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Some remarks on the occurrence, host-specificity and validity of *Myxobolus rotundus* Nemeczek, 1911 (Myxozoa: Myxosporea)

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Abstract *Myxobolus rotundus* Nemeczek, 1911 is a common and specific parasite of the common bream *Abramis brama* (L.). Small, round or ellipsoidal shaped plasmodia of this species develop in the gill and exhibit strong histotropism to the secondary gill lamellae with plasmodial development in their capillary network. *M. rotundus* is frequently found in mixed infection with *M. bramae* Reuss, 1906, a parasite of the afferent arteries of gill filaments. The round spores of *M. rotundus* resemble several other *Myxobolus* spp., but can be distinguished from these by their small subunit ribosomal RNA gene sequence (GenBank accession no. EU710583), which also differs from the sequence for '*M. rotundus*' from the skin of Chinese goldfish *Carassius auratus auratus* (L.), which we suggest has been misidentified. The SSU rRNA gene sequence of *M. rotundus* myxospores from bream corresponded to Triactinomyxon type 4 actinospores (AY495707) isolated from *Tubifex tubifex* (Müller) by Hallett et al. (2005), and we infer from this that these are alternate life stages.

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Introduction

Until the general acceptance of molecular biological methods in myxozoan research, spore morphology was the primary means of identification of different myxosporean species. The basic question of whether morphologically similar spores in different fish species belonged to one or more parasite species could not be solved conclusively without experimental infections with live hosts. Due to the complicated and long-term development of myxosporeans in an oligochaete and a fish, these experiments could be performed only in a few laboratories. A helpful but less accepted approach for identification was the consideration of host, organ and tissue specificity (Lom & Arthur 1989). This was based on the accumulated knowledge that members of some myxosporean genera, such as *Myxobolus* Bütschli, 1882, infect only a single host or closely related fishes, prefer development in a single organ and are strictly specific to a host tissue type. The combination of morphological and molecular biological methods, with a consideration of host range and tissue tropism, provides a holistic approach to discern valid species from synonyms or erroneously identified taxa.

Myxobolus rotundus Nemeczek (1911) is regarded as one of the most common species of *Myxobolus*. Donec & Shulman (1984) report it from 27 cyprinid fishes and from different organs; however, their work is primarily a compilation of earlier records of *M. rotundus* based on the morphology of spores scattered in different organs. Less well-trained parasitologists

often identify *Myxobolus* infections exclusively by the shape and the size of the myxospores and neglect the study of vegetative stages and their location in the fish body. Such authors tend to assign identities of species to commonly known taxa (i.e. *M. muelleri* Bütschli, 1882, *M. cyprini* Doflein, 1898 and *M. dispar* Thélohan, 1895) or based on some common adjectives, such as *oviformis, ellipsoides, cycloides, rotundus, parvus* or *magnus*.

Myxobolus rotundus was originally described by Nemeczek (1911) from the gills of the common bream *Abramis brama* (L.) in the Hungarian stretch of the River Danube near Komárom. Although he provided a good description of the species, Nemeczek's depiction of the spores is poor. Miroshnichenko (1980) provided an adequate line drawing, but failed to name the host fish. Molnár (1994) pointed out that most species of *Myxobolus* have a relatively strict host, organ and tissue specificity, and infect only genetically closely related fishes.

Myxobolus infections in bream are relatively well studied. In Hungary, seven species (*M. bramae* Reuss, 1906, *M.* (s.l.) *dogieli* Bykhovskaya-Pavlovskaya & Bykhovski, 1940, *M. hungaricus* Jaczó, 1940, *M. impressus* Miroshnichenko, 1980, *M. macrocapsularis* Reuss, 1906, *M. pseudodispar* Gorbunova, 1936 and *M. squamaphilus* Molnár, 1997) have been recorded (Molnár, 1997; Molnár & Baska, 1999; Molnár & Székely, 1999; Molnár et al., 2002; Molnár et al., 2008). For three of these, *M. hungaricus, M. bramae* and *M. macrocapsularis*, there is both histological and developmental cycle data (El-Mansy & Molnár, 1997; Eszterbauer et al., 2000; Székely et al., 2002). Molnár & Székely (1999) studied histo-pathological changes in the gill and described a *M. bramae* infection with small cysts in the lamellae, as well as an infection with large cysts in the arteries of the filaments. The above authors later realised that the lamellar infection was actually *M. rotundus* and recognised that this species needed redefinition. Thus, herein we give a redescription of the myxozoan species *Myxobolus rotundus* and support the validity of the species with DNA sequence data.

Materials and methods

Collection of host fish

Between 2002 and 2006, common bream *Abramis brama* and other cyprinid fishes from Hungary were studied within a broad investigation of *Myxobolus bramae* versus *M. rotundus* infections. Common bream (8–41 cm in total length) were collected from Lake Balaton (172 fish), Kis-Balaton water-reservoir (37), River Danube (12) and River Tisza (7). In addition, from Lake Balaton, 80 white bream *Blicca bjoerkna* (L.), and from the River Danube six blue-bream *Abramis ballerus* (Pallas) and four white-eyed bream *Abramis sapa* (L.) were examined.

Fish were harvested by a fine meshed seine or purchased from fishermen. All fish were transported to the laboratory alive and held in aquaria for up to four days prior to examination. All organs were checked for myxosporean infection, with special attention being paid to the gills.

Morphological methods

Parasites were collected from freshly killed fish. When mature plasmodia were found, a subsample of spores was studied live, another subsample was refrigerated then transferred to ethanol for molecular examination and a third subsample was preserved in glycerine jelly for slide preparation. The presence of an iodinophilic vacuole was checked by adding a drop of iodine solution to spores under a coverslip. The vitality of spores was checked by adding spores to a 0.4% solution of urea, and spores of a given plasmodium were regarded as mature when at least 90% extruded their polar filaments. Fresh spores were studied using differential interference contrast optics with an Olympus BH2 microscope. Spores were recorded on video, from which digitised images were obtained according to the method of Székely (1997), and measurements taken. Spores measured were collected from bream specimens of three different sizes. Enlarged photographic images of spores on the computer screen were used to determine spore dimensions. Tissue samples from infected organs which contained 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.

Molecular methods

The small subunit ribosomal RNA (SSU rRNA) genes of myxospores from two bream collected from Lake Balaton during 2004 and 2006 (samples 12 and 13, respectively) were sequenced. The ethanol-fixed samples were spun for 3 min at 14,000 rpm to pellet the spores, the ethanol was removed and the

pellet air-dried. Total DNA was extracted with a QIAGEN DNeasy™ tissue kit (animal tissue protocol; QIAGEN Inc., Valencia, California) and eluted in 2 steps of 30 and 20 µl molecular grade (MG) water.

The gene was amplified using primers ERIB1 and ERIB10 (Barta et al., 1997) in a 20 µl reaction which comprised: 0.5 µl extracted genomic DNA, 0.2 mM dNTPs, 0.25 µM of each primer, 2 µl 10X Taq buffer, 1.5 mM $MgCl_2$, 1 µl Rediload loading dye (Invitrogen, Karlsbad, California), 1.25 U Taq polymerase (Promega, San Luis Obispo, CA) and MG water. The PCR cycle profile was performed in a PTC-200 (MJ Research Inc., Watertown, MA, USA) and consisted of an initial denaturation step of 95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 120 sec and finished with a terminal extension at 72°C for 10 min, then rested at 4°C.

For sequencing, second-round PCRs were used to generate two overlapping templates with primer pairs ERIB1 and ACT1r (Hallett & Diamant, 2001), and MyxGen4f (Diamant et al., 2004) and ERIB10. Reagent amounts were scaled up to 50 µl reactions, included 1.25 µl of the ERIB1/ERIB10 template, and the above cycling profile was used with the extension step shortened to 60 sec. Aliquots of the resultant PCR products were electrophoresed through a 1% agarose 1X tris-acetate-EDTA buffer (TAE) gel stained with SYBR Safe (Invitrogen Inc., Madison, WI, USA) alongside a 1 kb + DNA ladder (Invitrogen) to confirm only a single amplicon of the expected size was present.

To amplify products for sequencing, 50 µl reaction volumes were used with 1.0 µl of first-round template with the cycle profile: 95°C for 2 min, followed by 35 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 45 sec and finished with a terminal extension at 72°C for 10 min, then rested at 4°C.

Products were purified using a QIAquick PCR purification kit (QIAGEN Inc.). DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and fragments were sequenced in both directions using the amplification primers and ABI Big Dye Terminator chemistry on an Applied Biosystems Capillary 3100 Genetic Analyzer (Foster City, CA, USA) at the OSU sequencing facility (Center for Genome Research and Biocomputing, Core Laboratories). The various forward and reverse sequence segments were aligned in BioEdit (Hall, 1999) and, where possible, ambiguous bases clarified using corresponding ABI chromatograms. A consensus sequence was submitted to GenBank. A standard nucleotide-nucleotide BLAST (blastn) search was conducted (Altschul et al., 1997).

Results

Myxobolus rotundus plasmodia, which contained mature spores, were found in the capillary network of the gill lamellae in 37 (16.2%) common bream *Abramis brama*. In 24 (64.9%) cases, a mixed infection with *M. bramae* was recorded. Spores morphologically resembling *M. rotundus* were also found in two (2.5%) white bream *Blicca bjoerkna* and in one (25%) white-eyed bream *Abramis sapa*. Only spores from common bream were characterised in detail.

Myxobolus rotundus Nemeczek, 1911

Syn. *M. bramae* of Molnár & Székely (1999) in part

Host: Common bream *Abramis brama* L. (Cyprinidae).
Locality: Lake Balaton, Hungary.
Other locality: River Danube (type–locality).
Site of tissue development: Capillary network of the gill lamellae.
Material: Voucher spores and histological sections deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-17832. The SSU rRNA gene sequence of *M. rotundus* deposited in GenBank under Acc. No. EU710583.

Description (Figs. 1–6)

Vegetative stages: Round or short-ellipsoidal plasmodia of 60–180 µm in diameter located inside gill lamellae.
Spores: Spores round or roundish in frontal view (Figs. 1a, 2a), lemon-shaped in lateral view (Figs. 1b, 2b). Valves thin, symmetrical, smooth. Sutural rim around spore without distinct marginal markings, protrudes 0.5–1.0 µm over surface of spore. Spores (N = 50) 10.6 ± 0.8 (9–12) µm long, 9.5 ± 0.8

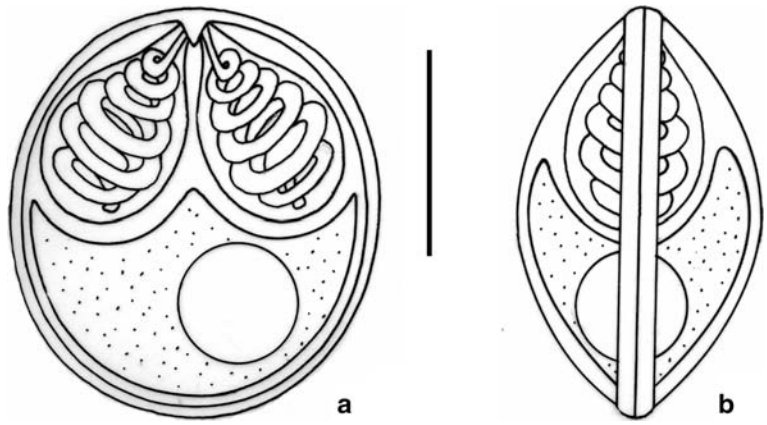


Fig. 1 Schematic drawings of *Myxobolus rotundus* myxospores: a. frontal view; b. sutural view. *Scale-bar*: 10 μm

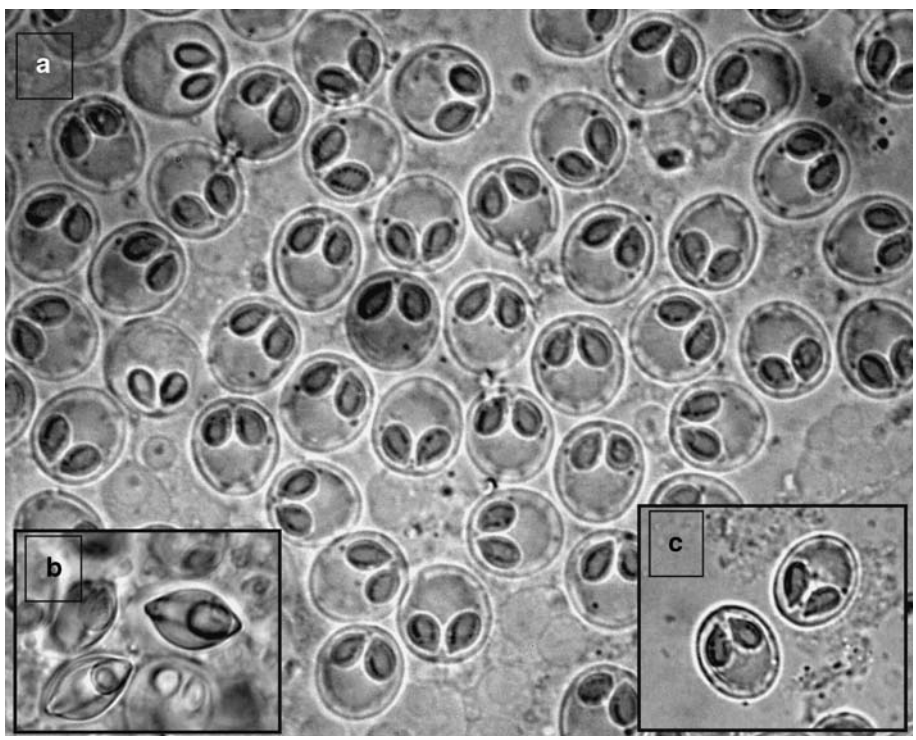
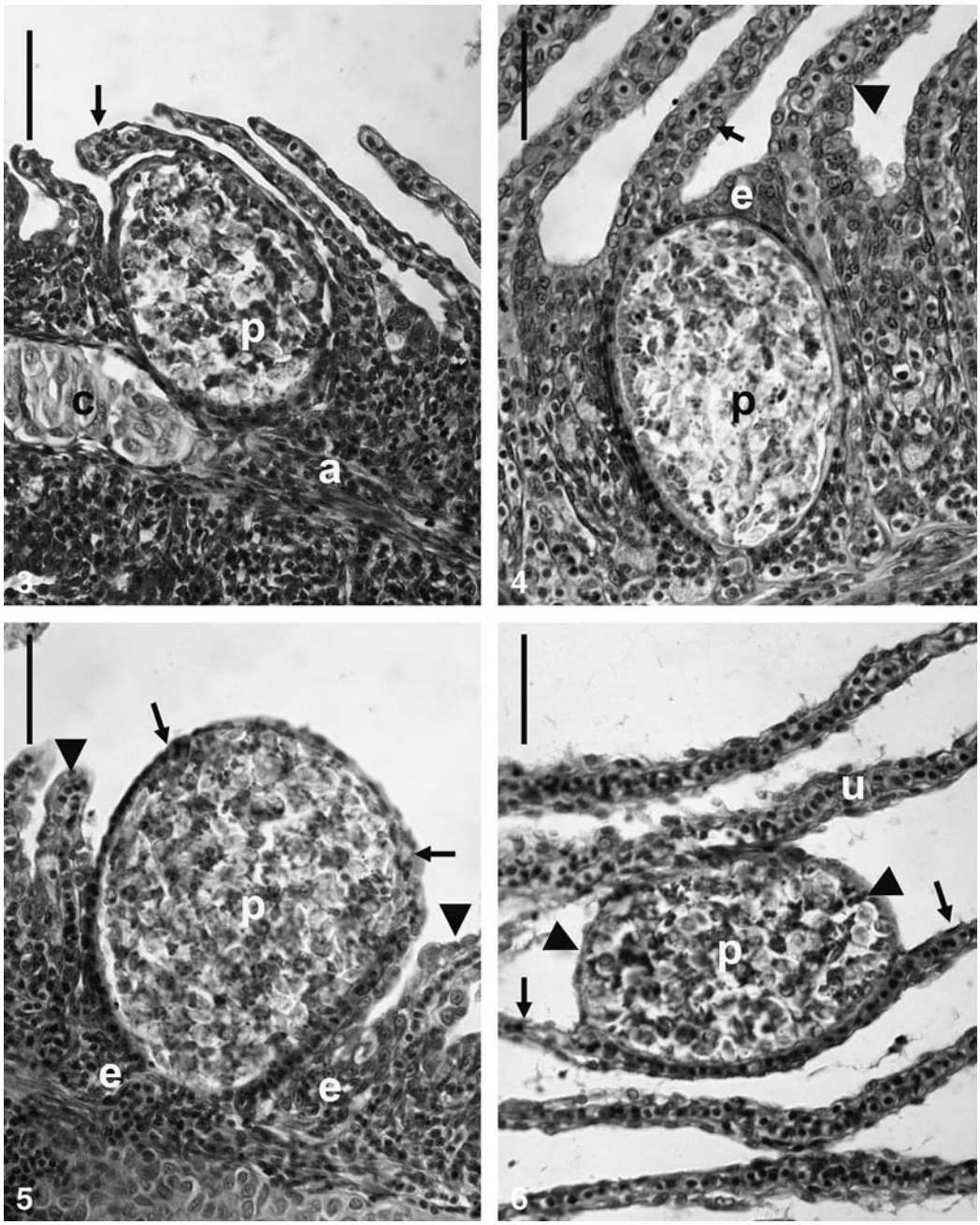


Fig. 2 Fresh, unstained myxospores: a. *Myxobolus rotundus* in frontal view; b (inset). *M. rotundus* in sutural view; c (inset). *M. bramae*. *Scale-bar*: 10 μm

(8–11) μm wide, 6.2 ± 0.3 (5.5–6.5) μm thick. Polar capsules, 2, pyriform, equal in size, 4.9 ± 0.2 (4.5–5) μm long and 3.2 ± 0.2 (3–3.5) μm wide, taper toward discharging canals of polar filaments. Polar filaments coiled, with 6 turns, situated perpendicularly to longitudinal axis of polar capsule. Spore has short-triangular or protuberant, 0.5 ± 0.1 μm long intercapsular appendix at anterior end. Sporoplasm nuclei indiscernible; large iodinophilous vacuole present in sporoplasm.

Histology: Small, round plasmodia of *M. rotundus* developed in the capillary network of the gill lamellae (Fig. 3). In medium and large bream, plasmodia were found only in the basal part of the lamellae, and the distal sections of the lamellae showed no changes (Figs. 3, 4). In smaller fish, plasmodia filled the total



Figs. 3–6 Histological sections (H & E) of plasmodia (p) of *Myxobolus rotundus.* 3. Filled with spores in the capillary network of a lamella (arrow) of the gill of a medium-sized (15 cm long) bream. The less damaged part of the network borders the plasmodium on one side only: (c) a part of the cartilaginous gill rays; (a) afferent artery of the gill filament. 4. Filled with spores in the capillary network of a lamella (arrow) in the gill of a large (25 cm long) bream. Arrowhead indicates uninfected neighbouring gill lamella; (e) compressed interlamellar epithelium. 5. Located centrally in a gill lamella of a small-sized (12 cm long) bream. The plasmodium is surrounded by the capillary network (arrows). Arrowheads indicate uninfected neighbouring gill lamellae; (e) compressed interlamellar epithelium. 6. Cross-sectioned lamellar region of the gill filament of a large bream. The plasmodium develops under the epithelial layer (arrowhead). The capillary network of the lamella is filled with red blood-cells. Parts of the infected lamella close to the afferent and efferent arteries are free from the plasmodium (arrows). The plasmodium only has physical contact with the uninfected lamella (u). *Scale-bars*: 10 μm

volume in the capillary network of the lamellae (Fig. 5). Less frequently, the plasmodium developed in the central region of the lamellae and the capillary network surrounded it on both sides (Fig. 6). In most cases, however, the plasmodium occupied only one side of the lamellae and protruded into the interlamellar space (Figs. 3, 4). This part of the plasmodium was bordered only by a single epithelial cell layer. On the other side of the plasmodium, the blood stream inside the capillary network appeared to be undisturbed. The location of plasmodia inside the lamellae was especially obvious in cross-sectioned lamellae (Fig. 6). In these sections, the plasmodia were intralamellar and infected only a single lamella. The regions of the lamellae close to the afferent and efferent arteries were not occupied by plasmodia. In both cases of plasmodial development, cells of the interlamellar epithelium were compressed (Figs. 4, 5).

In light infections, the small plasmodia of *M. rotundus* caused only minor pathological changes, with only compression of the neighbouring lamellae.

Molecular results

The SSU rRNA gene of *M. rotundus* was determined to be 2031nt long, inclusive of primers ERIB1 and ERIB10, and the consensus sequence was deposited in GenBank (acc. no. EU710583). The sequence contained 2 unresolved regions: 50 nt between positions 1,517 and 1,566, whose length was inferred by alignment with that of the closely related myxosporean *M. parviformis* Kallert, Eszterbauer, Erséus, El-Matbouli & Haas, 2005 (AY836151); and the final 12 nt of the sequence adjacent to primer ERIB10, whose length was inferred by alignment with *M. cerebralis* (Hofer, 1903) (AY479924). The SSU rRNA gene sequences of myxospore samples 12 and 13 were genetically identical over 1936 nt. The sequencing runs were both inhibited *c*.500 nt from the 3′ end of the gene. Sequencing would fail in this region when reading commenced from either the 5′ or 3′ end of the fragment. The OSU sequencing lab. suggested that this part of the gene may have either a poly-base repeat or some undenatured secondary structure that interfered with the sequencing read. Three polymorphic sites were present in sequence chromatograms of both samples (Fig. 7). These indicate the presence of multiple SSU rRNA alleles within the parasite population.

A BLAST search revealed that the SSU rRNA sequence of *M. rotundus* was identical to the sequence of Triactinomyxon type 4 of Hallett et al. (2005)(AY495707; over 1,456 nt). Re-examination of the original Triactinomyxon type 4 chromatograms showed they too exhibited the same polymorphic loci, which suggested the allelic mix within the parasite population remained constant from 2002 (actinospore sample) to 2006 (latest myxospore sample). The sequencing reads for both the triactinomyxon actinospore and myxospore failed at the same points. The SSU

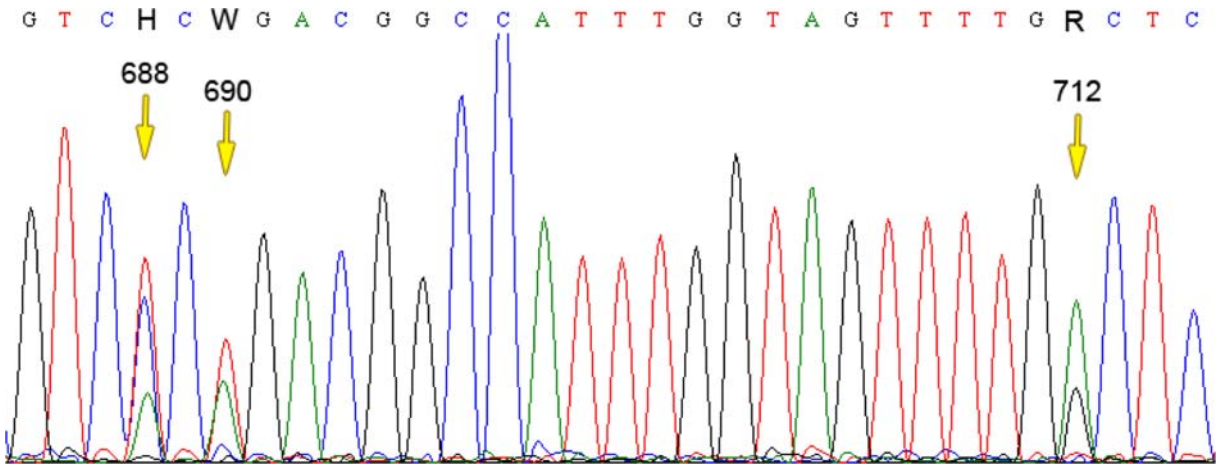


Fig. 7 Section of the sequencing chromatogram of the *Myxobolus rotundus* SSU rRNA gene showing three consistent and characteristic polymorphic loci (arrows)

rRNA gene sequence of *M. rotundus* did not match any other species in GenBank, including the Chinese '*M. rotundus*' (AY165179; 78% over 726 nt).

Remarks

The morphometrics of *M. rotundus* myxospores correspond to data given by Nemeczek (1911) and Miroshnichenko (1980) (Table 1). No morphological differences were found between plasmodia and spores collected from Lake Balaton and the River Danube. According to Nemeczek's original description, only *Abramis brama* can be regarded as the type-host, although Landsberg & Lom (1991) listed another cyprinid fish, *Gobio gobio* (L.). With round spores, *M. rotundus* morphologically resembles a great number of *Myxobolus* spp., but it shows closest affinity with *M. parviformis,* being similar in shape, location and measurements (Table 1), but the intercapsular appendix in *M. parviformis* appears smaller than in *M. rotundus*. There is a 96% sequence similarity between the SSU rRNA genes of the two species, which suggests they are distinct but closely related within the Myxozoa (*M. parviformis* AY836151; over *c*.1.580 nt). It was also similar to another gill-infecting species, *M. impressus* Miroshnichenko, 1980 (AF507970; *c*.94% over 1,509 nt), but this latter species has a different location in the gills; it infects the multilayered epithelium between the gill lamellae. Of the other *Myxobolus* species which commonly infect bream, spores of *M. bramae* resemble some of the less flattened spores of *M. rotundus*, but the two species can be readily distinguished by their site of development in the host and by their SSU rRNA gene sequences (86% similarity over *c*.1,590 nt; *M. bramae* AF507968).

Discussion

Although *Myxobolus rotundus* has been recorded from several fish species and from different sites in the fish body by several authors, this myxosporean actually seems to be a specific gill parasite of the common bream *Abramis brama*. The parasite forms small plasmodia and prefers the capillary network of gill lamellae. We suppose that the host range of *M. rotundus* might at most include some leuciscine fishes, such as *A. sapa, A. ballerus* or *Blicca bjoerkna*.

In our surveys of Hungarian bream, *M. rotundus* frequently occurred in mixed infection with *M. bramae*. The two species can be distinguished with careful scrutiny, as the latter species has oval to ellipsoidal spores which developed in large elongate plasmodia inside the arteries of the gill filaments. Before realising the importance of the minor differences in shape and size of the spores and plasmodia, and the different location in the gills, two of the present authors (Molnár & Székely, 1999) regarded *M. bramae* and *M. rotundus* as a single species, and they described both spores developing in small cysts inside gill lamellae and spores in large cysts in the filament arteries as *M. bramae*.

The simple, roundish spores of *M. rotundus* resemble several known and undescribed *Myxobolus* spp. that infect different fishes; correct identification is possible only after considering host-specificity or

Table 1 Morphometrics of myxospores of *Myxobolus rotundus* and some related *Myxobolus* spp

Parasite Source of data	*M. rotundus* Present study	*M. rotundus* Nemeczek (1911)	*M. rotundus* Miroshnichenko (1980)	*M. parviformis* Kallert et al. (2005)	*M. bramae* Eiras et al. (2005)
Spore shape	roundish	roundish	roundish	roundish	oval
Spore length	10.6 (9–12)	10	9–10.8	11.2 (9.9–12.1)	10–12
Spore width	9.5 (8–11)	9.8	8.1–9.8	–	8–10
Spore thickness	6.2 (5.5–6.5)	3	–	–	4.5–6.5
Polar capsule length	4.9 (4.5–5.0)	3.8–5	4.0–5.1	5.1 (4.6–5.8)	4–5.5
Polar capsule width	3.2 (3–3.5)	–	2–3	3.3 (2.7–3.8)	2.3–3.5
Polar filament length	–	40	40–50	–	–
No. filament turns	6	–	–	5	4–5
Intercapsular appendix	small	–	small	small	small

DNA sequences (from the type-host). By neglecting these aspects, the authors Wu & Wang (2000), Lu et al. (2002), Lu & Nie (2004) and Zhang et al. (2006) erroneously identified the species that infected goldfish *Carassius auratus auratus* (L.) as *M. rotundus*. In the present work, we obtained a different SSU rRNA sequence from spores from the type host *A. brama*, and we suggest this is compelling evidence that the *Myxobolus* species studied by Lu et al. (2002) and Zhang et al. (2006) is a different species, which may correspond to one of the great number of species listed from goldfish by Chen & Ma (1998) and Eiras et al. (2005). This distinction is important, since the species that infects goldfish is one of the most important myxosporean parasites of this fish in China (Wu & Wang, 2000).

The nomenclature of species in the older literature may have caused confusion. *Myxosoma rotundum* Meglitsch, 1937 was transferred to *Myxobolus,* which resulted in the combination *Myxobolus rotundus* (Meglitsch, 1937), but this was a secondary homonym of *M. rotundus* Nemeczek, 1911. To resolve this homonymy, Grinham & Cone (1990), followed by Landsberg & Lom (1991), assigned the new name *M. meglitschi* Grinham & Cone, 1990. However, this species was described from the gills rather than the skin of its cyprinid host.

Differences in DNA sequences of a given *Myxobolus* sp. (e.g. *M. muelleri* Bütschli, 1882), deposited in GenBank under the same name but from different fish hosts, indicate that there might be great genetic differences between samples identified only by the shape of the spores. We propose, therefore, that when DNA sequences are provided after species descriptions have been published (often many years), these should be obtained from parasite spores collected only from the type-host and, preferably, the type-locality. In the case of *M. rotundus*, only spores collected from *A. brama* should be regarded as representative material of this species, and samples collected from other cyprinids should be regarded as the same species only if spore morphology, host tissue tropism and gene sequences correspond.

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