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Description of *Myxobolus gayerae* sp. n. and re-description of *M. leuciscini* infecting European chub from the Hungarian stretch of the river Danube

K. Molnár*, Sz. Marton, E. Eszterbauer, Cs. Székely

Veterinary Medical Research Institute, Hungarian Academy of Sciences, PO Box 18, 1581 Budapest, Hungary

ABSTRACT: *Myxobolus gayerae* sp. n. and *M. leuciscini* González-Lanza & Alvarez-Pellitero, 1985 (Myxozoa: Myxobolidae) have been described and re-described from European chub *Leuciscus cephalus* L. from the Hungarian stretch of the river Danube. The ellipsoidal plasmodia of *M. gayerae* sp. n. were found in the mucosa of the intestinal wall, whereas the large, elongated plasmodia of *M. leuciscini* infected the afferent arteries of the gill filaments. The spores of *M. gayerae* sp. n. are relatively large, slightly oval and almost rectangular in shape. On the basis of spore morphology and 18S rDNA sequences, the most similar species was *M. cycloides* Gurley, 1893, but the 2 species differed in host and tissue tropism as well as in the size of the spores. The spores of *M. leuciscini* from *L. cephalus*, having no intercapsular appendix or occasionally a very small one, showed a high morphological similarity to spores collected from *L. cephalus cabeda*, *Chondrostoma polylepis* and *Rutilus arcasi* in Spain and described as *M. leuciscini* González-Lanza & Alvarez-Pellitero, 1985.

KEY WORDS: Myxozoa · New *Myxobolus* spp. · Morphology · Histology · Tissue tropism · 18S rDNA

INTRODUCTION

The European chub *Leuciscus cephalus* (L.), a common fish in Europe and in the Middle East, is regularly infected by several *Myxobolus* spp. (Shulman 1962, 1966, González-Lanza & Alvarez-Pellitero 1985, Landsberg & Lom 1991, Lom & Dyková 1992, 1995, Eiras et al. 2005, Feist & Longshaw 2006). In a recent paper, Molnár et al. (2006) recorded the occurrence of 8 species in this particular fish. Of the parasite species found, *M. muelleri* Buetschli, 1882, *M. dujardini* (Thélohan, 1892), *M. muellericus* Molnár, Marton, Eszterbauer, Székely, 2006 and *Myxobolus* sp. 2 were located in the gills; *M. cycloides* Gurley, 1893 was detected in the swimbladder serosa; *M. ellipsoides* Thélohan, 1892 in the fins; *M. pseudodispar* Gorbunova, 1936 in the muscles; and *Myxobolus* sp. 1 in the gut.

On the basis of the morphological examination of additional samples and the analysis of their 18S rDNA sequences, *Myxobolus* sp. 1 and *Myxobolus* sp. 2 were described in the present paper as *M. gayerae* sp. n. and *M. leuciscini* González-Lanza & Alvarez-Pellitero, 1985, respectively.

MATERIALS AND METHODS

Myxobolus samples from the chub *Leuciscus cephalus* in the present study were derived partially from 87 specimens (5 to 27 cm in length) collected from the Danube and from its tributary creeks by Szentendre (north of Budapest) between 1998 and 2005, and were studied previously by the authors of the present paper (Molnár et al. 2006). In 2006, additional materials were collected from 37 chub specimens and approximately the same number of samples from other leuciscine fishes (*Leuciscus idus*, *Rutilus rutilus*, *Scardinius erythrophthalmus*). The 1 and 2 yr old fish specimens (6 to 18 cm in length) were seined or collected by means of an electro-fishery device. The fish were carried to the laboratory alive in oxygenated plastic bags, kept in aerated aquaria, and subjected to a com-

*Email: molnar@vmri.hu

plete parasitological dissection within 3 d. In the course of the dissections, special care was taken to find the plasmodia of *Myxobolus* sp. 1 and *Myxobolus* sp. 2.

The dissection of fish, collection and preservation of spores, histology, photo-recording, as well as most of the molecular methods, were previously described by Molnár et al. (2006). Measurements were taken in micrometers on the basis of 30 fresh spores. A nested PCR system with the 18e to 18g' primers, followed by the MX5/MX3 primer pair was used for amplification. The purified PCR products were sequenced directly in both directions using the ABI BigDye Terminator V3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer.

RESULTS

During dissections performed in 2006, the occurrence of *Myxobolus pseudodispar* was the most frequent, since all of the 37 specimens were infected by this parasite. In most cases, mixed infections were found. *M. muellericus* was recorded in 27; *M. muelleri*, in 14; *M. dujardini* in 13; and *M. cycloides* and *M. ellipsoides* in 12 fish specimens. Only a single specimen was infected with *Myxobolus* sp. 1, and 3 specimens were infected with *Myxobolus* sp. 2.

On the basis of the *Myxobolus* spores collected up to 2005 (referred to by Molnár et al. 2006 as *Myxobolus* sp. 1 and sp. 2) and on the basis of the additional samples collected in 2006, the description of the novel species and the re-description of *M. leuciscini* from the new host are as follows:

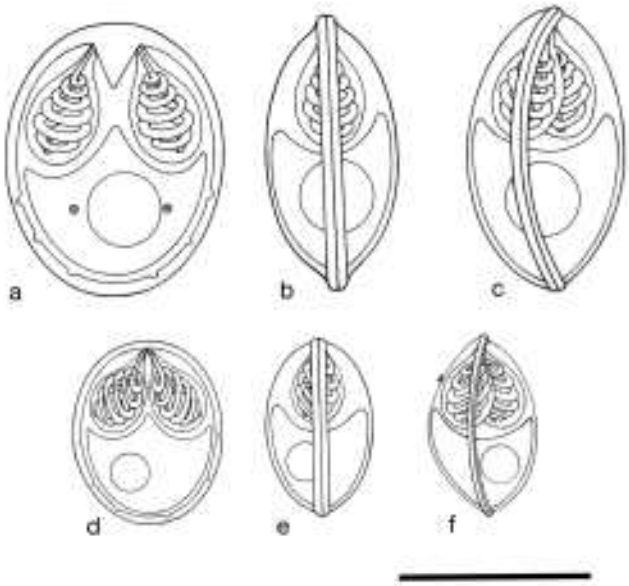


Fig. 1. Schematic drawings of (a to c) *Myxobolus gayerae* sp. n. and (d to f) *M. leuciscini*. (a,d) Frontal view, (b,e) sutural view and (c,f) semi-lateral view. Scale bar = 10 µm

Myxobolus gayerae sp. n. (syn. *Myxobolus* sp. 1 by Molnár et al. 2006)

In the course of dissection, pinhead-sized, round or ellipsoidal plasmodia up to 1 mm in length and 0.7 to 0.8 mm in width were observed in the first part of the intestine. Plasmodia were usually located in groups of 3 to 8. Spores develop in disporic pansporoblasts. Spores (Figs. 1a & 2a) are relatively large, short-ellipsoidal, and almost rectangle in frontal view and elongated-ellipsoidal, slightly lemon-shaped in sutural view (Fig. 1b). Length of the spores was 15.1 ± 0.62 µm (13.7 to 16.5 µm), width was 12.7 ± 0.65 µm (11.5 to 14.0 µm) and thickness was 7.9 ± 0.6 µm (7.3 to 8.7 µm). Polar capsules were equal in size, pyriform slightly converging anteriorly, 6.1 ± 0.62 µm (5.0 to 7.5 µm) in length and 4.0 ± 0.45 µm (3.2 to 4.5 µm) in width. There were 6 polar filament coils, arranged perpendicular to the capsule length. A large, elongated, triangular intercapsular appendix, measuring 2.4 ± 0.44 µm (2.0 to 3.0 µm) was located anteriorly between the capsules. Sutural protrusion forms a circular rim around the spore, emerging about 0.7 to 0.8 µm over the surface of the spore (Fig. 1c). In frontal view, it appears as a collar around the spore. The thickness of the rim in sutural view measures about 0.8 µm. Sutural edge markings (Nos. 6 and 7) are fairly visible in fresh spores. Single binucleated sporoplasm with a large, round iodinophilous vacuole is present. A mucous envelope was not found.

Type host: European chub *Leuciscus cephalus* L. (Cyprinidae).

Type locality: Bükkös Creek, a tributary of the river Danube (north of Budapest).

Site of tissue development: Subepithelial in the intestinal lamina propria.

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-17658. The 18S rDNA sequence of *Myxobolus gayerae* was previously deposited in GenBank under accession number DQ439809 as *Myxobolus* sp. 1 EE-2006.

Prevalence of infection: 2.4% (3 of 124).

Etymology: The species is named after Éva Kovács-Gayer, the eminent Hungarian fish pathologist.

Histology of infection: Plasmodia were located inside the propria and submucosa layers of the intestinal wall (Fig. 3). Semi-mature plasmodia contained mature spores in the central region and young sporogonic stages at the periphery. Ectoplasm of the plasmodia formed a thick eosinophilic wall around the endoplasm filled with spores (Fig. 4); 2 or 3 layers of dense connective tissue surrounded the plasmodium. In most parts, the connective tissue capsule was bor-

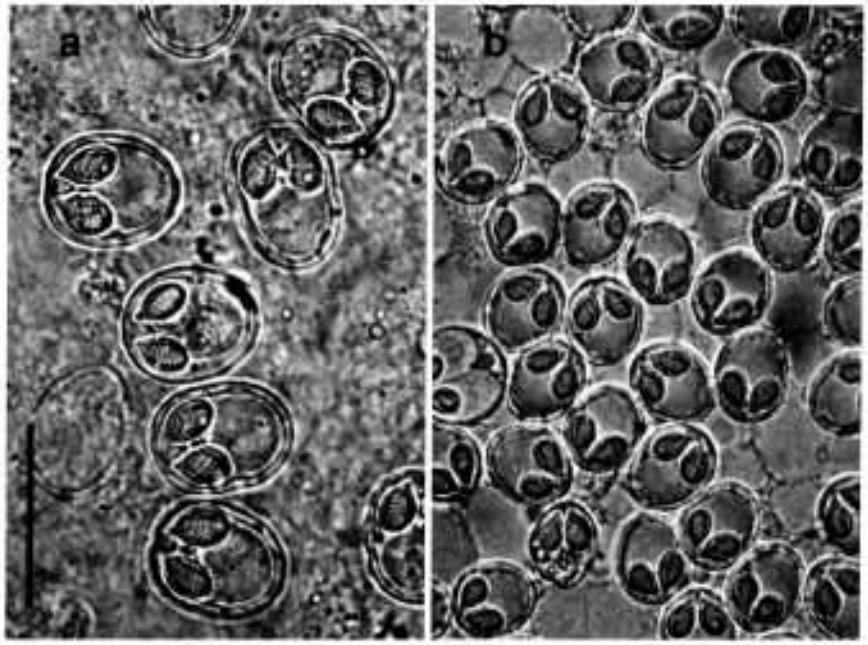


Fig. 2. Spores of new *Myxobolus* spp. from chub *Leuciscus cephalus*. (a) Spores of *Myxobolus gayerae* sp. n. and (b) spores of *M. leuciscini*. Scale bar = 20 µm

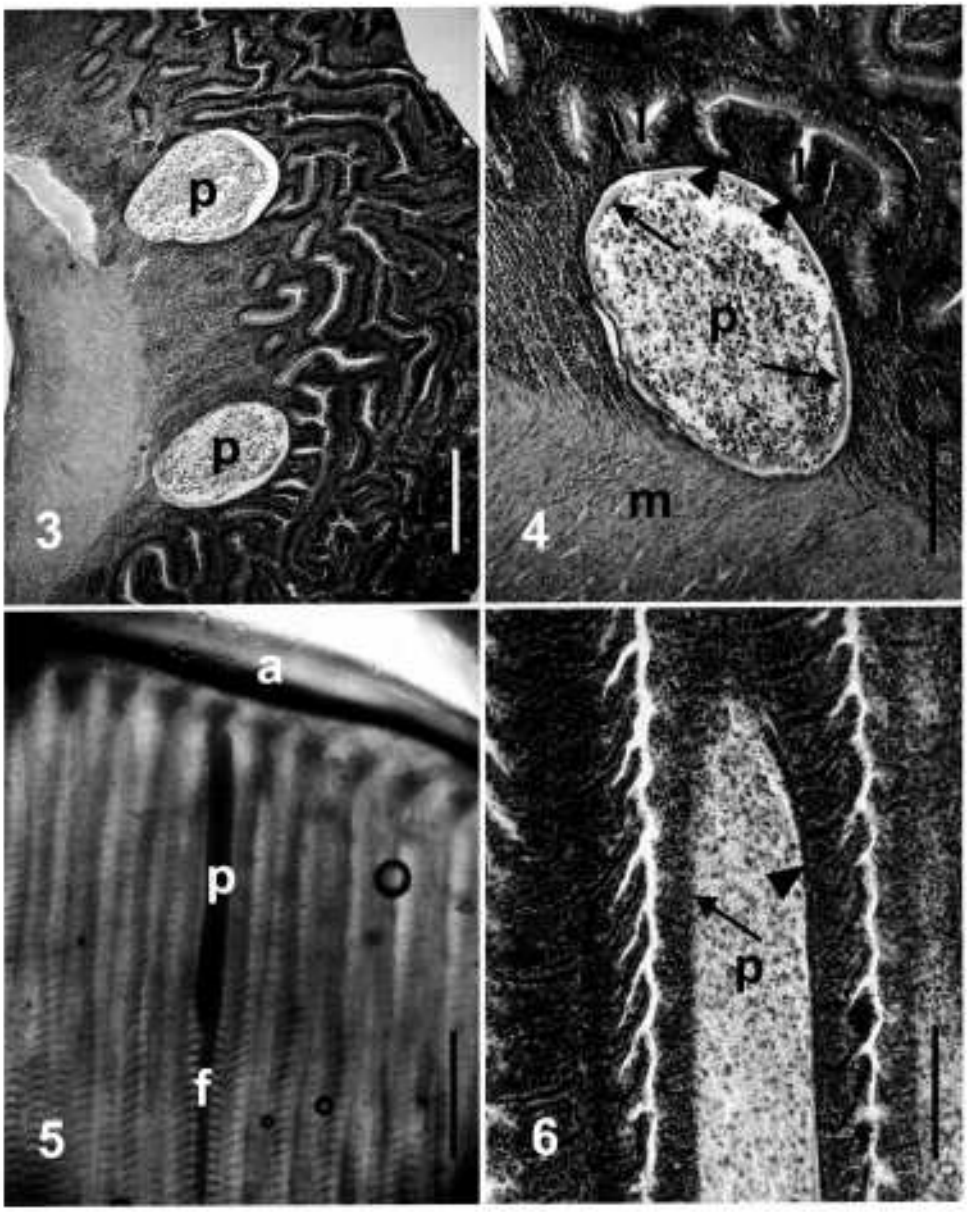
dered by a less dense connective tissue of the propria rich in capillaries. On the apical side, the plasmodium was in close contact with the intestinal epithelium; in the basal part, however, the cyst contacted the circular layer of the intestinal musculature (Fig. 4).

Molecular data: The DNA sequences of 2 samples of this species were 99.9% identical, as only a single nucleotide difference was detected over approximately 1550 bp long 18S rDNA sequences. However, they were rather different from the species *Myxobolus cyprinicola*, which is morphologically similar and develops plasmodia in the same location, although in a different host species. The genetic similarity of the 2 species was 86.7%. DNA sequences of this species also differed from sequences of other *Myxobolus* species collected from chub. The most closely related species was *M. cycloides*, with 96.9% similarity in their 18S rDNA fragment.

Remarks: This species resembled, in both spore morphology and location of plasmodia, *Myxobolus cyprinicola* Reuss, 1906 from the common carp *Cyprinus carpio* L., but the spores of *M. cyprinicola* were smaller in size. Despite the morphological similarities and identical location, the 2 species clearly differed at the DNA level. Morphologically, this species resembles *M. cycloides* ex *leuciscus*, a typical parasite of the swimbladder of chub, but differs from it by being larger in size. In its size, *M. gayerae* sp. n. also resembles *M. rutili* Donec & Tozyjakova, 1984, a parasite which causes cysts to develop on the fins of roach *Rutilus rutilus*, but differs from the latter species in the 18S rDNA of spores collected from roach in Hungary (authors' unpubl. data).

Myxobolus leuciscini González-Lanza & Alvarez-Pellitero, 1985 (syn. *Myxobolus* sp. 2 by Molnár et al. 2006)

Large, elongated, vascular and intrafilamental type plasmodia of this species were located in the afferent arteria of gill filaments, typically occupying the arteria segment close to the gill arch (Fig. 5). Spores were ellipsoidal in frontal view (Figs. 1d & 2b), measured 10.1 ± 0.75 µm (9.0 to 11.0 µm) in length and 9.0 ± 0.24 µm (8.5 to 9.5 µm) in width. Spores were lemon-shaped in sutural view (Fig. 1e), measuring 5.3 ± 0.49 µm (5.0 to 6.0 µm) in thickness. Polar capsules were equal in size, pyriform, slightly converging anteriorly with a bottleneck part at the end, 4.2 ± 0.26 µm (4.0 to 4.5 µm) in length and 2.9 ± 0.29 µm (2.5 to 3.5 µm) in width. Six polar filament coils were arranged obliquely to the long axis of the capsule. Only on a few occassions was a very small intercapsular appendix seen. In a number of fresh spores, sutural edge markings (Nos. 6 and 7) were observed. The suture forms a ~0.5 µm thick rim around the spore (Fig. 1f), which protrudes about 0.7 to 0.8 µm over the



Figs. 3 to 6. *Myxobolus gayerae* or *M. leuciscini* in *Leuciscus cephalus*. Fig. 3. *M. gayerae* sp. n. Plasmodia (p) in the gut of chub. Histological section, hematoxylin and eosin staining (H&E), scale bar = 400 µm. Fig. 4. *M. gayerae* sp. n. plasmodium (p) in the gut of chub. The plasmodium with an eosinophil ectoplasm (arrows) towards the intestinal lumen (l) contacts the intestinal epithelium (arrowheads) and basally lays on the intestinal musculature (m). Histological section, H&E, scale bar = 200 µm. Fig. 5. *M. leuciscini*. Plasmodium (p) in the gill filament (f) of chub close to the gill arch (a). Fresh mount, scale bar = 500 µm. Fig. 6. *M. leuciscini*. Plasmodium (p) in the artery of a gill filament in chub. On one side, the plasmodium fits tightly to the endothelium (arrow), on the other side, a thin row of red blood cells (arrowhead) shows the possible passage of blood. Histological section, H&E, scale bar = 100 µm

surface of the wall. A single, binucleated sporoplasm with small, round iodinophilous vacuole was present. A mucous envelope was absent.

Host: European chub *Leuciscus cephalus* L. (Cyprinidae).

Type locality: Bükkös Creek, a tributary of the river Danube (north of Budapest).

Site of tissue development: Afferent arteries of the gill filaments.

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-17656. The 18S rDNA sequence of *Myxobolus leuciscini* was previ-

ously deposited in GenBank under accession number DQ439811 as *Myxobolus* sp. 2 EE-2006.

Prevalence of infection: 7.2% (9 of 124).

Histology of infection: Plasmodia were located in the afferent arteries of the gill filaments, close to the gill rakers occupying about half of the filament in length (Fig. 6). Old plasmodia included mature spores; some younger ones also contained sporogonic stages. Ectoplasm of the plasmodia formed a thin eosinophilic wall around the endoplasm filled with spores. No degeneration of the affected gill filaments was recorded.

Molecular data: Three of the 4 sequenced samples showed 100% similarity, whereas the fourth sample was 99.5% similar to the other 3. The morphologically similar *Myxobolus muelleri* and *M. muellericus* differed from *M. leuciscini* at the DNA level (88.9 to 89.6% and 89.8 to 90.1% similarity, respectively). Genetically, the most closely related species was *M. ellipsoides* (94.4 to 94.7%).

Remarks: In its spore morphology and location within the host, our *Myxobolus leuciscini* best resembles the original description of *M. leuciscini* by González-Lanza & Alvarez-Pellitero (1985), who found this species in 3 different fishes (*Chondrostoma polylepis*, *Leuciscus cephalus cabeda* and *Rutilus arcasi*) in Spain. *M. leuciscini* has also been re-described by Iglesias et al. (2001), who observed this species in *C. polylepis*. The latter authors found no intercapsular appendix, but reported the presence of a small, nipple-like projection at the anterior end of the spore. On the spores we collected, this projection was not seen. Unfortunately, the *M. leuciscini* species described and re-described in Spain has not been studied at the DNA level up to now; therefore, a molecular comparison with our *M. leuciscini* species is not possible. *M. leuciscini* possesses similar spore morphology to *M. muelleri*, but also differs from it and from *M. muellericus* by lacking a well-observable intercapsular appendix and by also differing in its 18S rDNA. *M.* (*Lentospora*) *cabedae* described by Ghittino (1962) from the gill filaments of *L. cephalus cabeda* in the Po River differs from *M. leuciscini* by its well-observable intercapsular appendix. *M. leuciscini* seems to be morphologically and genetically closely related to *M. ellipsoides*, but differs by its smaller size and by its tissue tropism.

DISCUSSION

More than 30 *Myxobolus* species have been recorded from the European chub *Leuciscus cephalus* (Donec & Shulman 1984, Landsberg & Lom 1991, Eiras et al. 2005). Of these species, only 7 (*M. cabedae* Ghittino, 1962; *M. impressus* Miroshnichenko, 1980; *M. infundibulatus* Donec & Kulakovskaya, 1962; *M. isakovi* Shaova, 1969; *M. leuciscini* González-Lanza & Alvarez-Pellitero, 1985; *M. muelleri* Buetschli, 1982; and *M. muellericus* Molnár, Marton, Eszterbauer, Székely, 2006) have been described from the chub as the typical host. Comparative morphological and molecular studies on *Myxobolus* spp. from chub are available only in the paper by Molnár et al. (2006).

Two factors complicate the reliable identification of the morphologically similar *Myxobolus* species: the incomplete descriptions of the already known species and the sparse knowledge about their host specificity. Data available in the latter respect suggest that most *Myxobolus* spp. can infect only a single host or some closely related fishes (Molnár 1994, Molnár et al. 2002, Longshaw et al. 2003, Blazer et al. 2004, Eszterbauer 2004, Cone & Easy 2005, Cone et al. 2005). In a previous study (Molnár et al. 2006) and in an additional examination (Molnár et al. unpubl. data) some leuciscine fishes (*Leuciscus cephalus*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, *Abramis brama* and *Blicca bjoerkna*), only *M. pseudodispar* was found to infect all of the above fish species. Other *Myxobolus* species infecting the gills, kidney, swimbladder and skin of leuciscine fish species proved to be different, both in their morphology and in their 18S rDNA. Because of its spore morphology and its clearly visible triangular intercapsular appendix, *M. gayerae* sp. n. best resembled *M. cycloides*, the common parasite from the chub's swimbladder. These species also showed the closest relation on the basis of 18S rDNA sequences. However, the specific location of *M. gayerae* sp. n. in the gut and its larger spore size, as well as DNA sequence data, indicated a well-defined difference and supported the description of *M. gayerae* sp. n. as a novel species.

On the basis of the original description by González-Lanza & Alvarez-Pellitero (1985), *Myxobolus leuciscini* is found to be morphologically the most similar species to the one we studied, although neither the type host nor the exact tissue location was determined in the original description. Lacking the 18S rDNA sequence of *M. leuciscini* from the original hosts, the identity of the original *M. leuciscini* and the one we found cannot be excluded; therefore, we identified the collected spores as the above species. On the other hand, the examined spores differ from *M. leuciscini* re-described by Iglesias et al. (2001), having considerably different spore morphology and site selection. These findings suggest that the species known as *M. leuciscini* might cover >1 valid species. Of the species studied at the DNA level, *M. ellipsoides* best resembled *M. leuciscini* in its spore morphology as well, but the 2 species differed from each other in their locations within the host and in their 18S rDNA sequences.

Eiras et al. (2005) registered 751 valid *Myxobolus* spp. in their synopsis. The number of the species is expected to increase, as species identified only by spore morphology, as 'pyriformis, ovalis, rotundus, ellipsoides, etc.' might be attributed to several different species. On the other hand, a number of known species, especially those described by scattered spores without data on plasmodial stages being provided and those whose original description is poor and insufficient for exact species identification, should be regarded as nomen nudum.

Molnár (2002a,b) pointed out the importance of site selection of *Myxobolus* spp. in the host. The plasmodia of *M. leuciscini* have about the same specific developmental site as that of *M. muelleri* in the afferent arteries of the gill filaments, but data obtained up to this time suggest that the plasmodia of *M. leuciscini* tend to occupy the arterial region close to gill arches, whereas the plasmodia of *M. muelleri* prefer regions close to the tip of filaments.

Although several *Myxobolus* spp. have been described from the gut of fish, relatively little is known about the nodular infection in the intestinal wall. This kind of development characterizes *M. nodulointestinalis* Massoumian et al., 1986 and *M. cyprinicola* Reuss, 1906. In its site selection *M. gayerae* sp. n. belongs to this group, but in its morphology it differs from the above 2 species in several respects.

In our description, we reported for both species a well-observable sutural rim, which protruded over the surface of the spore. This rim gives the virtual effect of a thick wall. Spores of different *Myxobolus* spp. are generally regarded as having a relatively thick spore wall. This is correct if they are compared to some members of other myxosporean genera. The virtual thickness, however, measuring up to 0.4–0.6 µm in frontal view in some species, comes from the thick rim of the suture running around the spores (Fig. 1c,f). This rim has been well demonstrated in the scanning electron microscopic studies by Desser & Paterson (1978) on *Myxobolus* sp. from *Notropis cornutus* and by Adriano et al. (2002) on *M. porophilus*. The actual thickness of the mature spores measured at other parts of the wall was only 0.01 to 0.02 µm, as has previously been determined by transmission electron microscopic studies (Desser & Paterson 1978, Casal et al. 2002, Tajdari et al. 2005).

The present study and a previous survey (Molnár et al. 2006) show that a single fish species such as chub can be infected by several site-specific *Myxobolus* spp. Further studies on the parasitic myxozoan fauna of related cyprinid fishes are necessary to decide whether the *Myxobolus* spp. found during these examinations are specific parasites of chub or whether they can also infect other cyprinid fishes. In the case of *M. leuciscini*, molecular studies on the spores collected from the type hosts in Spain would be able to conclusively determine whether our identification as *M. leuciscini* was correct or whether the species we studied is new.

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