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Infection of the heart of the common bream, *Abramis brama* (L.), with *Myxobolus* s.l. *dogieli* (Myxozoa, Myxobolidae)

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Abstract

Myxobolus dogieli Bykhovskaya-Pavlovskaya & Bykhovski, 1940 is regarded as a site specific myxosporean, infecting the heart of cyprinid fish. During a survey of the myxosporean fauna of Lake Balaton fish, heart myxobolosis was found in the common bream, *Abramis brama*, with heavy infection of the ventricle and the bulbus arteriosus in some infected bream. Developing and mature plasmodia were mostly in the connective tissue of the subepicardium and subendocardium. Plasmodia developing in the subendocardium protruded into the lumen of the heart, while plasmodia developing in the subepicardium protruded over the epicardium forming large sausage-like outgrowths. Plasmodia with mature spores were found in the summer. The shape and size of the spores corresponded to those of the original description. Phylogenetic analysis based on the 18S rDNA sequence of *M. dogieli* showed that this species was basal in the genus *Myxobolus*. As no molecular data are available on spores from the type host, common carp, the species studied by us is temporarily designated as *Myxobolus* s.l. *dogieli*.

Keywords: common bream, heart infection, histology, molecular phylogeny, *Myxobolus*, Myxozoa, spore morphology, tissue location.

Introduction

Most *Myxobolus* spp. show specific site selection. Of these *Myxobolus dogieli* Bykhovskaya-Pavlovskaya & Bykhovski, 1940 is one of the best-known species infecting the heart of its fish host. The species was originally described from wild carp, *Cyprinus carpio* L., in the bays of the Azov Sea, with subsequent reports from cyprinid fish in the region of the Caspian and Black Seas (Donec & Shulman 1984). Of the other species infecting the heart as a typical site organ Eiras, Molnár & Lu (2005) recorded in their synopsis *Myxobolus baueri* Chernova, 1970 from *Tinca tinca* (L.) *M. bulbocordi* Masoumian, Baska & Molnár 1996 from *Barbus sharpeyi* (Günther); *M. cordis* Keysselitz 1908 from *Barbus barbus* (L.) *M. hearti* Chen & Ma, 1998 from *Carassius auratus* (L.)*; M. heteromorpha* Ma, 1993 from *Cyprinus carpio*; *M. hwangshihensis* Nie & Lie, 1992 from *Xenocypris argentea* (Günther) *M. kiangtshingensis* Ma, 1998 from *Rhodeus ocellatus* (Kner)*; M. kuleminae* Donec, 1984 from *Aspius aspius* (L.) *M. paralintoni* Li & Desser, 1985 from *Lepomis gibbosus* (L.) *M. suturalis* Shulman, 1962 from *Schizothorax intermedius* McClelland and *M. tuberculus* Nie & Lie, 1962 from *Carassius auratus*.

Bykhovskaya-Pavlovskaya & Bykhovski (1940) described *M. dogieli* causing large sausage-like cysts in and outside the heart. Nowak (1972) and Bauer, Voronin & Yunchis (1991), who regarded *M. dogieli* as a highly pathogenic agent of cultured common carp, found a similar infection in the heart. Masoumian *et al.* (1996) recorded a similar location for the cysts of *M. bulbocordis. Myxobolus* infection of the heart region of non-cyprinid fish was reported by Cone & Overstreet (1998) who found three different species in centrarchid fish.

This paper reports on the occurrence and histopathology of *Myxobolus* s.l. *dogieli* infection of the common bream, *Abramis brama* (L.), in Lake Balaton, and supplements the species diagnosis with information on spore morphology and molecular

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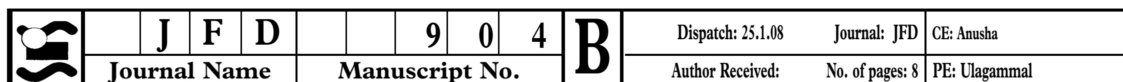

characterization of the 18S rDNA region of material from this host.

Materials and methods

Common bream and other cyprinid fish harvested from Lake Balaton, Hungary were studied in 2005 and 2006 during a survey of myxosporean infection of these fish. During the 2-year period, 92 common bream 8–41 cm in total length were examined. Fish were harvested by a fine meshed seine net or purchased from fishermen. All fish were transported to the laboratory alive and held in aquaria for 2–3 days prior to necropsy. Except for 24 specimens where only the heart was examined, all organs were examined for myxosporean infection.

When mature plasmodia were found, one piece was examined in a wet mount, a second was frozen for later molecular taxonomy, and a third prepared as a glycerine-jelly reference slide. The presence of an iodinophilious vacuole was checked by adding a drop of iodine solution to spores placed under a cover slip. Vitality of spores was checked by placing spores in a 0.4% solution of urea. Spores from a given plasmodium were regarded as mature when at least 90% of the spores extruded polar filaments in this solution. Unfixed spores were studied by Nomarski differential interference contrast with an Olympus BH2 microscope. Spore measurements were determined from spore video images obtained according to the method of Székely (1997). 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).

Molecular methods

For DNA extractions, samples preserved in ethanol were centrifuged at 5000 **g** for 5 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 50 μL buffer AE.

The 18S rDNA was amplified using the primers 18e and 18g' (Table 1) in a 25 μL reaction mixture, which comprised 1 μL extracted genomic DNA, 5 μL 1 mM deoxy-ribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.25 μL each primer, 2.5 μL 10X Taq buffer (MBI Fermentas), 1.25 μL 25 mM $MgCl_2$, 1 μL Taq polymerase (2 U)(MBI Fermentas) and 12 μL DEPC water. The Polymerase chain reaction (PCR) cycle consisted of an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 50 s, 56 °C for 50 s, 72 for 80 s and finished with a terminal extension of 72 °C for 7 min followed by rest at 4 °C.

This procedure was followed by a second round PCR with the SphF-SphR primer pair (Table 1). The total volume of the nested PCR reaction mixture was 50 μL, which contained 1 μL amplified DNA, 10 μL 1 mM deoxy-ribonucleotide triphosphates (dNTPs; MBI Fermentas), 0.5 μL of each primer, 5 μL 10X Taq buffer (MBI Fermentas), 2.5 μL 25 mM $MgCl_2$, 2 μL Taq polymerase (2 U);(MBI Fermentas) and 28.5 μL DEPC water. Amplification conditions in the second round were: 94 °C for 50 s, 56 °C for 50 s, 72 °C for 60 s for 35 cycles with an extension period at 72 °C for 10 min, followed by rest at 4 °C. Both PCR cycle profiles 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 a PCR-M™ Clean Up System (Viogene).

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

Table 1 Primers used for Polymerase chain reaction (PCR) or sequencing in this study

Primer	Sequence	Source
18e	5′-CTG GTT GAT TCT GCC AGT-3′	Hillis & Dixon (1991)
18g'	5′-CGG TAC TAG CGA CGG GCG GTG TG-3′	Hillis & Dixon (1991)
SphF	5′- ACT CGT TGG TAA GGT AGT GGC T-3′	Eszterbauer & Székely (2004)
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)

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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 (Higgins, Thompson, Gibson, Thompson, Higgins & Gibson 1994). The alignment was corrected manually using the alignment editor of the software MEGA 3.1 (Kumar, Tamura & Nei 2004). DNA sequence similarities were calculated with the Sequence Identity Matrix of BioEdit. Phylogenetic calculations were performed with MEGA 3.1. The data were analysed with neighbour-joining using the Tamura–Nei model. *Ceratomyxa shasta* was chosen as an outgroup.

Results

Myxobolus dogieli was first found in July 2005 in two bream 13 cm long. Three and four sausage-shaped outgrowths containing several elongate plasmodia each were evident on the surface of the heart which, after dissection, were found to be in the wall of the ventricle and bulbus arteriosus. Of 40 other bream examined that year only a single 15-cm-long specimen was infected. Eight of 23 bream examined from July to August 2006, had trophozoites within ventricular and bulbous tissues that did not extend into pericardium. The parasite was not found in any other season, nor in any other cyprinid examined during the same period.

Description of *Myxobolus s.l. dogieli* (based on 25 spores) from bream

Vegetative stages were round or elongated plasmodia measuring 120–250 μm in diameter and 500–3000 μm in length in the ventricle of the heart, in the bulbus arteriosus and in outgrowths of the epicardium of the heart.

Spores in frontal view are round or shortly ellipsoidal (Figs 1 & 2). A strongly protruding suture with three to five distinct edge markings forms a wide rim around the surface proper of the spore. Spores in sutural view have an elongated lemon shape with a suture protruding at the anterior and posterior end. Spore valves thin, symmetrical and smooth.

Length of the spores together with the wide rim 14 ± 0.41 (13.2–14.4) μm, width of the spores with the rim 12.7 ± 0.57 (12–13.5) μm, thickness 6.2 ± 0.72 (5,7–6.7) μm. Width of the rim in frontal view 1.54 ± 0.12 (1.4–1.7) μm, thickness in sutural view 1.2 ± 0.11 (1.1–1.3) μm. In sutural view the tapering ends of the spore with the rim forms a protrusion of 1.7 μm at the anterior and 2.1 μm at the posterior end.

The two polar capsules are pyriform, nearly equal in size, 4.13 ± 0.22 (4–4.5) μm long and 2.2 ± 0.26 (2–2.5) μm wide, tapering towards the discharging canals of the polar filaments. Polar filaments coiled with five turns in polar capsule, situated perpendicularly to the longitudinal axis of the capsule. Spores have a large triangular

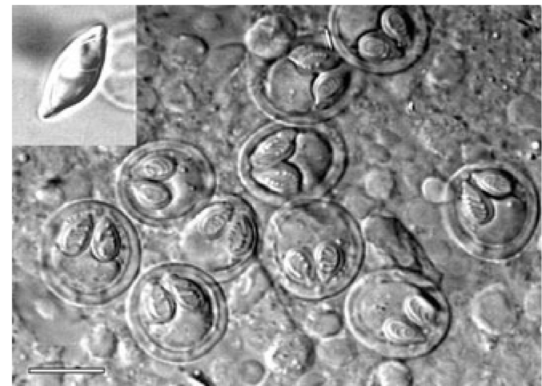

Figure 2 Spores of *Myxobolus* s.l. *dogieli* in frontal view. Inset: spore in sutural view (bar = 10 μm).

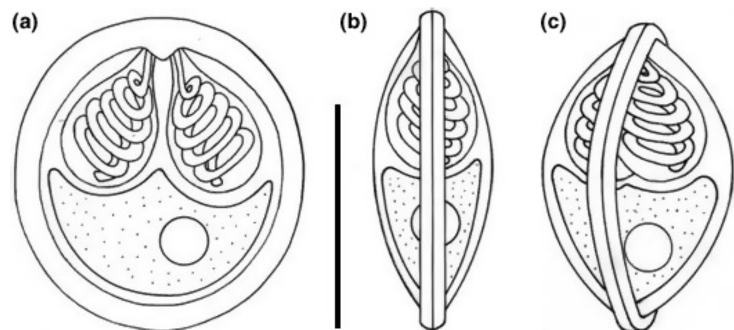

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Figure 1 Schematic drawings of *Myxobolus* s.l. *dogieli* spores. (a) Frontal view; (b) sutural view; (c) semilateral view (bar = 10 μm).

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intercapsular appendix at the anterior end. Sporoplasm nuclei are indiscernible. A small iodinophilous vacuole is present in the sporoplasm.

Type host: *Abramis brama* L.

Site of infection: Ventricle, bulbus arteriosus and outgrowths of subepicardium on the heart.

Locality: Lake Balaton, Keszthely, Hungary.

Type material: Syntype spores in glycerine-gelatine were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-17810. The 18S rDNA sequence of *Myxobolus* s.l. *dogieli* was deposited in Genbank under the accession number (EU003977–EU003978).

Etymology: S.l. indicate that the parasite was collected from fish other than the type host.

Remarks: Spores of *Myxobolus.* s.l. *dogieli* differ from spores of *M. cordis* and other *Myxobolus* spp. infecting the heart of cyprinid fish by their round or roundish spores as well as the large sutural rim emerging over the surface of the spore wall. The gross picture of infection was the same as depicted in the original description of *M. dogieli* from the type host, common carp, by Bykhovskaya-Pavlovskaya & Bykhovski (1940). Measurements of spores from the bream also fit into the range given by these authors. The gross picture and histology of the infection resembles most closely *M. bulbocordis* infection but spores of the latter species have an oval shape and are bordered by only a narrow rim.

Histology of infection

Normal structure of the bream heart

The ventricle of the bream has a central cavity and a wall characterized by large lacunae and a system of small luminae (Figs 3 & 4) between trabecular sheets of myocardia radiating outward from the central lumen. The endocardium is a flat endothelium joined to the myocardial cell bundles by the subepicardium composed of a connective tissue rich in capillaries and round cell elements. The wall of the bulbus arteriosus contains elastic collagenous chordae bordered by a similar subepicardium and subendocardium as in the ventricle (Fig. 5).

Histological changes in the Myxobolus-infected heart

Plasmodia round or oval in cross-section were found in the subendocardium of the ventricle of the

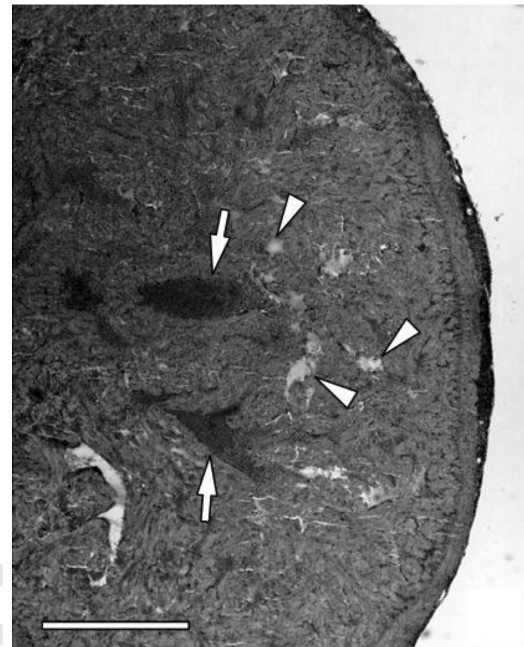

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Figure 3 Part of a cross-section of the ventricle wall of an uninfected heart of bream. Large lacunae (arrows) filled by blood and small luminae (arrow heads) are found between myocardial trabecules (H&E, bar = 100 μm).

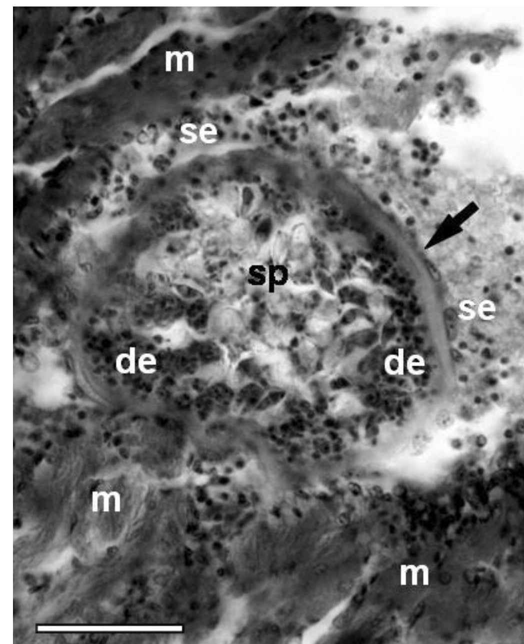

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Figure 4 Partially mature *Myxobolus* s.l. *dogieli* plasmodium in the ventricle of bream. The plasmodium located among myotrabeculae (m) is surrounded by cells of the subendocardium (se). Plasmodium bordered by a thick ectoplasm (arrows) contains spores (sp) in the centre and sporogonic developmental stages (de) at its periphery (H&E, bar = 10 μm).

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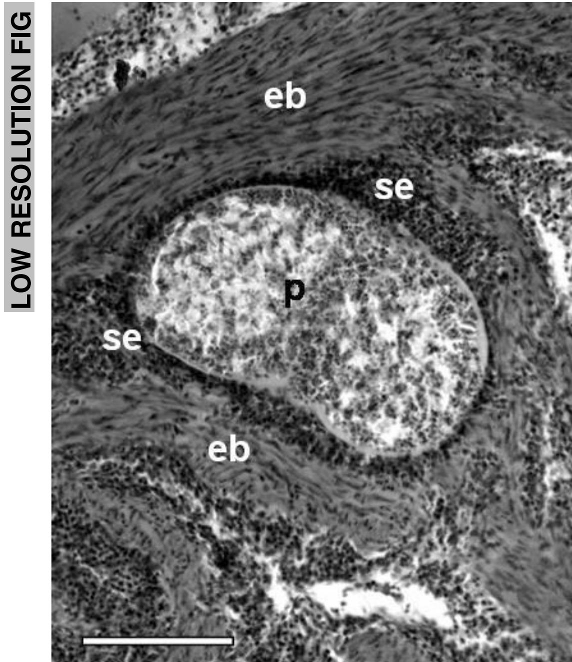

Figure 5 *Myxobolus* s.l. *dogieli* plasmodium in the wall of the bulbus arteriosus of bream. Plasmodium (p) is surrounded by a thick layer of subendocardium (se) bordering the elastic bundles (eb) of the collagen histiocytes (H&E, bar = 10 μm).

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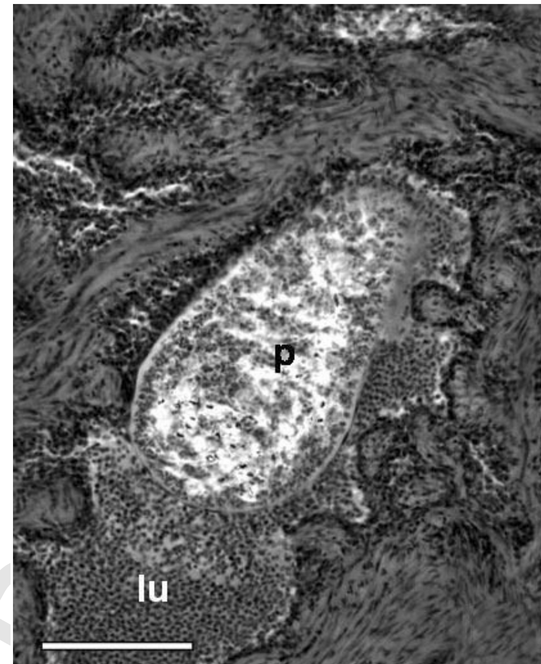

Figure 6 *Myxobolus* s.l. *dogieli* plasmodium in the wall of the bulbus arteriosus of bream. The larger part of the plasmodium (p) is in the lumen of the bulbus (lu) among erythrocytes (H&E, bar = 10 μm).

heart (Fig. 4) and in the bulbus arteriosus (Fig. 5). In both the ventricle and the bulbus plasmodia were located inside the connective tissue of the subendocardium separated from the myocardial bundles and elastic fibres, respectively. Less frequently plasmodia were only partly bordered by the subendocardium and the ectoplasm of the plasmodium was in direct contact with the blood in the lumen of the bulbus (Fig. 6). In heavily infected fish the subepicardium was also affected and large bundles of sausage-like forms grew out of the subepicardium and attached to the epicardial surface of the bulbus and the ventricle (Fig. 7). In these outgrowths plasmodia or in advanced cases groups of mature spores (Fig. 8) were recovered.

Molecular analysis

DNA sequences of three *Myxobolus* s.l. *dogieli* samples (EU003978) from different bream specimens showed 100% identity based on 1265 aligned base pairs, while a fourth sample (EU003977) showed 99.7% similarity to these three. The most closely related species to *Myxobolus* s.l. *dogieli* was a parasite of the chub, *Leuciscus cephalus* (L.),

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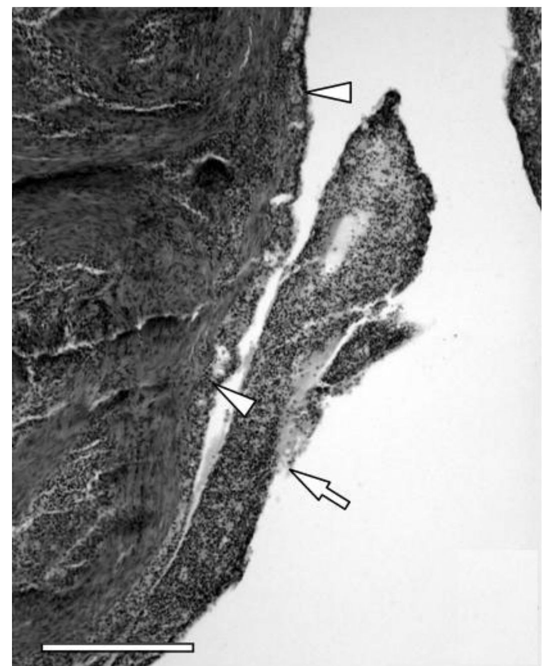

Figure 7 An outgrowth of the subepithelium (arrow) on the surface of the ventricle of bream (arrowheads) (H&E, bar = 10 μm).

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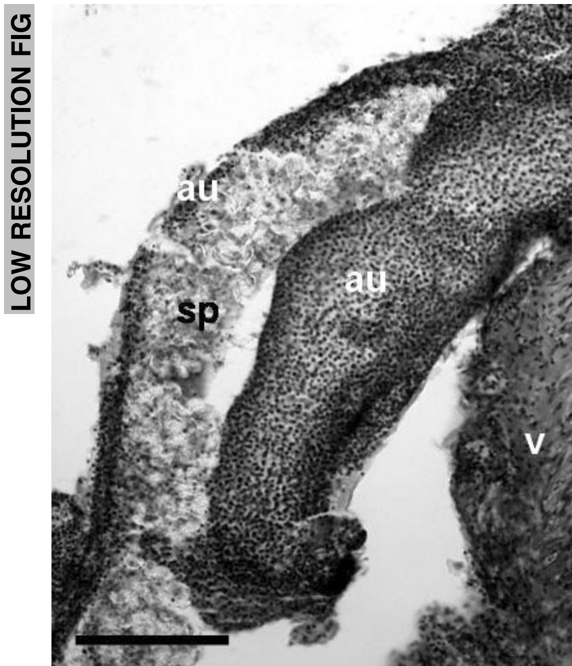

Figure 8 An elongated plasmodium of *Mxyobolus* s.l. *dogieli* with mature spores (sp) in a outgrowth (au) on the wall of the ventricle (v) of bream (H&E, bar = 10 μm).

reported by Molnár, Marton, Eszterbauer & Székely (2006) as *Myxobolus* sp. 2, which showed 93.8–94.0% similarity. Close similarity was also found to *Myxobolus ellipsoides* ex *Leuciscus cephalus* which was 92.9–93.5% similar to *M.* s.l. *dogieli* (Fig. 9).

Discussion

Myxobolus infection of the heart of different fish species is only rarely recorded, although *M. cordis* was described from its specific host, the barbel, in the early 1900s (Keysselitz 1908). Of these infections, *M. dogieli* is the best known. Donec & Shulman (1984) reported it from 22 fish species. Bauer *et al.* (1991), however, reported that this parasite is specific for the common carp. Masoumian *et al.* (1996), who found similar gross signs in *Barbus sharpeyi,* described *M. bulbocordis*, a species with different spore morphology. Spores collected in this study from the common bream were morphologically very close to the original description of *M. dogieli* but, because of the relatively large genetic distance between the bream and the common carp, until further molecular data is available the species found in this study will be designated as *Myxobolus* s.l. *dogieli*. The probability that heart and bulbus infections in different cyprinids, regarded up to now as caused by *Myxobolus dogieli,* represent more than one species is supported by the findings of Cone & Overstreet (1998) who described three different *Myxobolus* species from the bulbus arteriosus of closely related centrarchid fish.

Clinical signs caused by *Myxobolus* s.l. *dogieli* are rather characteristic. At lower intensities, plasmodia are apparently localized inside the ventricle and bulbus arteriosus of the heart, but as intensity increases large cysts may appear on the surface of the heart as sausage-like outgrowths. Little is known of the normal histological structure of the common bream heart. More detailed studies on the histology of the fish heart were performed by Icardo, Colvee, Cerra & Tota (1999a,b, 2002a,b) and Icardo, Imbrogno, Gattuso, Colvee, Cerra & Tota (2005), who examined the structure of the ventricle, bulbus arteriosus and the conus valves of the sturgeon and some marine fish with special regard to the subendocardium and subepicardium. Scanning electron microscopy observations by Icardo *et al.* (2005) on *Sparus aurata* L. showed that besides the main ventricular lumen, myocardial trabecular sheets formed several lacunae inside the ventricle. The ventricle of the common bream showed a similar structure to that of *Sparus aurata.* In the wall of the ventricle large lacunae filled with blood and small lacunae among myocardial trabeculae were readily found. The wall of the bulbus arteriosus in the bream was built up from wavy collagen bundles and fibroblast-like cells but smooth muscle cells as found by Icardo *et al.* (1999a,b) were not observed in this species. Both the subendocardium and subepicardium in common bream were wide and, besides capillaries, contained a lympho-haemopoietic tissue similar to the structure found by Icardo *et al.* (2002a,b) in *Acipenser nacardi.*

Plasmodia of *Myxobolus* s.l. *dogieli* seem to always develop in the connective tissue of the subepicardial and subendocardial layers and never affect the myocardial bundles in the ventricle or the collagenous trabeculae in the wall of the bulbus arteriosus. In infections of the subepicardium of the bulbus, plasmodia surrounded by a thick layer of subepicardial connective tissue cells and covered by the epicardial epithelium protruded over the surface of the heart and appeared at the bulbus region as sausage-like formations. During this study only developing plasmodia and mature spores from advanced cases were recorded but it cannot be

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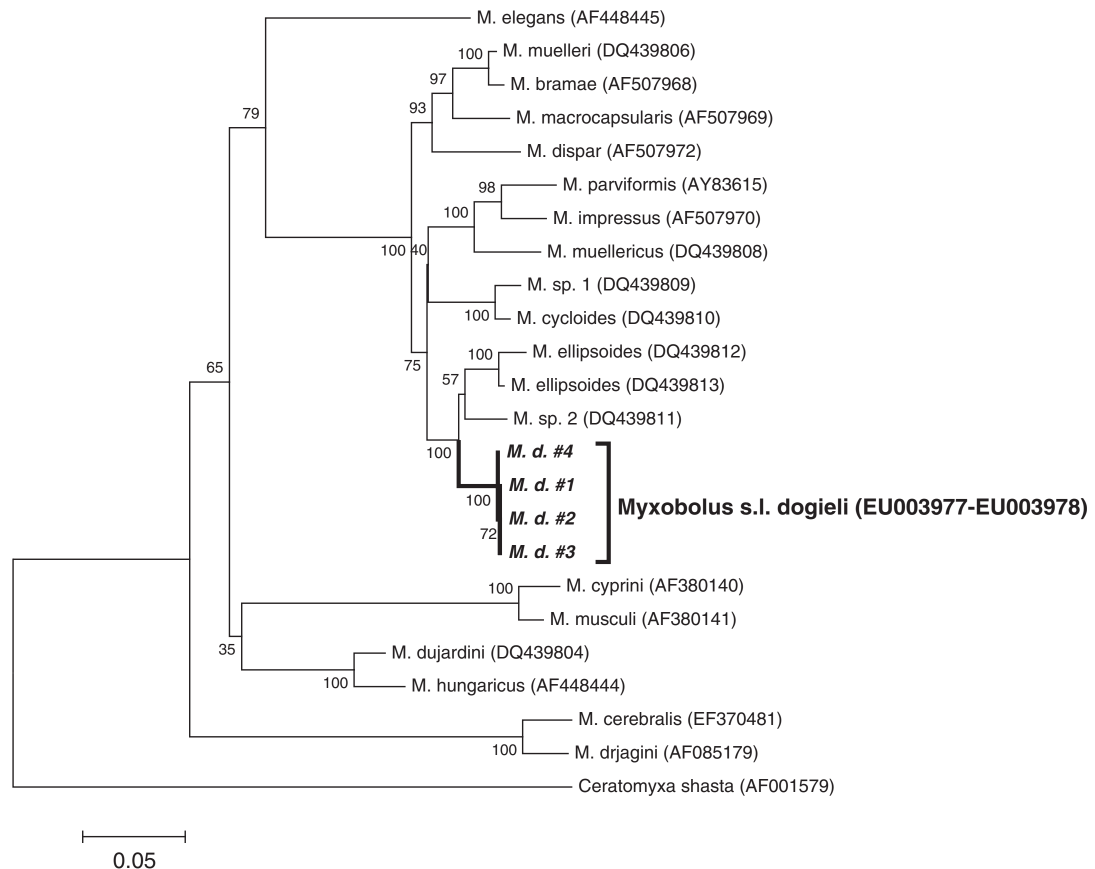

Figure 9 Phylogenetic tree generated by neighbour-joining analyses of the 18S rDNA sequences of myxosporeans, rooted at *Ceratomyxa shasta*. Numbers at nodes indicate bootstrap confidence values (1000 replications). GenBank accession numbers are given in parentheses. Myxosporeans examined in this study are in bold.

excluded that the initial infection was originally in a capillary of the connective tissue.

Sequence analysis of 18S rDNA gene of *Myxobolus* s.l. *dogieli* spores shows that samples collected from four different bream were very similar to each other, but they differ considerably from the other known *Myxobolus* species of cyprinid fish.

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