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VERSITA

Molecular genetic studies on morphologically indistinguishable *Myxobolus* spp. infecting cyprinid fishes, with the description of three new species, *M. alvarezae* sp. nov., *M. sitjae* sp. nov. and *M. eirasianus* sp. nov.

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Abstract

While studying *Myxobolus* gill infections of cyprinid fishes, the authors found large, segmented plasmodia in three species: ide (*Leuciscus idus*), asp (*Aspius aspius*) and white bream (*Blicca bjoerkna*). As regards their size and morphology, the spores from these plasmodia corresponded to those of *M. dujardini* described from chub (*Leuciscus cephalus*). However, the 18S rDNA sequences of spores from the three cyprinids differed from those of *M. dujardini.* Based on molecular differences, this paper describes two new species: *M. alvarezae* sp. nov. from ide and asp, and *M. sitjae* sp. nov. from white bream. The two new species and *M. dujardini* had a similar tissue tropism, and infected the multilayered epithelium of the gill filaments. Histological examination of the infected filaments demonstrated that the large plasmodia with multiple buddings were formed from amalgamating small plasmodia. Besides carrying infection in the filamental epithelium, the three above fish species were infected by small intralamellar plasmodia as well. These plasmodia were filled by spores that resembled the roach parasite *M. intimus* both in morphology and seasonal development. The 18S rDNA sequences of 'intimus-like' spores from ide and asp differed only in some base pairs from spores found in the type host roach, and were identified as belonging to *M. intimus*. The spores found in white bream, however, showed 3.6–5.0% difference in DNA sequence from those of *M. intimus*; therefore, they have been described as *M. eirasianus* sp. nov. The aim of this paper was to demonstrate the importance of using molecular methods for separating and identifying morphologically corresponding or closely similar *Myxobolus* spp.

Keywords

Myxozoa, occurrence, histology, site selection, phylogeny

Introduction

Up to this time, about 800 *Myxobolus* species have been described (Eiras *et al.* 2005, Lom and Dyková 2006), most of them from cyprinid fishes. The majority of descriptions are based on spore characteristics (shape and size of the spores and polar capsules, presence or absence of the intercapsular appendix and iodinophilous vacuole, etc.). To increase the number of specific characteristics of species, recent works (Molnár 1994, Lom and Dyková 2006, Dyková and Lom 2007) have suggested that the host and site specificity and histotropism of the species should be studied. Exact identification of a species is especially difficult when morphologically similar spores develop in adequate organs or tissues of phylogenetically closely related fish hosts. In such cases, only an analysis of the 18S rDNA structure of spores can provide a reliable answer. Studying the DNA structure of spores of two morphologically similar species from bream (*Abramis brama*) and ide (*Leuciscus idus*), Eszterbauer (2002) found that these similar spores, having ridges running parallel with the suture on the spore surface, differed significantly in their molecular structure and belonged to the species *M. hungaricus* Jaczó, 1940 and *M. elegans* Kashkovski, 1966, respectively. On the other hand, Molnár *et al.* (2002) found plasmodia of the intramuscularly developing species *M. pseudodispar* in four cyprinids of the Leuciscinae subfamily. However, the same authors described that morphol-

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ogically very similar spores developing in the same location in common carp and barbel, two genetically less closely related cyprinids, belonged to *M. cyprini* and *M. musculi* spp. on the basis of their DNA sequences.

In the present work, spores of similar morphology and developing in the same location in three different cyprinid species were studied by morphological, histological and molecular methods. Based on the differences found in their 18S rDNA structure, spores morphologically resembling *M. dujardini* of the chub were described from ide and asp as *M. alvarezae* sp. nov. and from white bream as *M. sitjae* sp. nov. Molecular sequence analysis of the 'intimus-type' spores proved that spores infecting ide and asp belonged to *M. intimus*, but those found in white bream belonged to a new species to be described as *M. eirasianus* sp. nov.

Materials and methods

Fish material

The fish material showing *Myxobolus* infection of the gills was obtained from Hungarian natural waters. In Hungary, a long-term project has been conducted to survey the health condition of natural-water fishes. In the framework of this programme, parasitic infections of economically important and some commonly occurring fish species have been studied. Most of the fish studied originated from Lake Balaton and from reaches of the River Danube north of Budapest, and less frequently from the River Tisza. A complete parasitological examination was performed in most cases, but specific investigations on infections involving the gills were also carried out in 2005 and 2007.

Altogether 79 specimens of white bream (*Blicca bjoerkna* L.) from Lake Balaton and 20 from the River Danube, 29 ides (*Leuciscus idus* L.) from the Danube close to Budapest and 15 asps (*Aspius aspius* L.) from Lake Balaton and the River Tisza were examined. The total length of 1- to 6-year-old white breams varied from 6 to 22 cm. Of the ides, 14 fish measuring 8 to 13 cm and 15 specimens measuring 35 to 45 cm in length were examined, while all the asps were more than 25 cm (26 to 42 cm) long. White breams and small-sized ides were seined, large-sized ides and asps were caught by commercial fishermen with gill nets. Chub and roach specimens examined in previous studies (Molnár *et al*. 2006, 2010) were seined by the authors from the Danube River and the Lake Balaton, respectively. All fish were carried to the laboratory alive in oxygenated plastic bags, and kept in aquaria. Fish sedated with clove oil were killed with a cervical cut. They were examined within 4 days after being collected.

Light microscopy

In the framework of a complete parasitological examination, the hemibranchia of the gills and the fins were cut and examined under a dissecting microscope for the presence of *Myxobolus* plasmodia. Pieces from the kidneys, liver, spleen, testes and muscles were compressed between two glass plates, and examined first under a stereomicroscope, then at 200- to 400-fold magnification in a Zeiss compound microscope. The gut was first surveyed under a dissecting microscope as a whole, then it was cut open and compressed between two glass plates. *Myxobolus* spores from the isolated and opened cysts were first studied in a wet mount, and then some of the spores were placed in glycerine-jelly onto a slide under a cover slip and preserved as a reference slide. Another part of the spores collected from a single matured plasmodium were placed into Eppendorf tubes and stored at –20°C for a later molecular taxonomic study. The vitality of spores was checked by placing them into a 0.4% solution of urea. Spores of a given plasmodium were regarded as mature when at least 90% of the spores extruded polar filaments in that solution. Unfixed spores were studied by Nomarski differential interference contrast of an Olympus BH2 microscope. Fresh spores were photographed with an Olympus DP20 digital camera or recorded on videotapes; digitised images were obtained and measurements were taken from fresh spores and from digitised photos. All measurements are given in µm.

Histology

Tissue samples from infected organs containing developing and mature plasmodia were fixed in Bouin's solution, embedded in paraffin wax, cut into 4–5 µm sections, and stained with haematoxylin and eosin.

Molecular methods

For DNA extractions, samples preserved in ethanol were centrifuged at 8000 × *g* for 10 min to pellet the myxospores, then the ethanol was removed. The DNA was extracted using a Qiagen DNeasy™ tissue kit (animal tissue protocol, Qiagen) and eluted in 75 µl of buffer AE.

The 18S rDNA was amplified using the primers ERIB1 and ERIB10' (Table I) in a 25 µl reaction mixture, which comprised 1 µl extracted genomic DNA, 5 µl 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.25 µl of each primer, 2.5 µl 10X Taq buffer (MBI Fermentas), 1.25 µl 25 mM $MgCl_2$, 0.1 µl Taq polymerase (1 U) (MBI Fermentas) and 12 µl distilled water. The PCR cycle consisted of an initial denaturation step of 95°C for 3 min, followed by 35 cycles of 94°C for 50 s, 56°C for 50 s, 72°C for 80 s and finished with terminal extension at 72°C for 7 min, then rested at 4°C.

This was followed by a second round of PCR with the MYX1F-SphR primer pair (Table I). The total volume of the nested PCR reactions was 50 µl, which contained 1 µl amplified DNA, 10 µl 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.5 µl of each primer, 5 µl 10X Taq

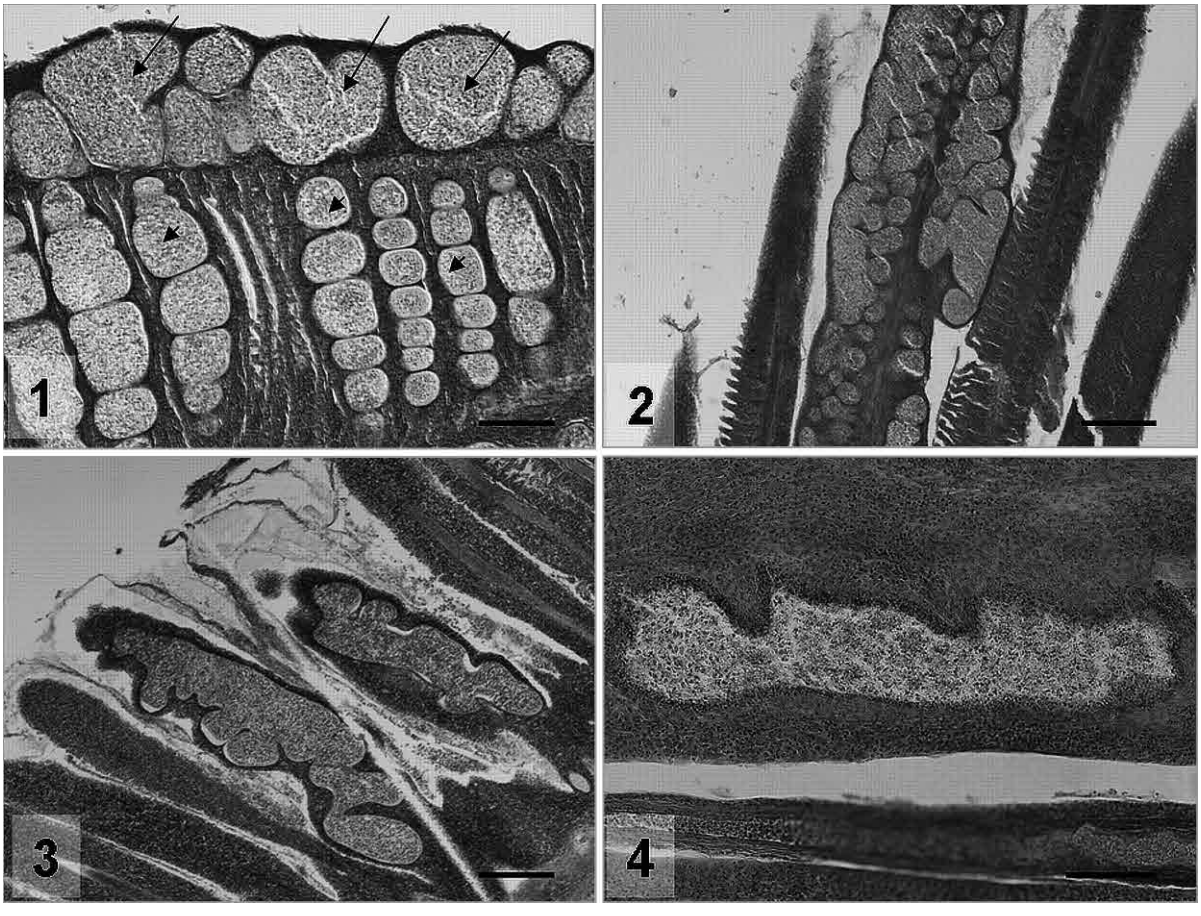
Table I. Primers used for PCR or sequencing

Primer	Sequence	Source
ERIB1	5'-ACC TGG TTG ATC CTG CCA G-3'	Barta *et al*. (1997)
ERIB10	5'-CTT CCG CAG GTT CAC CTA CGG-3'	Barta *et al*. (1997)
Myx1F	5'-GTG AGA CTG CGG ACG GCT CAG-3'	Hallett & Diamant (2001)
SphR	5'-GTT ACC ATT GTA GCG CGC GT-3'	Eszterbauer & Székely (2004)
MC5	5'-CCT GAG AAA CGG CTA CCA CAT CCA-3'	Molnár *et al*. (2002)
MC3	5'-GAT TAG CCT GAC AGA TCA CTC CAC A-3'	Molnár *et al*. (2002)
MB5r	5'-ACC GCT CCT GTT AAT CAT CAC C-3'	Eszterbauer (2004)
MB5f	5'-GAT GAT TAA CAG GAG CGG TTG G-3'	Eszterbauer (2004)

buffer (MBI Fermentas), 2.5 µl 25 mM $MgCl_2$, 0.2 µl Taq polymerase (2 U) (MBI Fermentas) and 28.5 µl water. Amplification conditions in the second round were: 94°C for 50 s, 56°C for 50 s, 72°C for 90 s for 35 cycles, and the cycle was terminated with an extension period at 72°C for 10 min, then rested at 4°C. Both PCR cycles were performed in a PTC-200 thermocycler (MJ Research). The PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA (TAE) buffer gel stained with 1% ethidium bromide and then purified with the EZ-10 Spin column PCR Purification Kit (Bio Basic Inc., Canada).

Purified PCR products were sequenced with primers listed in Table I using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyser.

The various forward and reverse sequence segments were aligned in BioEdit (Hall 1999) and ambiguous bases clarified using corresponding ABI chromatograms. Nucleotide sequences were aligned with the software CLUSTAL W



Figs 1–4. Plasmodia of *M. alvarezae* sp. nov. in the multilayered epithelium of the filaments of an ide. Plasmodia at the edge of filament (short arrows) and in the epithelium between gill lamellae (arrows). Histological section, haematoxylin and eosin (H & E). Bar = 500 µm (**1**). Lobulated *M. alvarezae* sp. nov. plasmodia in a gill filament of an asp. Histological section, H & E. Bar = 500 µm (**2**). Lobulated plasmodia of *M. dujardini* in the multilayered epithelium of the gill filaments of a chub. Histological section, H & E. Bar = 500 µm (**3**). *Myxobolus sitjae* plasmodium in the gill filaments of white bream. Histological section, H & E. Bar = 500 µm (**4**)

(Thompson *et al*. 1994). The alignment was corrected manually using the alignment editor of the software MEGA 5.0 (Tamura *et al.* 2011). DNA pairwise distances were calculated with the Mega 5.0 software using Kimura-2 substitution model. Maximum likelihood (ML) and Bayesian interference (BI) analyses were performed (Huelsenbeck and Ronquist 2001) and the resulting topologies were compared to each other. *Myxobolus cerebralis* was chosen as an outgroup.

The data set was tested using jModeltest 0.1 (Posada 2008) for the nucleotide substitution model of best fit, and the model shown by the Akaike Information Criterion (AIC) as the best-fitting one was chosen for each partition.

ML analyses were performed in Mega 5.0 under the GTR + G model. Bootstrap values based on 1000 resampled datasets were generated.

BI was computed by Topali 2.5 (Milne *et al*. 2008). The likelihood parameters for BI were based on the GTR + G model. Posterior probabilities (PP) were estimated over 1000000 generations via two independent runs of four simultaneous MCMCMC chains with every 100th tree saved. The burn in vas 25.

The ML and BI trees were visualised using the tree explorer of Mega 5.0.

Results

While studying myxosporean infections of cyprinid fishes, plasmodia and spores were found in three Danube fishes (*Leuciscus idus*, *Aspius aspius* and *Blicca bjoerkna*), which morphologically resembled *M. dujardini* (Thélohan, 1892) and *M. intimus* Zaika, 1965 found earlier in the chub (Molnár *et al*. 2006) and roach (Rácz *et al.* 2004), respectively. Molecular studies have proved that spores collected from the three recently studied fishes differ in their 18S rDNA sequences from the spores of *M. dujardini* collected earlier from chub and deposited in the GenBank. Based mostly on molecular data a species infecting ide and asp has been described as *M. alvarezae* sp. nov., while another species infecting the white bream has been recorded as *M. sitjae* sp. nov. of the spores resembling *M. intimus*, those found in the ide and asp were found to belong to *M. intimus*, while spores collected from the white bream proved to be a new species, to be described as *M. eirasianus* sp. nov. Description of the new species is as follows:

Description of M. alvarezae *sp. nov.*

Three specimens of large-sized ides and a three-year-old asp were infected with this *Myxobolus* species in the gill filaments. No infection was found among the one- or two-year-old ides of small size. In one of the ide specimens, large elongated but compact plasmodia were located in the multilateral epithelium of the non-lamellated part of the filamental edge. In another

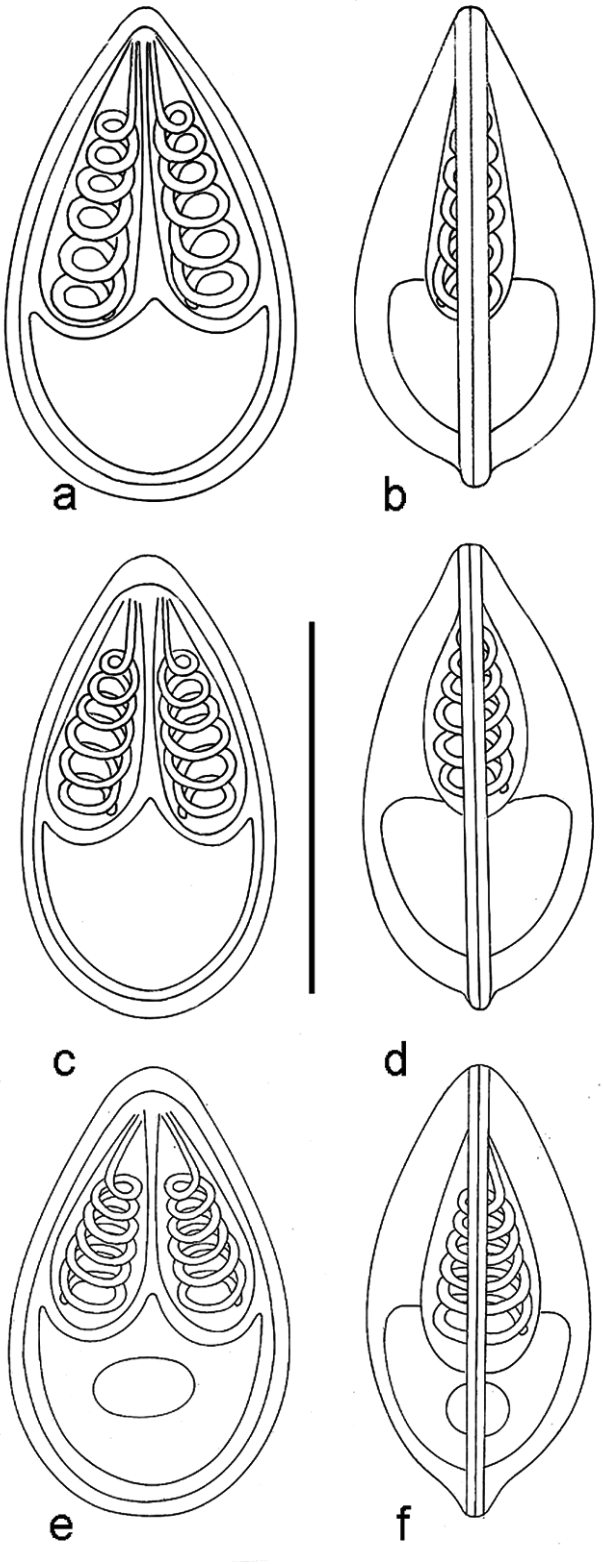


Fig. 5. Schematic drawings of spores. (**a**) *M. alvarezae* from ide in frontal view; (**b**) *M. alvarezae* from ide in sutural view; (**c**) *M. alvarezae* from asp in frontal view; (**d**) *M. alvarezae* from asp in sutural view; (**e**) *M. sitjae* in frontal view, (**f**) *M. sitjae* in sutural view. Bar = 10 μm

Table II. Comparison of the spore measurements of 'dujardini-type' *Myxobolus* spp. infecting cyprinid fishes. Mean ± SD and range in parentheses are expressed in μm. PC – polar capsule, n.d. no data

***Myxobolus* spp.**	***M. dujardini* by Thélohan, 1892**	***M. dujardini* by Donec and Shulman, 1984**	***M. dujardini* by Lom, 1961**	***M. dujardini* by Molnár *et al*., 2006**	***M. alvarezae* sp. nov. Present paper**	***M. alvarezae* sp. nov. Present paper**	***Myxobolus sitjae* sp. nov. Present paper**
Host	*Scardinius erythrophthalmus*	*Rutilus rutilus*	*Leuciscus leuciscus*	*Leuciscus cephalus*	*Leuciscus idus*	*Aspius aspius*	*Blicca bjoerkna*
Additional hosts	*Perca fluviatilis, Rutilus rutilus, Cyprinus carpio*	21 cyprinids	*L. cephalus*				
Spore length	(12–13)	(11–13)	(11–13)	11.5 ± 0.4 (11–12)	11.7 ± 0.42 (11.3–12.6)	13.6 ± 1.04 (12.6–14.7)	12 ± 0.72 (11–13)
Spore width	(7–8)	(5–8)	(5–7)	7.4 ± 0.55 (6.5–8)	6.8 ± 0.31 (6.5–7.6)	6.7 ± 0.77 (5.6–8.4)	7.5 ± 0.42 (6.8–8.1)
Spore thickness				7.1 ± 0.47 (6.5–7.3)	6.4 ± 0.41 (6.1–6.9)	6.5 ± 0.83 (5.9–8.4)	6.7 ± 0.22 (6.3–6.9)
PC length	6	(5–7)	(5–5.5)	5.5 ± 0.47 (5–6)	6.7 ± 0.32 (6.1–7.2)	6.8 ± 0.64 (6.3–7.7)	6.6 ± 0.36 (6.0–7.2)
PC width	3	(1–2)	2.2	2.3 ± 0.44 (2–3)	2.3 ± 0.16 (2–2.5)	2.9 ±0.59 (2.1–4.2)	2.9 ± 0.31 (2.3–3.2)
Thickness of sutural rim	n.d.	n.d.	n.d.	n.d.	(0.7–0.9)	0.7	(0.5–0.7)
No. coils in PC	n.d.	n.d.	n.d.	n.d.	6	6	6

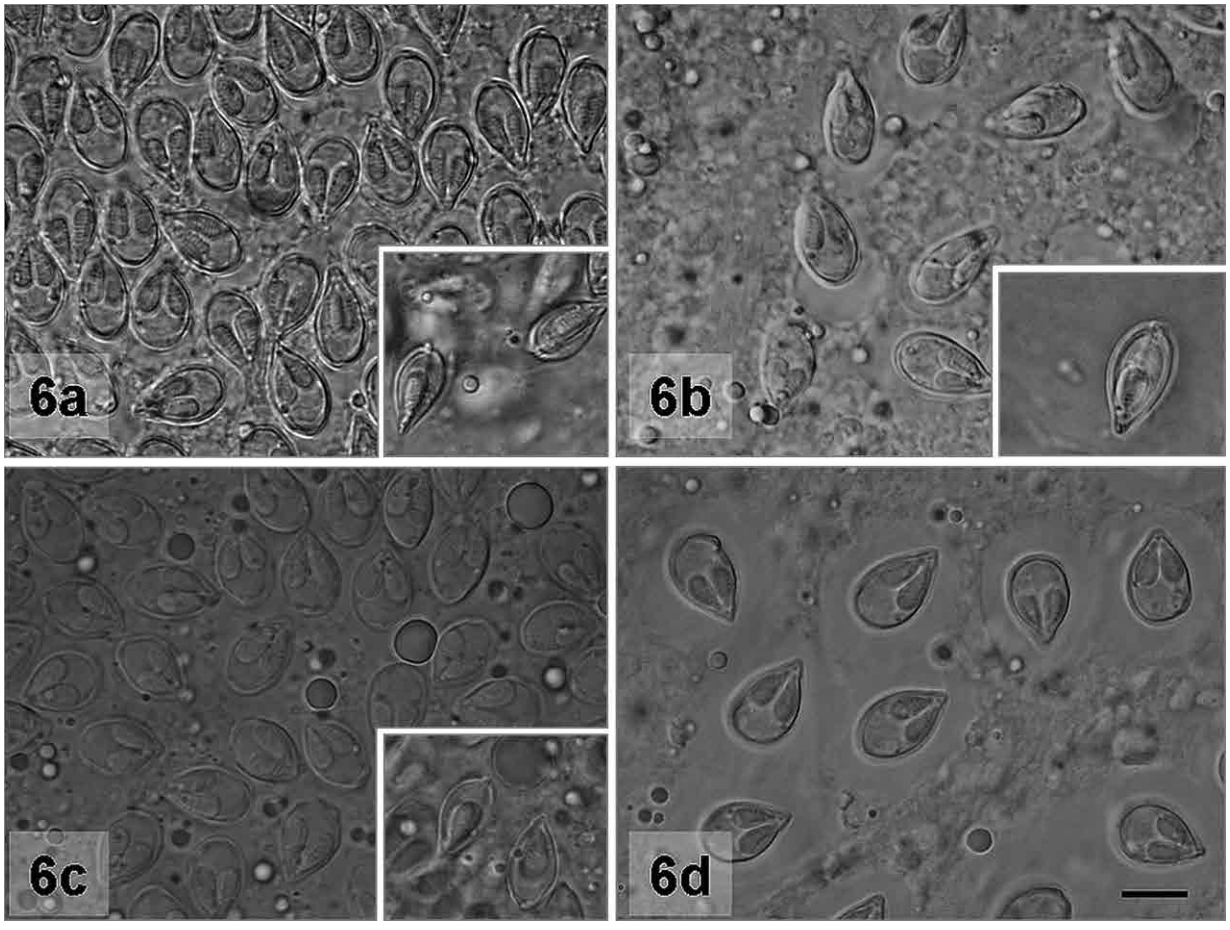


Fig. 6. Microphotographs of 'dujardini-type' spores. (**a**) *M. alvarezae* from ide, inset: a spore in sutural view; (**b**) *M. alvarezae* from asp, inset: a spore in sutural view; (**c**) *M. sitjae* from white bream, inset: a spore in sutural view; (**d**) *M. dujardini* from chub. Bar = 10 μm

Table III. Comparison of the spore measurements of *Myxobolus intimus* Zaika, 1966 given by different authors and *M. eirasianus* sp. nov. species from leuciscin cyprinids. Mean ± SD and range in parentheses are expressed in µm. PC – polar capsule, n.d. – no data

***Myxobolus* spp.**	***M. intimus* by Donec and Shulman, 1984**	***M. intimus* by Lom and Dyková, 1992**	***M. intimus* by Rácz *et al*., 2004**	***M. intimus* Present paper**	***M. intimus* Present paper**	***M. eirasianus* Present paper**
Host	*Rutilus rutilus*	*Rutilus rutilus*	*Rutilus rutilus*	*Leuciscus idus*	*Aspius aspius*	*Blicca bjoerkna*
Spore length	(11.2–12.6)	12.5	13.5 ± 0.6 (12.6–14.7)	11.5 ± 0.53 (10.8–12.6)	13.6 ± 0.6 (12.6–14.3)	12.8 ± 0.34 (12.6–13.5)
Spore width	(9.8–11.2)	10	9.2 ± 0.4 (8.6–9.7)	8.4 ± 0.68 (7.2–9.0)	10 ± 0.57 (9.1–10.8)	9.6 ± 0.42 (9.0–10.3)
Spore thickness	n.d.	n.d.	8.0 ± 0.4 (7.0–8.5)	7.0 ± 0.35 (6.5–7.6)	7.4 ± 0.5 (6.8–8.2)	7.9 ± 0.51 (7.2–9.0)
PC length	5.6	n.d.	5.5 ± 0.4 (4.8–6.2)	6.1 ± 0.53 (5.8–6.8)	6 ± 0.32 (5.5–6.6)	5.9 ± 0.28 (5.4–6.3)
PC width	4.2	n.d.	3.4 ± 0.5 (2.9–4.8)	3.3 ± 0.2 (3.0–3.6)	3.8 ± 0.34 (3.5–4.4)	3.5 ± 0.17 (3.2–3.7)
Thickness of sutural rim	n.d.	n.d.	n.d.	0.85 ± 0.07 (0.7–0.9)	0.7 ± 0.14 (0.5–0.9)	0.7 ± 0.12 (0.6–0.8)
No. coils in PC	n.d	n.d.	5–6	6	5–6	6

ide, 3–5 mm long, large mature plasmodia were located in the same place but they had several lateral buddings which penetrated into the multilayered epithelium between neighbouring lamellae. In the third ide and in the asp roundish plasmodia, 31 to 200 µm in diameter, containing spores and disporoblastic pansporoblasts were found in the epithelial edge of the filaments and in the epithelium between the lamellae (Figs 1 and 2). Description of the species is based on spores collected from the ide. Data on spores from asp are presented in Table II.

Spores (Figs 5a-d and 6a,b) were pyriform both in frontal and sutural view. Length of the spores was 11.7 ± 0.42 (11.3–12.6) (n = 50), width 6.8 ± 0.31 (6.5–7.6) (n = 50), thickness 6.4 ± 0.41 (6.1–6.9) (n = 11). Polar capsules were elongated, equal in size, slightly converging anteriorly, 6.7 ± 0.32 (6.1–7.2) long (n = 50) and 2.3 ± 0.16 (2–2.5) wide (n = 50). Six filament coils arranged perpendicular to the capsule length wound less densely in the polar capsule. No intercapsular appendix was found in the spores. Sutural protrusion formed a circular rim around the spore emerging about 0.7 to 0.9 over the surface of the spore. At the posterior end of the spore the rim of the suture emerged from the spore surface 0.7 to 0.9. Sutural edge markings were not seen. The single binucleated sporoplasm had no iodinophilous vacuole in the spore. Mucous envelope was not found.

Type host: Ide, *Leuciscus idus* (L.) (Cyprinidae).

Additional host: Asp, *Aspius aspius* (L.) (Cyprinidae).

Type locality: River Danube, Hungary.

Site of tissue development: Multilayered epithelium of the gill filaments.

Type material: Syntype spores in glycerine-jelly and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18248 for spores from *Leuciscus idus* and Coll. No. HNHM-18249 for spores from *Aspius aspius*. The 18S rDNA sequence of *M. alvarezae* sp. nov. was deposited in the GenBank under accession numbers FJ716096 from the ide and FJ716097 from the asp.

Prevalence of infection: 20% in ides older than 3 years; 6.6% in asp.

Etymology: The species is named after Dr. Pilar Alvarez-Pellitero, the well-known Spanish fish parasitologist.

Molecular data: 18S rDNA sequences of *M. alvarezae* (FJ716096) collected from the gill filaments of an ide specimen was 99.7% similar to sequences obtained for plasmodia from the asp (FJ716097). The similarity of *M. alvarezae* sequences from ide and asp to *M. sitjae* sp. nov. was 94.7% and 94.4%, respectively. The sequences also showed a close similarity (96.3% and 96.0%) to sequences of the species identified by Molnár *et al*. (2006) as *M. dujardini* (Thélohan 1892) collected from the gills of chub (*Leuciscus cephalus*) and deposited in the GenBank.

Histology: In the gills of the histologically studied third ide specimen 8 filaments were infected, but the infection was very intensive in each of these filaments (Fig. 1). Contrary to the other two cases, in this case a chain formed by dozens of plasmodia filled out the multilayered epithelium at the non-lamellated edge of the filaments and the space between lamellae. Most of the plasmodia had a round shape, but frequently two or three neighbouring plasmodia amalgamated and formed a shortly elongated or amorphous shape. In histological slides made from an infected asp, the seemingly homogeneous large plasmodium was composed of several small rounded and large amalgamated plasmodia which were located mostly in the multilayered epithelium of the non-lamellated edge of the filament (Fig. 2). A similar histological picture was seen in a chub with *M. dujardini* infection where non-separated small plasmodia, non-segmented large plasmodia and lobular plasmodia were equally seen (Fig. 3).

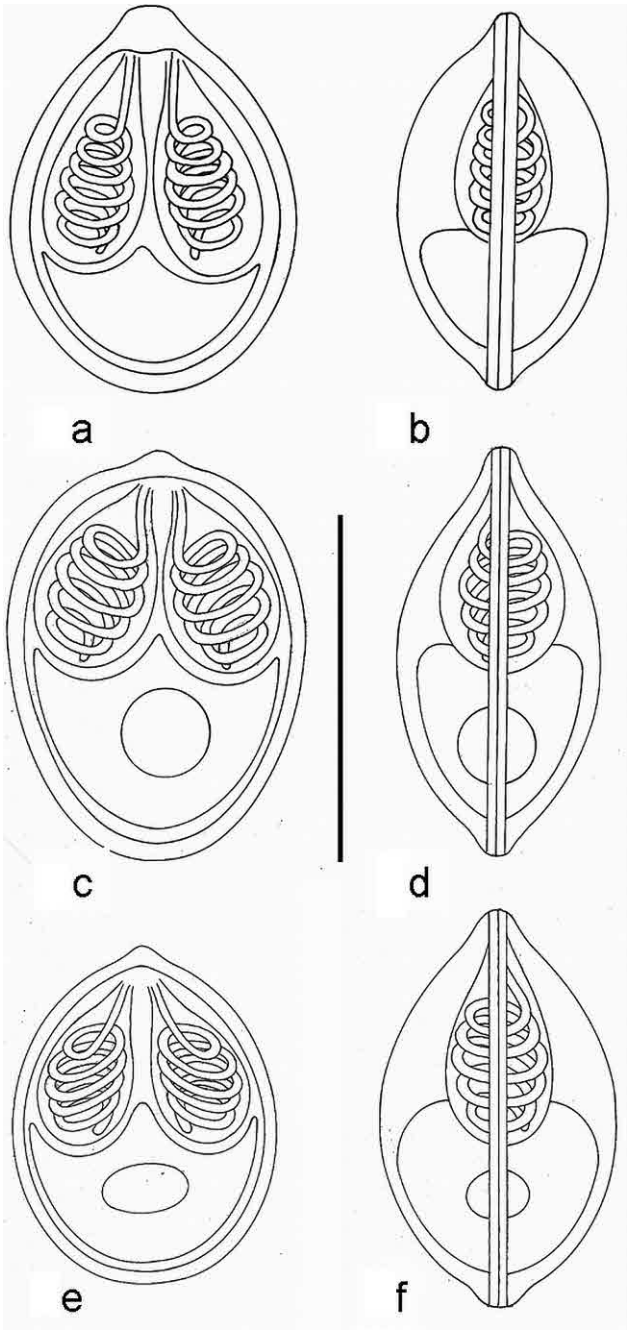


Fig. 7. Schematic drawings of spores: (**a**) *M. intimus* from ide in frontal view; (**b**) *M. intimus* from ide in sutural view; (**c**) *M. intimus* from asp in frontal view; (**d**) *M. intimus* from asp in sutural view; (**e**) *M. eirasianus* in frontal view; (**f**) *M. eirasianus* in sutural view. Bar = 10 µm

Remarks: The size and location of plasmodia as well as the shape and size of the spores of *M. alvarezae* from ide and asp and *M. sitjae* are highly reminiscent of *M. dujardini* infecting the gills of the chub (Table II, Fig. 6d), however, the 5.3 and 5.6% differences in the 18S rDNA sequences of the above species indicate the separation of the two new species and *M. dujardini*.

Description of M. sitjae *sp. nov.*

Two specimens of 22 cm long white bream collected from the River Danube in March were infected with this *Myxobolus* species, while in other seasons of the year no infection was found. Three large plasmodia measuring 1500 to 2000 in length and 150 to 210 in diameter were found in the epithelium of the gill filaments (Fig. 4). Spores (Figs 5e,f and 6c) were pyriform both in frontal view and sutural view. Length of the spores was 12 ± 0.72 (11–13) (n = 50), width 7.5 ± 0.42 (6.8–8.1) (n = 50), thickness 6.7 ± 0.221 (6.3–6.9) (n = 11). Polar capsules were elongated, equal in size, slightly converging anteriorly, 6.6 ± 0.36 (6–7.2) long (n = 50) and 2.9 ± 0.31 (2.3–3.2) wide (n = 50). Six filament coils arranged perpendicular to the capsule length wound less densely in the polar capsule. No intercapsular appendix was found in the spores. Sutural protrusion formed a circular rim around the spore emerging about 0.7 to 0.9 over the surface of the spore. At the posterior end of the spore the rim of the suture emerged from the spore surface 0.7 to 0.9. Sutural edge markings were not seen. The single binucleated sporoplasm had no iodinophilous vacuole in the spore. Mucous envelope was not found.

Type host: White bream, *Blicca bjoerkna* (L.) (Cyprinidae).

Type locality: River Danube, Hungary.

Site of tissue development: Multilayered epithelium of the gill filaments.

Type material: Syntype spores in glycerine-jelly and histological slides were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18250. The 18S rDNA sequence of *M. sitjae* sp. nov. was deposited in the GenBank under the accession number JF311898.

Prevalence of infection: 5% in white breams from the River Danube.

Etymology: The species is named after Dr. Ariadna Sitjà-Bobadilla, the well-known Spanish myxosporean specialist.

Molecular data: 18S rDNA sequences of *M. sitjae* (JF311898) collected from the gill filaments of a white bream specimen showed the closest similarity to *M. alvarezae* sp. nov. (94.3 and 94.6%). Its sequences also showed a close similarity (94.2%) to sequences of the species identified by Molnár *et al*. (2006) as *M. dujardini* (Thélohan 1892) collected from the gills of chub (*Leuciscus cephalus*).

Histology: Elongated plasmodia of this species were found in the multilayered epithelium at the non-lamellated edge of the filaments (Fig. 4).

Remarks: The size and location of plasmodia as well as the shape and size of the spores of this species are highly reminiscent of *M. dujardini* infecting the gills of chub (Table II, Fig. 6d); however, the 5.3% difference in 18S rDNA sequences between the two species indicates the separation of the two species.

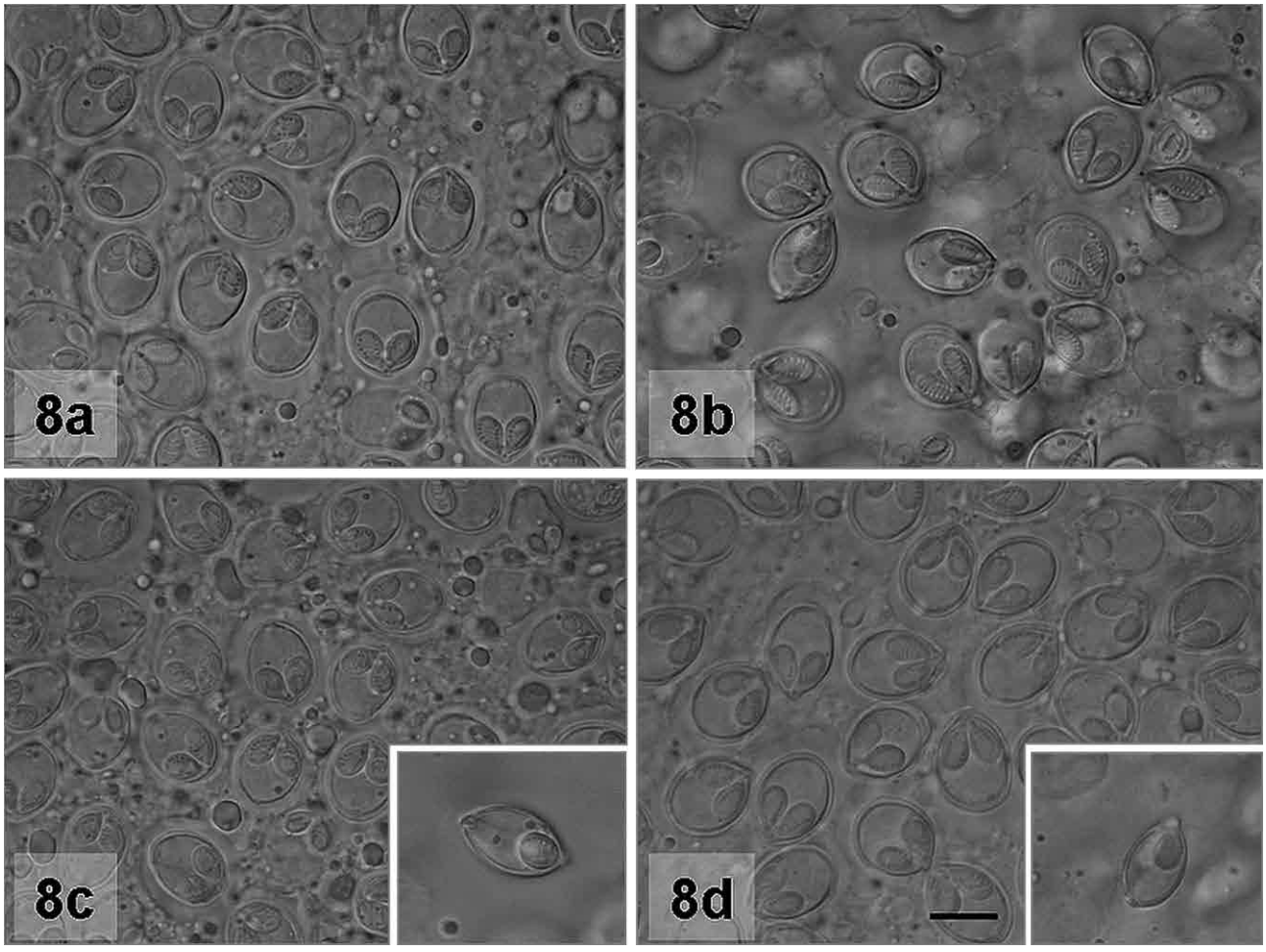


Fig. 8. Microphotographs of 'intimus-type' spores. (**a**) *M. intimus* from roach; (**b**) *M. intimus* from ide; (**c**) *M. intimus* from asp, inset: a spore in sutural view; (**d**) *M. eirasianus* from white bream, inset: a spore in sutural view. Bar = 10 µm

Occurrence of Myxobolus intimus *Zaika*, *1965 in the gills of* Leuciscus idus *and* Aspius aspius

Five specimens of large-sized ides and a single 30 cm long asp specimen collected from the River Danube in March and April were infected with this *Myxobolus* species, while in other seasons of the year and among the one- to two-year-old ides and asps of small size no infection was found. Small round or roundish plasmodia 50 to 150 in diameter, containing 3000 to 6000 spores, developed in the capillary network of the gill lamellae. Spores corresponding in shape and size to spores known from the type host roach, and from ide and asp, are presented in Table III and on Figs 7a-d and 8a-c).

Type material: Syntype spores in glycerine-jelly and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18252 for spores from *Leuciscus idus*, and Coll. No. HNHM-18253 for spores from *Aspius aspius*. The 18S rDNA sequences of *M. intimus* from ide and asp were deposited in the GenBank under the accession numbers FJ716098, JX390689, JX390690, JX390691 and JF311899.

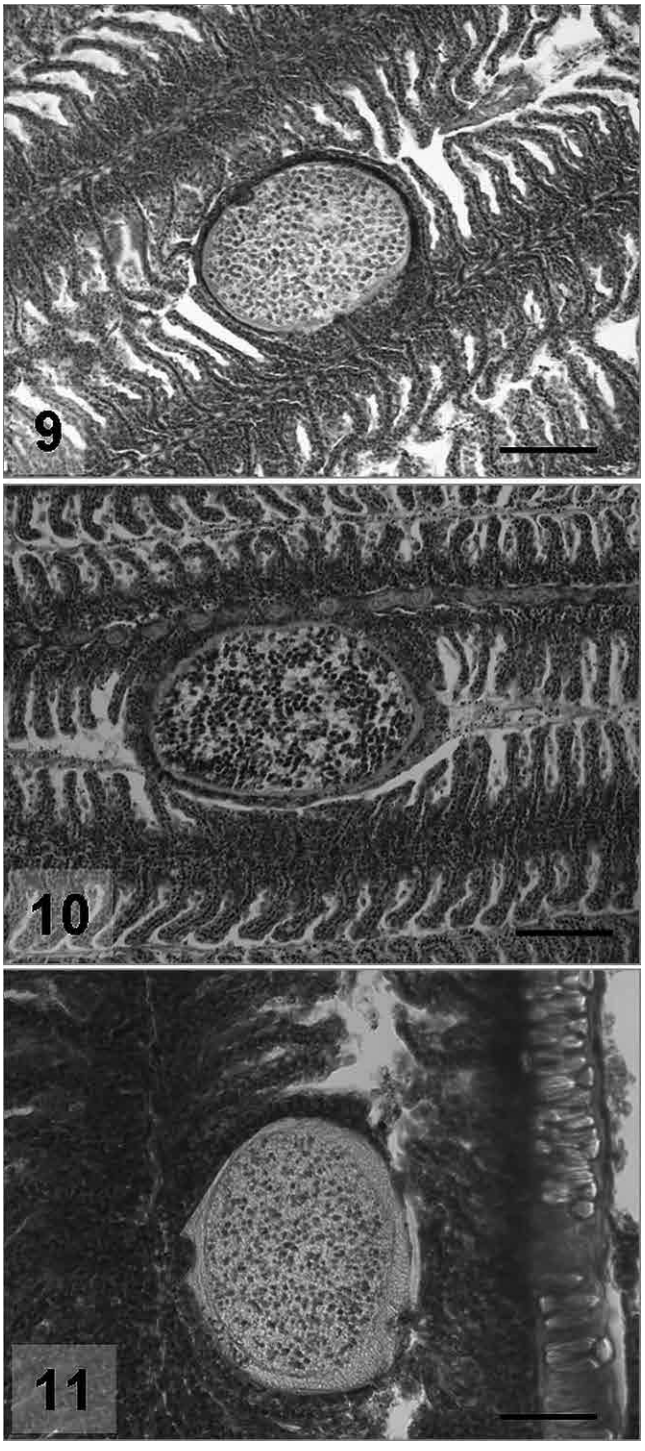
Prevalence of infection: 32% in ides older than 3 years.

Molecular data: The 18S rDNA sequences of four replicate samples of (FJ716098, JX390689, JX390690, JX390691) collected from the gill filaments of three ide specimens showed 99.2–99.7% similarity to *M. intimus* spores collected from the roach and 99.1–99.7% similarity to spores collected from the asp (JF311899). The inner distance between the samples from ide were 99.6%. The similarity to *M. eirasianus* sp. nov. was 95.3–96.4%.

Histology: In histological sections made from the gill of an ide round plasmodia developing in the capillary network of the gill lamellae were found (Fig. 9). Plasmodia filled most of the capillary network of the lamellae in a similar way as described in the roach (Fig. 10). The multilayered epithelium between neighbouring lamellae was compressed, and the neighbouring lamellae were pushed aside.

Remarks: The size and location of plasmodia as well as the shape and size of the spores from ide and asp corresponded to those of *M. intimus* infecting the gills of roach. The minor

differences in size and shape among spores from the three fish species and the less than 1% difference in the 18S rDNA sequences do not indicate a species differentiation.



Figs 9–11. *Myxobolus intimus* plasmodium in the gill lamella of ide. Histological section, H & E. Bar = 100 µm (**9**). *Myxobolus intimus* plasmodium in the gill lamella of roach. Histological section, H & E. Bar = 100 µm (**10**). *Myxobolus eirasianus* plasmodium in the gill lamella of white bream. Histological section, H & E. Bar = 500 µm (**11**)

Description of M. eirasianus *sp. nov.*

Eight specimens of 25–30 cm long white breams collected in April were infected with this *Myxobolus* species. Small round or roundish plasmodia 60 to 130 in diameter, containing 3500 to 5000 spores, developed in the capillary network of the gill lamellae (Fig. 11). Spores (Figs 7e,f and 8d, Table III) were pyriform in frontal view and lemon shaped in sutural view. Length of the spores was 12.8 ± 0.34 (12.6–13.5) (n = 50), width 9.6 ± 0.42 (9–10.3) (n = 50), thickness 7.9 ± 0.51 (7.2–9) (n = 11). Polar capsules were pyriform, equal in size, slightly converging anteriorly, 5.9 ± 0.28 (5.4–6.3) long (n = 50) and 3.5 ± 0.17 (3.2–3.7) wide (n = 50). Six filament coils arranged perpendicular or oblique to the capsule length wound densely in the polar capsule. No intercapsular appendix was found in the spores. Sutural protrusion formed a relatively thick circular rim around the spore emerging about 0.6 (0.5 to 0.7) over the surface of the spore. The rim of the suture emerged from the spore surface 0.7 (0.5 to 0.8) both at the anterior and at the posterior end of the spore. Sutural edge markings were not seen. The single binucleated sporoplasm had no iodinophilous vacuole in the spore. Some of the spores were surrounded by a thin, round mucous envelope.

Type host: White bream, *Blicca bjoerkna* (L.) (Cyprinidae).

Type locality: River Danube, Hungary.

Site of tissue development: Capillary network of the gill lamellae.

Type material: Syntype (Voucher) spores in glycerine-jelly and histological slides were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-18251. The 18S rDNA sequence of *M. eirasianus* sp. nov. was deposited in the GenBank under the accession number JF311900.

Prevalence of infection: 47% of white breams in April.

Etymology: The species is named after Dr. Jorge C. Eiras, the well-known specialist in myxosporean research.

Molecular data: 18S rDNA sequence of *M. eirasianus* (JF311900) showed similarity to spores of *M. intimus* from roach, ide and asp in 95.1%, 95.1–96.4% and 95.0%, respectively. It was, however, a surprise that its sequence showed the closest similarity to spores of *M. hungaricus* Jaczó, 1940 from the common bream in 99.4%, a species having different spore morphology.

Histology: Round plasmodia developed in the capillary network of gill lamellae (Fig. 11). Plasmodia filled most of the capillary network of the lamellae. The infected lamellae were almost filled by the plasmodium; however, the remnants of capillaries were often visible at their base and at their tips. The multilayered epithelium between neighbouring lamellae was compressed, and the neighbouring lamellae were pushed aside.

Phylogenetic aspects

The topology of both phylogenetic trees constructed by ML and BI were almost identical; therefore, only the ML tree is

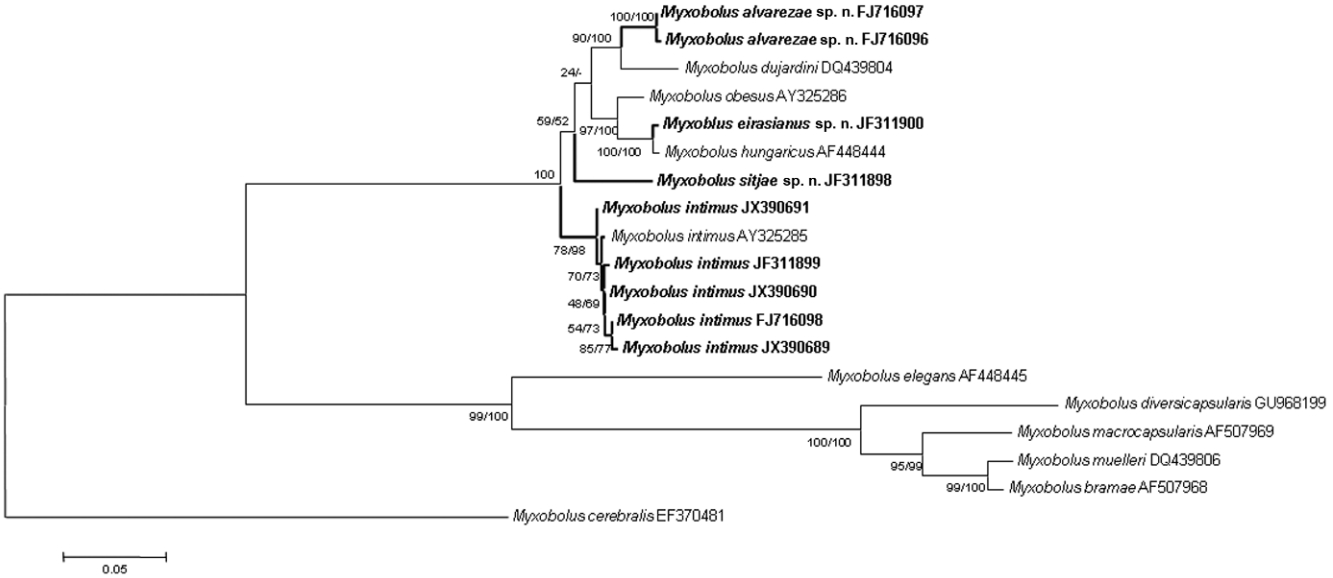


Fig. 12. Phylogenetic tree constructed on the basis of maximum likelihood analysis of the 18S rDNA sequences of selected myxozoans. Numbers at nodes indicate the bootstrap values (ML) and posterior probabilities (BI). *Myxobolus cerebralis* was used as the outgroup. Unsupported nodes by BI are marked with hyphen

presented here, complemented with the posterior probabilities of BI (Fig. 12). It can be observed that the two *M. alvarezae* sequences and the *M. dujardini* sequence are in the same clade, supported by relatively high bootstrap values. *M.sitjae* has branched surprisingly to *M. intimus* instead of *M. dujardini* and *M. alvarezae*, however, the bootstrap values of ML are quite low at the respecting node, moreover BI did not support it. *M. intimus* from ide and from asp were branched together with *M. intimus* described from roach, supported by high bootstrap values. *M. eirasianus* was grouped with *M. hungaricus* and *M. obesus.*

Discussion

The fact that the taxonomy of *Myxobolus* species had previously been based exclusively on spore morphology resulted in numerous misidentifications due to the similarity of spores originating from different fish species. These sources of error were reduced by taking into account the species, host and tissue specificity; however, accurate species identification was rendered possible only by adding an analysis of the DNA sequences of the species involved. The close to 100% identity of the 18S rDNA sequences undoubtedly indicates that the two species examined are identical, but a 98–99% sequence identity already makes it difficult to decide whether we are faced with a single or multiple species. In such cases, only the result of cross-infection experiments can confirm the diagnosis. Unfortunately, within the *Myxobolus* genus such experiments have only been performed with the species *Myxobolus cerebralis* and *M. arcticus* and *M. pseudodispar*. By infection experiments conducted with *M. cerebralis* actinospores, Hedrick *et al.* (1999, 2001) *M. cerebralis* proved that this species is capable of infecting at least four different salmonids (*Oncorhynchus mykiss*, *O. clarki*, *O. tshawitscha* and *Salvelinus confluentus*) but it cannot colonise *Thymallus arcticus*, which is also a salmonid fish. In view of the fact that the original host of *M. cerebralis* was the brown trout (*Salmo trutta*), this parasite can infect salmonids of at least three different genera. Unfortunately, these experiments were not followed by the analysis of gene sequences, and GenBank data are available for the rainbow trout only. Regarding *M. cerebralis* infection of this latter fish species, Andree *et al*. (1999) established that between the 1700 base pairs (bp) long sequences of the European and the American rainbow trout there was a 14 bp difference, which indicates a 99.2% identity.

A series of cross infection experiments were performed in the years of 2000–2001 by Székely (unpublished). He successfully infected *Tubifex tubifex* oligochaete specimens with *M. pseudodispar* spores collected from the muscles of different cyprinid fishes (rudd – *Scardinius erythrophthalmus*, bream – *Abramis brama*, white bream – *Blicca bjoerkna* and roach – *Rutilus rutilus*). In all cases typical *M. pseudodispar* triactinomyxon type actinospores developed and were released from oligochaetes. Interestingly enough, at challenging the actinospores originating from the four other cyprinid fish species with the test fishes, only the roach specimens became infected in the cross infection experiments. When triactinospores originated from roach were used for infection of fishes, only the roach became infected and other cyprinids [rudd, bream, white bream, barbel, common carp (*Cyprinus carpio*), bleak (*Alburnus alburnus*), ide (*Leuciscus idus*), vimba bream (*Vimba vimba*) and nase (*Chondrostoma nasus*)] remained negative. Unfortunately, these experiments were not

followed by the analysis of gene sequences. Results of the cross-infection experiments suggest that of the cyprinids investigated the roach is the most susceptible fish host for *M. pseudodispar*.

A more detailed study was performed by Urawa *et al*. (2011) on *M. arcticus*. Myxospores of *M. arcticus* developing in masu salmon (*Oncorhynchus masu*) showed 100% similarity to actinospores received from *Lumbriculus variegatus* in Japan, and they were also very similar to myxospores from the sockeye salmon and actinospores from *Stylodrilus heringianus* (99.95%). Nevertheless the actinosporean isolate from *L. variegatus* did not experimentally infect the type host sockeye salmon. The above authors concluded that the American and Japanese *M. arcticus* were unique strains. During our studies, we observed similar, more than 99.2% identities in the case of morphologically similar spores collected from different fish species. In our paper, we regard these spores as specimens of a single species. At the same time, in cases when we find identities lower than 98%, we conclude that the parasites examined represent two species that undoubtedly originate from the same root but have become distinct species during phylogeny.

During our morphological studies, in ide, asp and white bream we found spores that were morphologically identifiable with, or highly similar to, the species *M. dujardini* known from the type host chub, or the species *M. intimus* common in the roach as type host. The plasmodia of these spores occurred in the same location in the gills of the corresponding hosts. The DNA sequence of spores collected from ide, asp and white bream and resembling *M. dujardini* showed 4.0%, 3.7% and 5.7% difference, respectively, from the DNA sequences of *M. dujardini* spores collected from chub and deposited in the GenBank, although, in addition to the morphological identity of the spores, the plasmodium development restricted to the multilayered epithelium of the gill also showed a high degree of similarity. The 4.0%, 3.7% and 5.7% difference found in the nucleotide sequences of the DNA exceeds the limits allowed for identification as a single species, and justifies our decision that the species detected from ide, asp and white bream should not be identified with the species *M. dujardini*. At the same time, between spores detected from ide and asp there was only 0.3% difference in the 18S rDNA sequences, which suggests that the parasite described as *M. alvarezae* sp. nov. is a common parasite of these two closely related fish species. In the white bream, which belongs to the tribe Abramini of the Leuciscinae subfamily and is genetically slightly more distant from the asp and ide, we also found a species that resembled *M. dujardini* in spore morphology and in the tissue location of plasmodia, and describe it here as *M. sitjae* sp. nov. Regarding its gene sequences, this latter parasite differed from *M. dujardini* originating from the type host chub in 5.8% and from the species *M. alvarezae* from ide and asp in 5.3% and 5.6%, respectively. Unfortunately, so far we could not detect the species *M. dujardini* from the rudd, a species considered a type host.

A substantial (4.8%) difference was found also between the GenBank-deposited DNA sequences of the species *M. intimus* originating from the type host roach and the DNA structure of the species described by us as *M. eirasianus* sp. nov. developing in the white bream in the same location and producing spores of similar morphology in the gill lamellae. At the same time, the spores collected from plasmodia developing in similar location in ide and asp showed high morphological similarity and a high degree of 18S rDNA sequence identity (99.2–99.7%) to the species *M. intimus* described from the type host; therefore, we identified these species with the above species. We interpreted the less than 1% difference as a sign of identity, as a difference of similar degree (0.3–0.7%) could be demonstrated also between the GenBank-deposited sequences of the species *M. cerebralis* from rainbow trout. Surprisingly, less than 1% difference in DNA sequence could be demonstrated also between the species *M. eirasianus* and *M. hungaricus*, although the spores of these two species are morphologically well distinguishable. Namely, the spores of *M. hungaricus* have two ribs on the spore surface running parallel with the suture.

The morphological similarity of the spores, the identical location, the seasonal spore formation in March and April, the identical histotropism and the relative similarity in DNA structure suggests that ‘dujardini-type’ and ‘intimus-type’ species have common phylogenetic origin. The data obtained so far indicate that the occurrence of a given *Myxobolus* species can be expected with the greatest likelihood, besides the type host and the typical host, in the fish species most closely related to the host. It could be assumed that the species *M. intimus* would infect, in addition to the type host roach, also other fish species belonging to the same tribe, e.g. the ide. However, the present studies indicate that, despite the indisputable morphological similarity of the spores and the fact that the spore formation of the parasite in the gill lamellae was restricted to the spring months, the fish species belonging to the same tribe but two different genera have two closely related but distinct *Myxobolus* species. A similar conclusion was drawn by Cone and Overstreet (1998), who studied *Myxobolus* species living in the bulbus arteriosus of centrarchid fishes. They suggested that the *Myxobolus* species found in identical organ and tissue locations in closely related centrarchids became distinct species parallel with the species separation of their hosts. Also, it may seem surprising that species distinguishable from *M. dujardini* known from *Leuciscus cephalus* could be detected in *Leuciscus idus* assigned to the same genus, as well as in asp and white bream. Studying the morphology of *M. dujardini* spores isolated from dace (*Leuciscus leuciscus*) and chub (*L. cephalus*), Lom (1961) reported that spores of similar shape but different width occurred in the two fish species. These apparent differences are relatively easily interpretable if we consider the results obtained by sequence analysis of the cytochrome b gene during molecular studies published in the field of fish taxonomy during the past two decades (Briolay *et al.* 1998, Zardoya and Doadrio 1999). In the light of the re-

cently elucidated genetic relatedness of cyprinids, the similarities and differences found by us in their parasitic infections are not so surprising. Zardoya and Doadrio (1999), Briolay *et al*. (1998) and Costedoat *et al.* (2006) are of the opinion that, because of the substantial genetic distance between *Leuciscus cephalus* and *L. idus*, the above species should be classified into two genetically rather distant genera (*Squalius* and *Leuciscus*); at the same time, the species *Aspius aspius* prove to be rather close to the *Leuciscus* genus (Perea *et al*. 2010). The results of our studies suggest that from the genetic relatedness of fishes we can draw conclusions not only on the likely occurrence of a given *Myxobolus* species or several morphologically similar species in another (closely related) fish species, but on the basis of the shared parasites the science of parasitology may provide valuable data to ichthyologists investigating the phylogenetic origin of the fish hosts.

When studying the very large and ramifying plasmodia of *M. dujardini* that occasionally fill an entire gill filament, for a long time it was difficult to explain how these plasmodia of substantial size developed. Studying the plasmodia of *M. nodulointestinalis*, Masoumian *et al*. (1996) found that the cysts of substantial size, observed in the intestinal wall of *Barbus sharpei*, were actually formed by the fusion of several smaller plasmodia. In the present study, we could histologically process a relatively early developmental stage of *M. alvarezae* sp. nov., when a confluent chain of multiple smaller plasmodia was observed instead of the commonly seen single, large plasmodium. A similar case was described by Feist and Longshaw (2006), who observed a large number of closely linked smaller plasmodia in the case of the species identified by them as *M. macrocapsularis* but probably identifiable with *M. dujardini*, among the gill lamellae of chub. We suppose that these plasmodia, undergoing gradual fusion, form a single integrated plasmodium complex by the end of spore formation, as was observed several times by Molnár *et al*. (2006) for the species *M. dujardini* and also noticed by us on two occasions for *M. alvarezae* sp. nov. earlier. The studies also demonstrated how the lateral lobes developed on the plasmodia long stretched out in the multilayered epithelium. In our opinion, these lateral lobes are not the result of plasmodium budding; rather, they represent remnants of plasmodia developing in the epithelium between the lamellae, which have fused with the main branch of the plasmodium that develops along the edges of the gill filament.

The present studies further confirm earlier observations (Molnár 1994; Eszterbauer 2002, 2004) suggesting that *Myxobolus* species have much stronger host and organ specificity and histotropism than supposed earlier, and that the morphologically similar spores found in different hosts or in the different organs and tissues of the same hosts represent distinct and probably hitherto not described *Myxobolus* species in the majority of cases. The fact that the *Myxobolus* species hitherto found in different fish species but diagnosed as a single species may actually correspond to several distinct species, raises numerous problems in the designation of the type hosts. Thélohan (1892), who originally described the species *M. dujardini*, designated four fish species (*Scardinius erythrophthalmus*, *Perca fluviatilis*, *Rutilus rutilus* and *Cyprinus carpio*) as its original hosts. Of them, only *S. erythrophthalmus* and *R. rutilus* belong to the Leuciscinae subfamily, and only these two species, or one of them, can be regarded as the host of *M. dujardini* with certainty. Therefore, it is doubtful whether the sequences originating from the chub and deposited in the GenBank under the name *M. dujardini* indeed represent the type species.

While studying the species examined, we made observations also on seasonality. The development of the species *M. intimus* and *M. eirasianus* sp. nov. shows an expressed seasonality, and the spore formation and detectability of these species are restricted to the early spring months, like those of some species having an anteriorly tapering spore (*M. hungaricus* and *M. elegans*). At the same time, in the case of *M. dujardini*, *M. alvarezae* sp. nov. and *M. sitjae* sp. nov. we could not observe any signs indicative of seasonality. The present studies also suggest that the number of species morphologically identifiable as a single species but proving to be distinct by molecular methods can be rather high. Because of the above considerations, the great majority of *Myxobolus* spores found in different hosts and identified with a certain known species are likely to represent hitherto unknown species and, therefore, several thousand hitherto not identified distinct species are likely to exist also in the fish species already studied, in contrast to the about 800 *Myxobolus* species described so far.

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