects no. OTKA F45908 and K71837).

References

Andree K.B., Székely Cs., Molnár K., Gresoviac S.J. & Hedrick R.P. (1999) Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvulidae) based on small subunit ribosomal RNA sequences. *Journal of Parasitology* **85,** 68–74.

Corpet F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Research* **16,** 10881–10890.

Donec Z.S. & Shulman S.S. (1984) Cnidosporidia. In: *Key to the Parasites of Freshwater Fishes of USSR Fauna*, Vol. 1 (ed. by O.N. Bauer), pp. 88–251. Nauka, Leningrad (in Russian).

Eiras J.C., Molnár K. & Lu Y.S. (2005) Synopsis of the genus *Myxobolus* Bütschli, 1882 (Myxozoa: Myxosporea: Myxobolidae). *Systematic Parasitology* **61,** 1–46.

Ergens R., Gussev A.V., Izyumova N.A. & Molnár K. (1975) Parasite fauna of fishes of the Tisa River basin. *Rozpravy Československé Academie Ved, Rada Matematickych Ved* **85,** 1–117.

Eszterbauer E. (2004) Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity. *Diseases of Aquatic Organisms* **58,** 35–40.

Eszterbauer E. & Székely Cs. (2004) Molecular phylogeny of the kidney-parasitic *Sphaerospora renicola* from common carp (*Cyprinus carpio*) and *Sphaerospora* sp. from goldfish (*Carassius auratus auratus*). *Acta Veterinaria Hungarica* **52,** 469–478.

Felsenstein J. (1997) *PHYLIP – Phylogeny Inference Package v3.6a.* University of Washington, Seattle, WA.

Gorbunova M. (1936) *Changes in the Parasitic Fauna of Pike and Roach According to their Age.* Uchenie Zapiski Leningradskogo Ordena Lenina Gosudarstvennogo Universiteta. No. 7. (Biol. Ser.) Fasc. 3. Problems of Ecological Parasitology, 5–30 (in Russian).

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids. Symposium Series* **41,** 95–98.

Hillis D.M. & Dixon T. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66,** 411–453.

Lom J. (1969) On a new taxonomic character in Myxosporidia, as demonstrated in descriptions of two new species of *Myxobolus*. *Folia Parasitologica* **16,** 97–103.

Lom J. & Dyková I. (2006) Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitologica* **53,** 1–36.

Molnár K. (1971) Protozoon diseases of the fry of herbivorous fishes. *Acta Veterinaria Academiae Scientiarum Hungaricae* **21,** 1–14.

Molnár K. (1997) *Myxobolus squamaphilus* sp. n. (Myxozoa: Myxosporea), a common parasite of the scales of bream (*Abramis brama* L.). *Acta Protozoologica* **36,** 221–226.

Molnár K. (2000) Survey on *Myxobolus* infection of the bleak (*Alburnus alburnus* L.) in the River Danube and in Lake Balaton. *Acta Veterinaria Hungarica* **48,** 421–432.

Molnár K. & Baska F. (1999) Redescription of *Myxobolus hungaricus* Jaczó, 1940 (Myxosporea: Myxobolidae), a parasite of bream (*Abramis brama*). *Acta Veterinaria Hungarica* **47,** 341–349.

Molnár K. & Székely Cs. (1999) *Myxobolus* infection of the gills of common bream (*Abramis brama* L.) in Lake Balaton and in the Kis-Balaton reservoir, Hungary. *Acta Veterinaria Hungarica* **47,** 419–432.

Molnár K., Székely Cs., Csaba Gy., Láng M. & Majoros G. (2001) Pathological research and animal health problems of fishes from Lake Balaton. In: *A Balaton Kutatásának 2000. Évi Eredményei* (ed. by S. Mahunka & J. Banczerowski), pp. 158–166. Magyar Tudományos Akadémia, Budapest (in Hungarian).

Molnár K., Eszterbauer E., Székely Cs., Dán Á. & Harrach B. (2002) Morphological and molecular biological studies on intramuscular *Myxobolus* spp. of cyprinid fish. *Journal of Fish Diseases* **25,** 643–652.

Molnár K., Marton S., Eszterbauer E. & Székely Cs. (2006) Comparative morphological and molecular studies on *Myxobolus* spp. infecting chub from the River Danube, Hungary, and description of *M. muellericus* sp. n. *Diseases of Aquatic Organisms* **73,** 49–61.

Schuurmans-Stekhoven J.H. (1920) Myxosporidienstudien. II. Die multiplicative und propagative Entwicklung der Myxosporidien. *Archiv für Protistenkunde* **6,** 249–307 (in German).

Staden R. (1996) The Staden sequence analysis package. *Molecular Biotechnology* **5,** 233–241.

Whipps C.M., Adlard R.D., Bryant M.S., Lester R.J., Findlay V. & Kent M.L. (2003) First report of three *Kudoa* species from eastern Australia: *Kudoa thyrsites* from mahi mahi (*Coryphaena hippurus*), *Kudoa amamiensis* and *Kudoa minithyrsites* n. sp. from sweeper (*Pempheris ypsilychnus*). *Journal of Eukaryotic Microbiology* **50,** 215–219.

Received: 3 April 2008
Revision received: 30 May 2008
Accepted: 2 June 2008