Myxozoan infections of the three Indian major carps in fish ponds around Meerut, UP, India, with descriptions of three new species, *Myxobolus basuhaldari* sp. n., *M. kalavatiae* sp. n. and *M. meerutensis* sp. n., and the redescription of *M. catlae* and *M. bhadrensis*

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Abstract New myxosporean species are described from Indian fishes cultured in pond farms of Meerut, Uttar Pradesh (U.P.) state. Based upon plasmodia found in the Indian major carps (*Catla catla, Cirrhinus cirrhosus, Labeo rohita* and their hybrids) three new *Myxobolus* spp., *M. basuhaldari* sp. n., *M. kalavatiae* sp. n. and *M. meerutensis* sp. n. are described, and two species, *M. catlae* and *M. bhadrensis*, are redescribed. Plasmodia of *M. basuhaldari* sp. n., *M. kalavatiae* sp. n., *M. meerutensis* sp. n. and *M. catlae* developed in small cysts in the gill lamellae, while plasmodia and scattered spores of *M. bhadrensis* were found in the muscles and kidney, respectively. Plasmodia and spores found in these fishes differed from each other with respect to their morphology, tissue tropism and 18S rDNA sequence. No major pathological changes were found, but severe infections were observed.

Keywords Myxozoa · New *Myxobolus* spp. · Morphology · Histology · Tissue tropism · 18S rDNA · India

Introduction

Freshwater fish culture has old traditions in India and parasitic infections have always received special attention. In India, of the great number of fish species cultured in pond farms, major carps [*Catla catla* (Ham), *Cirrhinus cirrhosus* (Bloch) (syn. *Cirrhina mrigala* (Ham.) and *Labeo rohita* (Ham.) and their hybrids] are the most common due to their high economic importance as food fishes. On the Indian subcontinent, fish parasitoses caused by myxosporean parasites are very common (Tripathi 1952; Sanaullah and Ahmed 1980; Singh and Kaur 2012). At least 97 *Myxobolus* spp. have been described from Indian fishes, 29 of which infect the major carps and their hybrids (Kalavati and Nandi 2007).

Descriptions of *Myxobolus* spp. known from India are based mostly on the morphology of spores including their size, shape and polar capsules (Basu and Haldar 2003; Kaur and Singh 2010a, b, 2011; Banerjee et al. 2011; Kaur et al. 2012, 2013; Majumder et al. 2013; Madhavan et al. 2013). In a synopsis on the Indian species of the genus *Myxobolus* Bütschli, 1882, Kaur and Singh (2012) reported 131 nominal species. Because spores of different species can have similar morphologies, recent works (Molnár 1994; Cone and Overstreet 1998; Lom and Dyková 2006; Ferguson et al. 2008) suggest considering host and organ specificity and histotropism as well. Although this information contributes to species identifications, DNA sequence data is required for a complete description or definitive identification of a species There is an immense need for molecular data from Indian *Myxobolus* species to achieve this. To date, only one Indian *Myxobolus* species is available in GenBank, *M. carnaticus* (KF796620), a gill parasite from mrigal *(C. cirrhosus)*; however, the article describing it is yet unpublished.

In this paper, we report on investigations in which five *Myxobolus* species were collected from Indian major carps and their hybrids. Using morphological and molecular biological examinations three of the above species, *M. basuhaldari*, *M. kalavatiae* and *M. meerutensis* have been described as new species (sp. n.), whereas *M. catlae* and *M. bhadrensis* were identified as known species.

Materials and methods

Fishes were collected from the Parikshitgarh fish farm (28°59′N, 77°56′E) and from the Khajuri pond (28°99′N, 77′87′E), Meerut, Uttar Pradesh (UP) state, India. During a two-week period in November 2009 different size and age classes of *Labeo rohita* (N=10), *Cyrrhina*

cirrhosus (N=10) and *Catla catla* (N=10) were examined for myxozoan infections in the molecular taxonomy laboratory of the Department of Zoology, Chaudhary Charan Singh University, Meerut, UP, India. The gill filaments of each hemibranchium, the kidney and muscles were checked for myxozoan plasmodia under a Motic stereomicroscope (SMZ-168 series). Plasmodia were carefully removed from the tissues and opened with a fine needle on a slide. A subset of the spores obtained from mature plasmodia was studied as fresh preparations under a Motic DMBA300 digital microscope, and another subset was fixed in 70% ethanol in vials for further morphological and molecular biological examinations in Hungary. Pieces from the muscles were compressed between two glass slides and studied as squash preparations under a Zeiss compound microscope. Photos of fresh spores were taken with an Olympus BH-2 microscope. Infected organs (hemibranchia of gills, kidney and muscles) were fixed in Bouin's solution for 4 hours, washed in 80% ethanol several times, embedded in paraffin wax, cut into 5 to 8 µm thick sections and stained with haematoxylin and eosin. Photos of histological sections were taken with an Olympus BH-2 microscope equipped with a DP-10 digital camera. Measurements of 50 fresh myxospores from a single plasmodium were taken with a calibrated eyepiece micrometer according to the guidelines of Lom and Arthur (1989) or, in some exceptional cases, the myxospores were measured on the photomicrographs. Measurements are given in µm unless stated otherwise.

Molecular methods

Ten samples of five distinct species were analysed by molecular methods, in the case of two species (*Myxobolus bhadrensis* and *Myxobolus basuhaldari* sp. n.) more than one sample were analysed from two different fish species (Table 1). Parallel samples came always from different fish specimens. For DNA extraction, samples preserved in ethanol were centrifuged at 5000 × *g* for 5 min to pellet the myxospores, and then the ethanol was removed. The DNA was extracted using a QIAGEN DNeasy™ tissue kit (animal tissue protocol; Qiagen) and eluted in 50 µl AE buffer. The 18S rDNA was amplified using the 18S rDNA universal eukaryotic primers ERIB1 and ERIB10 (Barta et al. 1997). PCR was carried out in a 25 µl reaction mixture comprising 2 µl of extracted genomic DNA, 5 µl of 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.50 µl of each primer, 2.5 µl of 10× Taq buffer (MBI Fermentas), 0.1 µl of Taq polymerase (2 U; MBI Fermentas) and 14.4 µl of water. The PCR cycle consisted of an initial denaturation step of 95 °C for 3 min,

followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and was 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 (Hallett and Diamant 2001) and SphR (Eszterbauer and Székely 2004) primer pair. The total volume of the PCR reactions was 50 µl, which contained 1 µl of amplified DNA, the quantity of the other PCR components were double amount of the first reaction. Amplification conditions in the second round were 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 50 °C for 50 s, 72 °C for 1 min 40 s, 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. The amplified DNA was then purified with the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc). Purified PCR products were sequenced in both directions with the SPHr primer from the second round and various inner primers: ACT1fr (Hallett and Diamant 2001), MB5, MB3 (Eszterbauer and Székely 2004), MC5, MC3 (Molnár et al. 2002). Sometimes the use of additional sequencing primers was required: the PCR primer Myx1f from the second round and CR1F (5'-CGAAGACGATCAGATACCGTCCTAG-3'), CR1R (5'-CTAGGACGGTATCTGATCGTCTTCG-3') (This study), NSF573 (Li et al. 2013), 1200F (5'-GATYAGATACCGTCSTAGT-3') and 1700R (5'-GGCATCACWGACCTGYTAT) (Dyková et al. 2008, with small modifications). ABI Big Dye Terminator v3.1 Cycle Sequencing Kit was used and the sequences were read with an ABI 3100 Genetic Analyzer.

The various forward and reverse sequences were assembled in MEGA 6 (Tamura et al. 2013) and ambiguous bases clarified using corresponding ABI chromatograms. The assembled sequences and the reference sequences from the Genbank were aligned using the algorithm Clustal W (Thompson et al. 1994) DNA pairwise distances were calculated with MEGA 6 software using the Tamura–Nei substitution model. The phylogenetic positions of Indian myxozoans were estimated and compared with other topologies by maximum likelihood (ML) and Bayesian inference (BI) analyses. For ML analyses, model testing of the dataset was performed for the nucleotide substitution model of best fit by the Akaike Information Criterion and the best-fitting one (GTR + G + I) was chosen using MEGA 6. Bootstrap values based on 1,000 resampled datasets were generated. BI was computed by Topali 2.5 (Milne et al. 2008). The substitution models were tested by the Bayesian Information Criterion and GTR + G + I was chosen. Posterior probabilities were estimated over 1,000,000 generations via five independent runs of four simultaneous MCMCMC chains

with every 100th tree saved. The first 25% of the sampled trees were discarded as "burn in". *Myxobolus cerebralis* (EF370481) was chosen as an outgroup in the final alignment.

Results

Five different types of spores were collected. Three different types of spores obtained from the gills of *L. rohita*, *Catla catla* and *Cirrhinus cirrhosus* showed the typical characteristics of the genus *Myxobolus* Bütschli, 1882. No mixed infections were found in the specimens of *L. rohita* investigated. These newly isolated species morphologically differ from the known *Myxobolus* spp. and are described as new species under the name of *M. basuhaldari* sp. n., *M. kalavatiae* sp. n. and *M. meerutensis* sp. n., respectively. Elliptical spores with different sized polar capsules of *M. bhadrensis* Seenappa and Manohar 1981 were collected from the muscle and kidney of *Labeo rohita* and *Catla catla*. Elongated, large spores from the gills of *C. cirrhosus* were identified with *M. catlae* Chakravarty 1943. Descriptions of the species found follow below.

PCR amplification of the 18S rDNA generated ~1600 bps amplicons. The sequences of the various reads were usually between 500 and 800 bps, therefore almost every part of the amplicons were sequenced at least twice, except the a small portion of the 5' and 3' ends. The alignment of the different samples and the downloaded additional myxozoan sequences was 1714 bps long, of which 755 positions were variable and 602 parsimony-informative. ML and BI analyses of the sequences generated similar topologies, except for some branches with low support. Therefore, only the ML tree is presented here (Fig. 7), the posterior probabilities of the BI analyses are shown in the nodes (the nodes unsupported by BI are marked with a hyphen).

Myxobolus basuhaldari sp. n. (Figs. 1 a, b; 2a; 3)

Taxonomic summary

Type host: *Labeo rohita* (Ham.). Local name: rohu.

Additional host: *Catla catla* (Ham.). Local name: bhakur or katla. Type locality: Parikshitgarh Fish Farm, Meerut, UP, India

Site of tissue development: Gill lamellae.

Type material: Syntype spores in glycerine-gelatine and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-70445, 70446, 70447. The 18S rDNA sequences of *M. basuhaldari* sp. n. were deposited in GenBank: type specimen: KM029974, paratype specimens: KM029975 and KM029976.

Prevalence of infection: 3/10 in the first-year-old age group (fish 5–8 cm in size).

Intensity of infection: 8 to 20 plasmodia in each hemibranchia.

Etymology: The species name is a combination of the names of Drs. Basu and Haldar who described similar *Myxobolus* spp. in India.

Description

Trophozoites: Large disporoblastic, roundish plasmodia 2–3 × 0.2–0.4 mm with mature spores were found in the gills inside the multilayered epithelium of the gill filaments.

Spores: The spores both in frontal and sutural view were small, ovoidal, bluntly pointed with a small knob at the tapered anterior end (Figs. 1a, b and 2a). Length of the spores is 7.3 ± 0.31 (6.8–7.8) (N=25), width 5.5 ± 0.35 (5.1–6.2) (N=25), thickness 3.7 ± 0.09 (3.5–3.8) (N=3). The two polar capsules are equal or closely equal, pear shaped, slightly converging anteriorly. Capsules are 3.2 ± 0.31 (2.9–3.7) long (N=50) and 2.1 ± 0.08 (2.0–2.2) wide (N= 25). Five filament coils arranged perpendicularly to the capsule length are wound in the polar capsule. The wall of the spore in frontal view measured 0.5 (0.4–0.6). No intercapsular appendix was present. The tapered anterior end of the spore is compact. Sutural edge markings were not seen. A single binucleated sporoplasm with round iodinophilous vacuole was present. A mucous envelope was not found.

Histology: Intralamellar plasmodia were located in the capillary network of the gill lamellae. The plasmodia found were filled with matured spores (Fig. 3).

Molecular data: The DNA sequence of *M. basuhaldari* sp. n. sample from *Labeo rohita* (KM029974) is most closely related to other two Indian species, i.e. *M. kalavatiae* sp. n. (96.1%) and *M. catlae* (90.5%), and was placed on the same branch on the phylogenetic tree supported by high bootstrap value (Fig. 7).

Remarks: The location of plasmodia as well as the measurements of spores of *M. basuhaldari* sp. n. isolated from *Labeo rohita* correspond in several respects to *M. shantipuri* described from the hybrid of *Catla catla* × *Labeo rohita* by Basu and Haldar (2002) from West Bengal, but the schematic drawings given by the latter authors significantly differ from those depicted by us. The latter authors mentioned that a part of the spores had unequal polar capsules. Such

spores were found also among our samples, although less frequently. The three parallel samples of *M. basuhaldari* sp. n. (KM029974, KM029975 and KM029976, Table 1.) from three different hosts (two from *Labeo rohita* and one from *Catla catla*) were highly similar to each other (99.7–100%), which confirms that they are the representatives of the same species..

Myxobolus kalavatiae sp. n. (Figs. 1c, d; 2b; 4)

Taxonomic summary

Type host: Mrigal carp *Cirrhinus cirrhosus* (Bloch). Local name of the host: mrigal.

Type locality: Khajuri Fish Pond, Meerut, UP, India.

Site of tissue development: Gill lamellae.

Type material: Phototype spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM- 70442. The 18S rDNA sequence of *M. kalavatiae* sp. n. was deposited in GenBank under the accession number KM029973.

Prevalence of infection: 3/10 in the first-year-old age group (fish 5–8 cm in size).

Intensity of infection: Plasmodia were found in most gill filaments.

Etymology: The species was named in honour of the famous Indian myxosporean specialist, Prof. C. Kalavati.

Description

Trophozoites: Small disporoblastic, round plasmodia less than 2 mm with mature spores were found in the capillary network of the gill lamellae.

Spores: The spores are small, ellipsoidal both in frontal and sutural view (Figs. 1c, d and 2b). Length of the spores is 7.3 ± 0.27 (6.8–7.7) (N=50), width 5.3 ± 0.5 (4.8–5.8) (N=25), thickness 3.7 ± 0.09 (3.5–3.8) (N=3). The two polar capsules are equal or closely equal, pear shaped, slightly converging anteriorly. Capsules are 3.1 ± 0.16 (2.9–3.4) long (N=25) and 1.9 ± 0.18 (1.6–2.0) wide (N=25). Filament coils in the capsule were not observable. The wall of the spore in frontal view measured 0.45 (0.4–0.5). No intercapsular appendix was present. Sutural edge markings were not seen. A single binucleated sporoplasm with round iodinophilous vacuole was present. Mucous envelope was not found.

Histology: Intralamellar plasmodia were located in the capillary network of the gill lamellae. The plasmodia found were filled with mature spores. Small cysts of this species filled only the basal part of the lamellae, so that the tips of the infected lamellae were regularly seen (Fig. 4).

Molecular data: The DNA sequence of *M. kalavatiae* sp. n. (KM029973) sample from *Cirrhinus cirrhosus* is also most closely related to Indian gill-infecting *Myxobolus* species, like the above-detailed *M. basuhaldari* sp. n. (95.9–96.2%) and *M. catlae* (91.7%), respectively (Fig. 7).

Remarks: The location of plasmodia as well as the measurements of spores of *M. kalavatiae* sp. n. described from *Cirrhinus cirrhosus* corresponded to *M. basuhaldari* sp. n., but the oval spores with pointed ends of the latter species differed in their morphology from the elliptical spores of *M. kalavatiae*, and there were also 3.08–4.01% distances between the DNA sequences of the two species. From the other 30 species described from India from the major carps, *M. kalavatiae* differed by the smaller size of its spores, by the equal size of its polar capsules and by lacking an intercapsular appendix.

Myxobolus meerutensis sp. n. (Figs. 1e, f; 2c)

Taxonomic summary

Type host: *Labeo rohita* (Ham.). Local name of the host: rohu.

Type locality: Parikshitgarh Fish Farm, Meerut, UP, India.

Site of tissue development: Gill lamellae.

Type material: Phototype spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-70448. The 18S rDNA sequence of *M. meerutensis* sp. n. was deposited in GenBank under the accession number KM029977.

Prevalence of infection: 3/10 in the first-year-old age group (fish 5–8 cm in size).

Intensity of infection: 4-8 plasmodia in a hemibranchium,

Etymology: The species was named after the locality of the fish pond Meerut in India.

Description

Trophozoites: Small plasmodia less than 2 mm in size located in the capillary network of the gill lamellae. Plasmodia contained spores.

Spores: Spores were ellipsoidal in frontal view and lemon shaped in sutural view (Figs. 1e, f and 2c). Spores were 9.1 ± 0.32 (8.8–9.6) long (N=50), 6.9 ± 0.41 (6.4–7.2) wide (N=25), and 5.1 ± 0.11 (4.9–5.2) thick (N=14). Elongated polar capsules converging only at the end were equal in size, 4.3 ± 0.17 (4.1–4.5) long (N=25) and 2.1 ± 0.28 (1.6–2.4) wide (N=25). Six polar filament coils were poorly seen. A small knob-like intercapsular appendix, measuring 0.4–0.5 μm (N=16), was located anteriorly between the capsules. A sutural protrusion formed a circular rim around the spore which emerged about 0.6 to 0.8 μm over the surface of the spore. In sutural view the suture formed an about 0.8 to 0.9 μm small protrusion at the anterior end. Sutural edge markings were rarely seen in fresh spores. A single binucleated sporoplasm was present. Iodinophilous vacuole was not found.

Histology: No histology was done.

Molecular data: *M. meerutensis* sp. n. (KM029977) was placed in a clade containing mostly gill-infecting myxozoans; however, there were no closely related species regarding the 18S rDNA sequence similarities. The highest similarity was shown by *Henneguya doneci* (88.5%) and *M. hearti* (88.8%) (Fig. 7). *Myxobolus carnaticus* (KF796620), a parasite from the gill of mrigal was also placed in this clade.

Remarks: Location of plasmodia of *M. meerutensis* sp. n. described from *Cirrhinus cirrhosus* corresponded to both *M. basuhaldari* sp. n. and *M. kalavatiae* sp. n., as all the three species formed small intralamellar plasmodia. The spores of *M. meerutensis* were larger than those of the latter two species, and the more elongated polar capsules also differed from them. The molecular analyses do not support any relevant connection between the above-mentioned species, and thus *M. meerutensis* sp. n was placed on a distinct branch of the phylogenetic tree. The differences between their DNA sequences were about 25%.

Myxobolus bhadrensis Seenappa and Manohar 1981 (Figs 1g, h; 2d; 5)

Taxonomic summary

Host: *Labeo rohita* (Ham.). Local name of the host: rohu.

Additional host: *Catla catla* (Ham.). Local name of the host: bhakur or katla.

Locality: Parikshitgarh Fish Farm, Meerut, UP, India.

Site of tissue development: muscles, however, scattered spores were found in the kidneys too.

Material: Digitized photos of syntype spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-70443, 70444. The 18S rDNA sequences of *M. bhadrensis* were deposited in GenBank: type specimen: KM029971, paratype specimens: KM029968, KM029969, KM029970, and KM029972

Prevalence of infection: 1/10 of the 20–25 cm size group.

Intensity of infection: 8-12 plasmodium in a hemibranchium.

Redescription

Trophozoites: Plasmodia. Elongated plasmodia were detected intracellularly in muscle cells.

Spores: The spores (Figs. 1g, h and 2d) in frontal view are ellipsoidalwith two different or somewhat differing polar capsules. Length of the spores is 10.0 ± 0.41 (9.2–10.4) (N=25), width 6.6 ± 0.37 (6.0–7.2) (N=25), thickness 4.5 ± 0.56 (4.0–5.3) (N=3). Polar capsules are pear shaped, filling most of the spore cavity. Larger capsule 5.5 ± 0.37 (4.8–6.0) long (N=25) and 3.0 ± 0.34 (2.6–3.3) wide (N=25). Smaller capsule 4.2 ± 0.41 (3.6–4.8) long (N=25) and 2.0 ± 0.29 (1.6–2.6) wide. Loosely arranged filament coils run obliquely to the length of the polar capsules. They number 4 in the larger and 3 in the smaller polar capsules. The thickness of the wall in frontal view measures 0.45 (0.4–0.5). Intercapsular process is not present. Sutural edge markings were not seen. A single, very small binucleated sporoplasm with round iodinophilous vacuole was present. Mucous envelope was not found.

Histology: Large plasmodia of this species filled with spores were found in the muscles, located intracellularly in muscle cells (Fig. 5). In some parts of the musculature the host cells became damaged and spores infiltrated by melanomacrophage cells were located free among undamaged muscle cells. Spores belonging to this species were also found in the kidneys. These spores were mostly found in the melanomacrophage centres but individual spores were detected also in the epithelium of the urinary channels.

Molecular data: Based on the phylogenetic analyses, *Myxobolus bhadrensis* (KM029971) is related to other intramuscular *Myxobolus* spp. of cyprinid fishes (*M. terengganuensis*, *M. pseudodispar*, *M. musculi*, *M. cyprini*, *M. artus* and *M. stanlii*), its sequences showed the closest similarity with *M. terengganuensis* (94.3%) from Malaysia (Fig. 7), while the similarity with the other intramuscular species was between 91.6-94.9%.

Remarks: The characteristic ellipsoidal or somewhat deformed spores of this species with different sized polar capsules resemble those of other *Myxobolus* species infecting the

muscles of cyprinid fishes. By its unequal polar capsule *M. bhadrensis* also resembles the species *M. hosadurgensis* Seenappa and Manohar 1981 and *M. indicum* Tripathi 1952 described from *Cyrrhina cirrhosus*. The measurements of the two latter muscle-dwelling species correspond to *M. bhadrensis* but due to the poor illustration of the spores their identification as a synonymous species needs further investigations. The unequal polar capsules of this species also resemble *M. mrigalae* Chakravarthy 1939 but the latter species had been described from the scales of its mrigal host. The presence of spores in different parts of the body, but mainly in the kidney, resembles other muscle-dwelling species (*M. cyprini, M. pseudodispar, M. terengganuensis*) where spores from intramuscularly developing plasmodia are carried by the blood stream to different organs, first of all to the kidneys, where they are either captured and destroyed by melanomacrophage cells or released through renal tubules into the urine (Molnár and Kovács-Gayer 1985; Baska 1987; Székely et al. 2009). Five parallel samples of *M. bhadrenis* were sequenced (Table 1), two were isolated from the muscles of *Labeo rohita* and *Catla catla*, and three were isolated from the kidneys of the two species (two specimes from *Catla catla*) The 18S rDNA sequences (KM029968–KM029972) were almost identical (99.4–100%), the small distances are mostly due to some ambiguous nucleotide positions.

Myxobolus catlae Chakravarty 1943 (Figs. 1i, j; 2e; 6)

Taxonomic summary

Host: *Cirrhinus cirrhosus* (Ham.). Local name of the host: mrigal.

Locality: Khajuri Fish Pond in India.

Site of tissue development: Gill lamellae.

Type material: Digitized photos of syntype spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-70441. The 18S rDNA sequence of *M. catlae* was deposited in GenBank under the accession number KM029967.

Prevalence of infection: 1/10 of the 20–25 cm size group.

Intensity of infection: 8-12 plasmodium in a hemibranchium.

Redescription

Trophozoites: Plasmodia, spherical or oval with a medium size of 45 × 150, were detected inside the capillary network of the gill lamellae.

Spores: Large spores (Fig. 1i, j and 2e) are elongated oval in frontal view, sharply tapering at the anterior poles. In sutural view they are spindle shaped, sharply tapering at the anterior end (Fig. 1d). Length of the spores is 17.1 ± 0.45 (16.6–17.6) (N=25), width 6.6 ± 0.05 (6.5–6.65) (N=25), thickness 5.4 ± 0.47 (4.8–6.0) (N=3). Polar capsules are equal or slightly differing in size. They are elongated, very long, slightly converging anteriorly. Larger capsule 10.4 ± 0.47 (8.4–11.2) long (N=25) and 2.5 ± 0.05 (2.5–2.6) wide (N=25). Smaller capsule 9.9 ± 0.62 (8.4–10.6) long (N=25) and 2.4 ± 0.05 (2.3–2.4) wide. Eleven to 15 filament coils arranged perpendicularly to the capsule length wound densely in the polar capsule. The thickness of the spore wall is 0.45 ± 0.1 (0.4–0.5). No intercapsular appendix was seen. Sutural edge markings were not seen. A single, very small binucleated sporoplasm with round iodinophilous vacuole was present. Mucous envelope was not found.

Histology: Intralamellar plasmodia of this species located asymmetrically in the lamellae (Fig. 6) so that the blood stream in the capillary network runs only at one side of the plasmodia. In the vicinity of plasmodia the interlamellar epithelium was compressed and deformed (Fig. 6).

Molecular data: The 18S rDNA sequence of *Myxobolus catlae* (KM029967) showed the closest similarity with the above-detailed *M. kalavatiae* sp. n. (91.7%) and *M. basuhaldari* sp. n. (90.2–90.5%) (Fig. 7).

Remarks: By its spores of elongated shape and by the large number of filamental turns in the polar capsules this species differs from most of the known *Myxobolus* spp. In its very long polar capsules it bears the closest resemblance to *M. catmrigalae* Basu and Haldar 2004 known from hybrids of *Catla* × *Cirrhinus* and *M. maruliensis* described from channid fishes by Sarkar et al. (1985). Spore sizes measured by us are somewhat larger than the data given by Chakravarty (1943) for *M. catlae* and by Sarkar et al. (1985) for *M. maruliensis,* but smaller than those given by Basu and Haldar (2004). Filamental turns in the polar capsules counted by us are about the same as those given by Chakravarty (1943) and Sarkar et al. (1985) but much less than described by Basu and Haldar (2004) for *M. catmrigalae*. Spores of *M. catmrigalae* and *M. maruliensis* differ from *M. catlae* the most by their unusually small sporoplasms. Recently a new species, *M. slendrii* has been described by Kaur and Singh (2010a) from *C. mrigala* which differed from the above species by the slender shape of the spores and by the lower number of filamental coils in the polar capsules. Of the non-Indian *Myxobolus* spp., *M. koi* Kudo 1919, a parasite of the common carp is also similar to *M. catlae*,

but its spores are somewhat thicker. *Myxobolus Leptobarbi*, a parasite of the mad barb *Leptobarbus hoevenii* also resembles this species but it is a parasite of the muscle cells. The DNA sequence data presented in this paper gives a further key for differentiating this species from others.

Discussion

During this survey, infections with three new and two known *Myxobolus* spp. were found in cultured Indian major carps. Plasmodia of four *Myxobolus* species, i.e. *M. basuhaldari* sp. n., *M. kalavatiae* sp. n., *M. meerutensis* sp. n. and *M. catlae*, were found in the gill lamellae of the hosts, *Labeo rohita*, *Catla catla* and *Cirrhinus cirrhosus*. Elongated cysts of *M. bhadrensis* infected the muscle cells of *C. catla*. In addition to spores in plasmodia, free spores of the above species inside macrophage centres of the kidney were collected from *L. rohita*. To date Fifty-seven *Myxobolus* spp. have been reported from Indian major carps (Kaur and Singh 2012). The majority of the species was described only by the morphology and size of the parasites without data on host specificity, site selection and histotropism. Moreover, very often the line drawings accompanying descriptions were not sufficiently informative. Therefore, the identification of *Myxobolus* spp. in Indian fishes is rather difficult. Guidelines for proper morphological description of myxozoan species were summarised by Lom and Arthur (1989). However, the occurrence of spores of similar size and shape in systematically far standing fishes and in different sites of the fish body suggests that morphological data are not sufficient for the correct identification of *Myxobolus* spp. To avoid incorrect identifications, histological studies and the consideration of host specificity, site selection and histotropism are also recommended when describing new *Myxobolus* spp. (Molnár 1994).

Precise identification can be achieved with the support of molecular data, which usually means 18S rDNA analysis in the case of myxozoans. Rocha and Azevedo (2012) suggested that, besides the above criteria, all descriptions should be supported by scanning electron microscopic data. Lacking facilities for electron microscopy we could not suit these requirements, but in our investigations we tried to meet the majority of requirements. Morphological data supported by DNA studies in our present investigations showed that five different DNA sequences were obtained from the samples collected from three major Indian carps. Relatively few data are available on the host specificity of *Myxobolus* spp. from Indian fauna. Some *Myxobolus* species are known only from a single host, while others seem to infect closely related fishes. Hedrick et al. (2001) described that *M. cerebralis* could infect

salmonid fishes belonging to the genera *Salmo, Oncorhynchus* and *Salvelinus*. In a recent study conducted on *Myxobolus* spp. of closely related cyprinids, Cech et al. (2012) concluded that a given *Myxobolus* sp. usually infects a single host species or some closely related fishes belonging to the same tribes (tribus).

There are many hybrids of the Indian major carp spp. and these are closely genetically related (Barman et al. 2003), thus they may share *Myxobolus* spp. Data obtained in the present study show that *Cirrhinus cirrhosus*, *Labeo rohita* and the genetically farther standing *Catla catla* are infected by the same *Myxobolus* sp. In our case, however, precise investigation of the host specificity was impeded by the large number of hybrids cultured in Indian ponds. We cannot exclude that among the fishes examined there were hybrid specimens which were susceptible from their maternal side to infection with a given *Myxobolus* sp. Our previous investigations (Molnár et al. 1984) made on *Dactylogyrus* spp. showed that these highly specific parasites infected only the type host and only those hybrids that were maternal descendants. Of the *Myxobolus* spp. studied by us, *M. catlae* is a representative of species with elongated spores. Similar *Myxobolus* spp. are common in Asian and South American fishes, but they are not known from European cyprinids (Eiras et al. 2005). Spores of *M. bhadrensis* developing in muscle cells are closely related to *M. cyprini* type *Myxobolus* spp. common in Europe and India (Molnár et al. 2002; Kalavati and Nandi 2007). The mature spores of this intracellular parasite group leave their host's muscle via the blood and most the spores are shed from the fish body through the kidney. Therefore, finding *M. bhadrensis* spores in the melanomacrophage centres of the kidney is not a surprise. Nevertheless, the kidney cannot be regarded as a site of original infection. The three new *Myxobolus* spp. described here are characterised by small intralamellar plasmodia and two of them (*M. basuhaldari* and *M. kalavatiae*) by small spores. Of them, *M. basuhaldari* sp. n. differs from most Indian *Myxobolus* spp. by its oval spores with a knob-like structure at the tapered end. *Myxobolus kalavatiae* sp. n. with its ellipsoidal spores is morphologically similar to the majority of myxobolids and is distinguished from them by its 18S rDNA sequences.

The molecular results and the phylogenetic analysis (Fig 7) supported the identification of three new species and the identification of two previously described species. *Myxobolus basuhaldari* sp. n., *M. kalavatiae* sp. n. and *M. catlae* were placed within the same clade with high bootstrap values adjacent to a clade containing gill-infecting myxozoans. The 3.9-4.1% distance between the 18S rDNA sequences of *M. basuhaldari* sp.n and *M. kalavatiae* sp. n and the cc. 8-9% between *M. catlae* and the former two species was remarkable enough to regard them as three separate species. *Myxobolus meerutensis* sp. n.,

and another gill parasite of *Labeo rohita* was placed within a different branch of *Myxobolus* species, separated from the above-mentioned myxozoans of *L. rohita, Catla catla* and *C. cirrhosus*. This clade also includes gill-infecting myxozoans; however, none of them showed a high similarity to *M. meerutensis* sp. n, the highest similarity was only 78.8% to *Myxobolus algonquinensis* (AF378335) and *Myxobolus hearti* (GU574808) and 78.5% to *Henneguya doneci* (HM146129). It should be noted that *M. carnaticus*, the gill parasite of *C. cirrhosus*, was also placed within this clade. *Myxobolus bhadrensis* develops in the muscles of fish and, accordingly, it branched with muscle parasites such as *M. terengganuensis and M. pseudodispar*, supported by high bootstrap values. The two species having parallel samples, *M. basuhaldari* sp. n. and *M. bhadrensis*, showed only low intraspecific variation in 18S rDNA sequences (0.3-0.6%); however, both of them were isolated from two fish species (*L. rohita* and *C. catla*), which supports the notion that these myxozoans can infect related fish species.

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Legend to the figures

Fig. 1 Schematic illustrations of spores: *Myxobolus basuhaldari* sp. n. spores (a) frontal view, (b) sutural view; *Myxobolus kalavatiae* sp. n. spores (c) frontal view, (d) sutural view; *Myxobolus meerutensis* sp. n. spores (e) frontal view, (f) sutural view; *Myxobolus bhadrensis* spores (g) frontal view, (h) sutural view; *Myxobolus catlae* spores (i) frontal view, (j) sutural view. Bar = 10 µm

Fig. 2 Ethanol-fixed spores: (a) *Myxobolus basuhaldari* sp. n.; (b) *Myxobolus kalavatiae* sp. n.; (c) *Myxobolus meerutensis* sp. n.; (d) *Myxobolus bhadrensis*; (e) *Myxobolus catlae*. Bar = 10 µm

Fig. 3 Plasmodia (p) of *Myxobolus basuhaldari* sp. n. in the gills of *Labeo rohita*. Histological section. Haematoxylin and eosin (H & E). Bar = 100 µm

Fig. 4 Plasmodia (p) of *M. kalavatiae* in the lamellae of the gill filaments of *Cirrhinus cirrhosus*. Small plasmodia fill only the basal part of the lamellae. Histological section. Haematoxylin and eosin (H & E). Bar = 50 µm

Fig. 5 A plasmodium of *Myxobolus bhadrensis* developing in the muscle cells of *Labeo rohita*. The plasmodium containing spores (s) is bordered by the ectoplasm of the muscle cell (arrow). Histological section. Haematoxylin and eosin (H & E). Bar = 10 µm

Fig. 6 Plasmodium (p) of *Myxobolus catlae* in the gill lamella of *Cirrhinus cirrhosus*. The plasmodium is located asymmetrically in the lamella, the rest of the capillary runs at one side of the lamella (arrow). Histological section. Haematoxylin and eosin (H & E). Bar = 50 µm

Fig. 7 Phylogenetic tree generated by maximum likelihood analysis of the 18S rDNA sequences of myxosporeans, rooted at *Myxobolus cerebralis*. Numbers at nodes indicate bootstrap confidence values (1000 replications) and posterior probabilities. Unsupported nodes by BI are marked with a hyphen. GenBank accession numbers are given in parentheses. Myxosporeans examined in this study are indicated in bold

Table 1. The list of the sequenced samples

Sample	Species	Host	Organ	Collecion site	Accession number
IN1	*Myxobolus basuhaldari* sp. n.	*Labeo rohita*	gill lamellae	Parikshitgarh Fish Farm	KM029974
IN5	*Myxobolus basuhaldari* sp. n.	*Labeo rohita*	gill lamellae	Parikshitgarh Fish Farm	KM029975
IN7	*Myxobolus basuhaldari* sp. n.	*Catla catla*	gill lamellae	Parikshitgarh Fish Farm	KM029976
IN3	*Myxobolus kalavatiae* sp. n.	*Cirrhinus cirrhosus*	gill lamellae	Khajuri Fish Pond	KM029973
IN6	*Myxobolus meerutensis* sp. n	*Labeo rohita*	gill lamellae	Parikshitgarh Fish Farm	KM029977
IN9	*Myxobolus bhadrensis*	*Catla catla*	kidney	Parikshitgarh Fish Farm	KM029968
IN10	*Myxobolus bhadrensis*	*Catla catla*	kidney	Parikshitgarh Fish Farm	KM029969
IN11	*Myxobolus bhadrensis*	*Labeo rohita*	kidney	Parikshitgarh Fish Farm	KM029970
IN14	*Myxobolus bhadrensis*	*Labeo rohita*	muscle	Parikshitgarh Fish Farm	KM029971
IN19	*Myxobolus bhadrensis*	*Catla catla*	muscle	Parikshitgarh Fish Farm	KM029972
IN2	*Myxobolus catlae*	*Cirrhinus cirrhosus*	gill lamellae	Khajuri Fish Pond	KM029967

Figure
Click here to download Figure: Indian-Myxobolu-Figures-corr.ppt

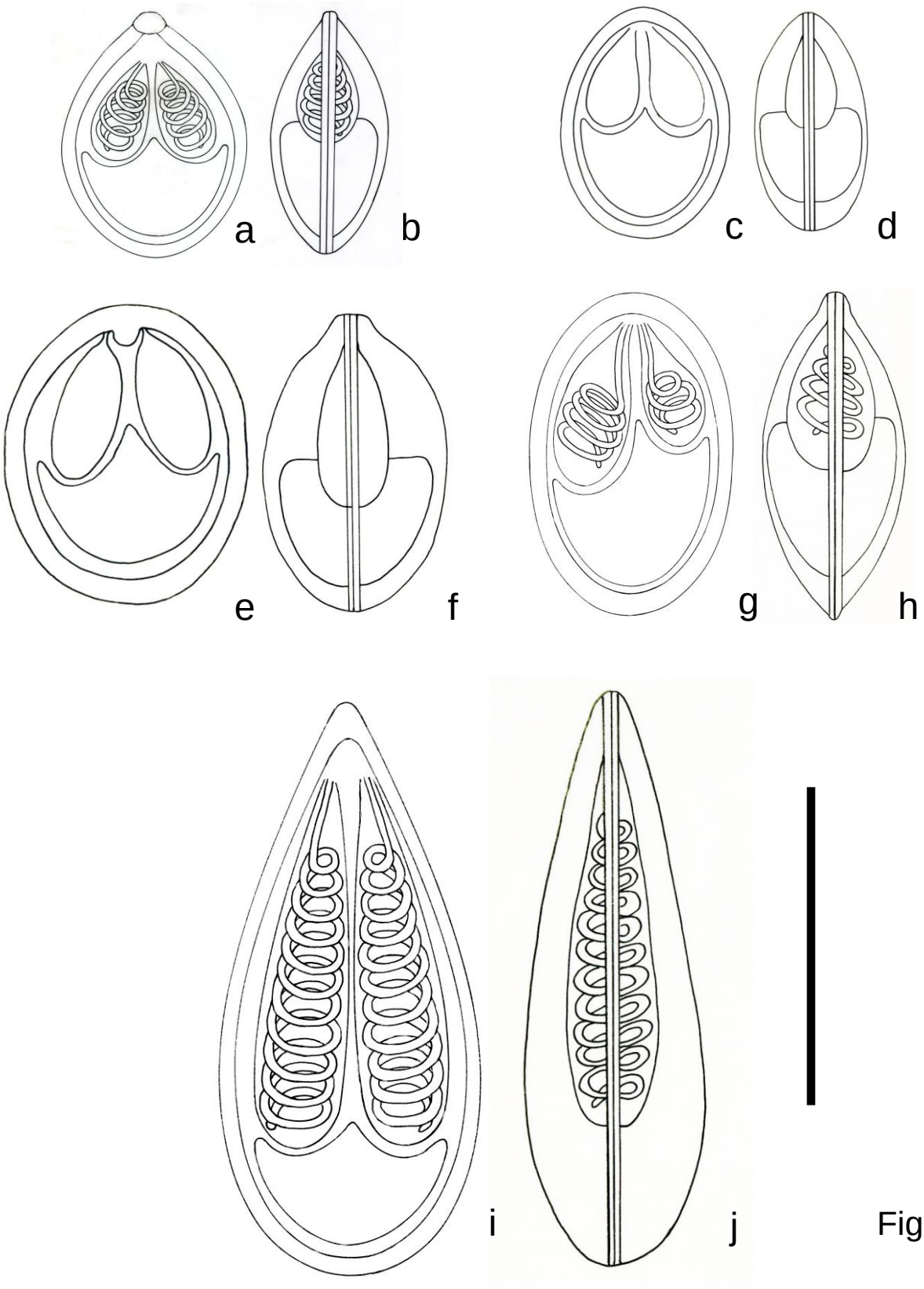


Fig.1

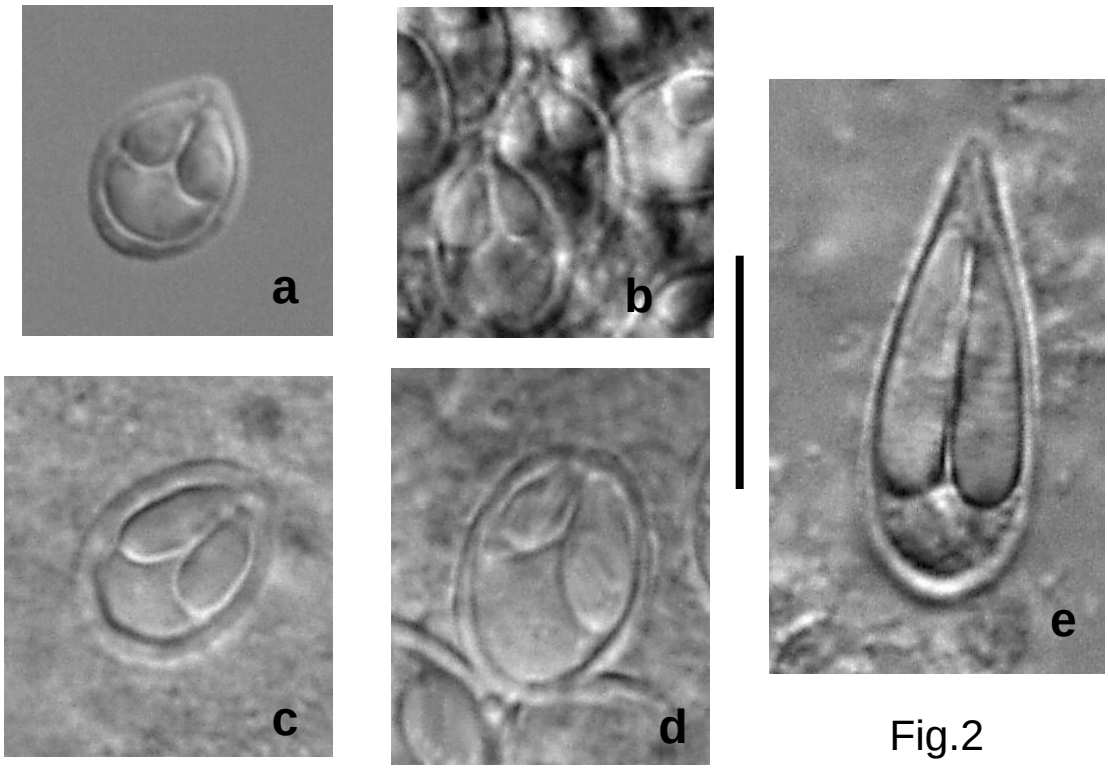
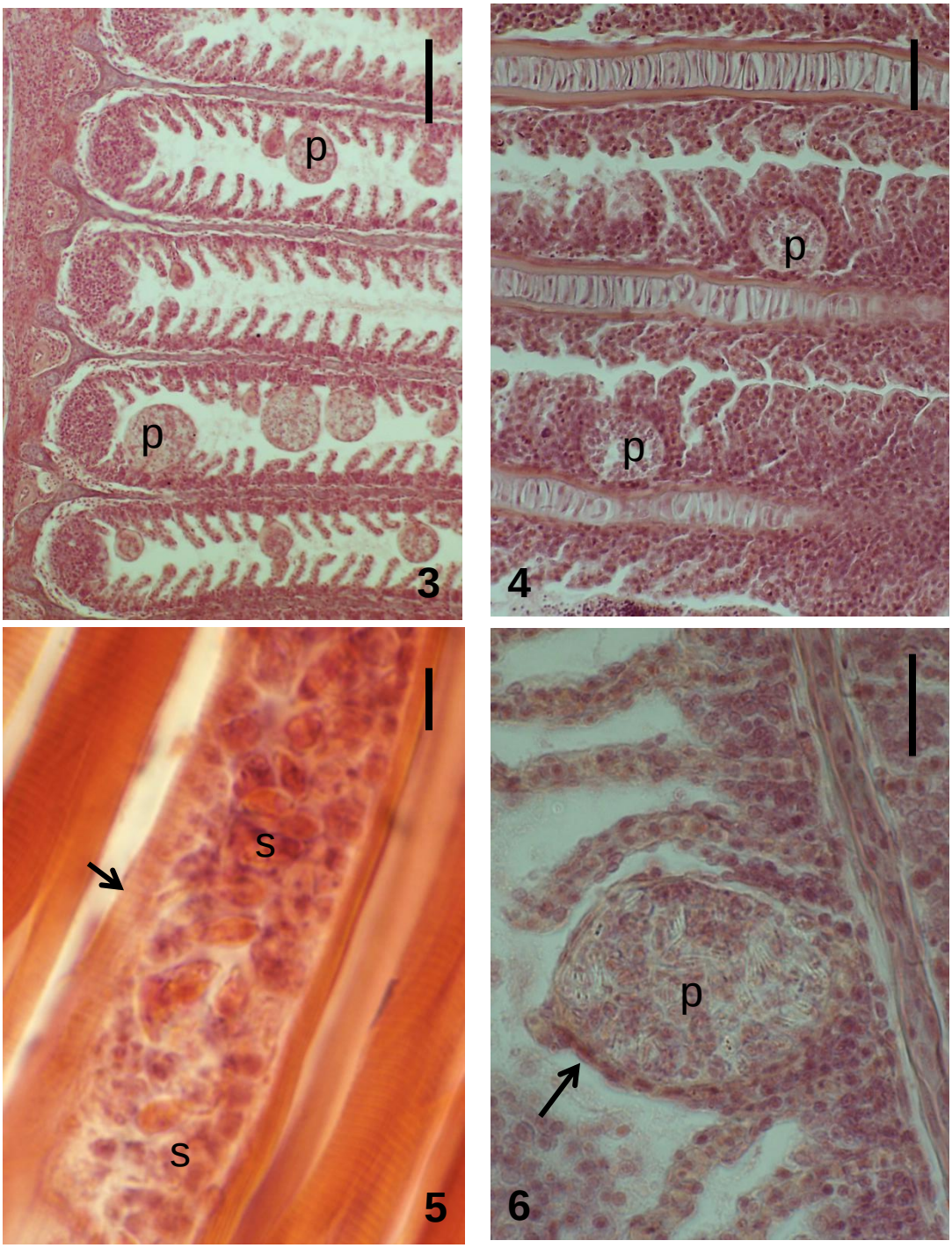


Fig.2



Figs. 3-6

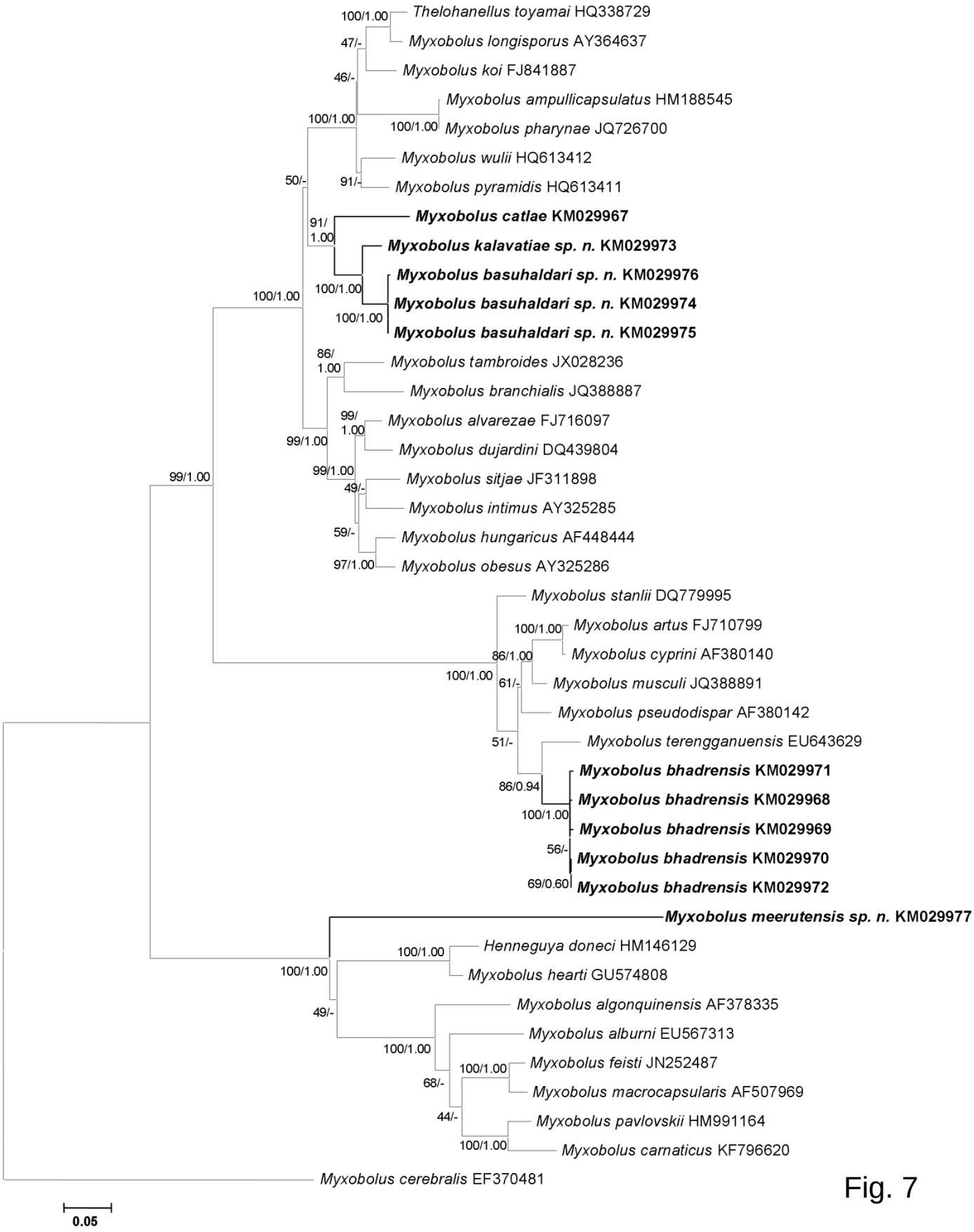

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Fig. 7