Journal of Fish Diseases 2002, **25,** 643–652

Morphological and molecular biological studies on intramuscular *Myxobolus* spp. of cyprinid fish

K Molnár[1], E Eszterbauer[1], C Székely[1], Á Dán[1,2] and B Harrach[1]

1 Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary
2 Central Veterinary Institute, Budapest, Hungary

Abstract

The validity of *Myxobolus* species infecting the skeletal muscles of six cyprinid fish species was studied by morphological and molecular biological methods. Intracellularly developing *Myxobolus* spores identified as *M. cyprini* from the common carp, *M. musculi* from the barbel, and *M. pseudodispar* from the roach, rudd, common bream and white bream were very similar in their shape and size. Nonetheless, in species identified as *M. pseudodispar*, the occurrence of spores with an asymmetrical shape was higher than in *M. cyprini*, while asymmetrical spores were only occasionally found in *M. musculi*. The DNA sequence analysis of the polymerase chain reaction (PCR)-amplified 18S rRNA gene of *Myxobolus* spores from these fish showed a similar phylogeny to that of their host species. As morphological studies and DNA sequence analysis demonstrated slight but real differences in the spores infecting muscles of the six cyprinid species, it is suggested that *M. musculi*, *M. pseudodispar* and *M. cyprini* are valid species.

Keywords: Myxobolus spp., Myxosporea, morphology, molecular phylogeny, 18S rDNA, Cyprinidae.

Introduction

Skeletal muscle has been recorded as a location for myxosporean parasites of freshwater fish since the end of the eighteenth century. Amongst others, *Myxobolus pfeifferi*, *M. musculi*, *M. pseudodispar*, *Henneguya zschokkei*, *Thelohanellus pyriformis* and *T. fuhrmanni* are common parasites in this tissue (Bykhovski 1962; Shulman 1966).

The intracellular location of myxosporeans in muscle cells was first described by Keysselitz (1908) for *M. musculi,* a parasite of the barbel. The pathogenic effect of muscle-dwelling myxosporeans of freshwater fish is best studied in the members of the genus *Myxobolus*. *Myxobolus pfeifferi* Thélohan causing the boil disease of the barbel, *Barbus barbus* (L.), *M. cyprini* Doflein the causative agent of pernicious anaemia in the common carp, *Cyprinus carpio* L., and *M. sandrae* Reuss producing intensive infections in the muscle of the pike perch, *Stizostedion lucioperca* (L.), are regarded as the most pathogenic species in the muscle of freshwater fish (Markevich 1951; Schäperclaus 1954; Kocylowski & Myaczynski 1960; Shulman 1966), but recently Ogawa, Delgahapytiya, Furuta & Wakabayashi (1992) reported heavy infections in the muscle of the common carp by *M. artus* Akhmerov.

Besides the above mentioned species, a muscle location has been described for several other *Myxobolus* spp. In most of these cases, only solitary scattered spores were observed in the muscle, and similar dispersed spores were simultaneously also found in other organs. *Myxobolus cyprini*, one of the most common parasites of the common carp, was considered as a species developing in different organs in small plasmodia, because of the presence of scattered spores in these organs. However, Molnár & Kovács-Gayer (1985) proved that this species was a typical intracellular parasite of muscle cells, and spores found in other organs had been carried there by the blood circulation after the maturation and disruption of the intramuscular plasmodia. A similar intramuscular development was described by Baska (1986) for *M. pseudodispar* Gorbunova, a frequent

Correspondence *Dr K Molnár, Veterinary Medical Research Institute, Hungarian Academy of Sciences, PO Box 18, H-1581, Budapest, Hungary*
(e-mail: kalman@vmri.hu)

© 2002
Blackwell Science Ltd

parasite of the roach, *Rutilus rutilus* (L.). Based on the asymmetrical spores and the different sized polar capsules, Shulman (1966) and Baska (1986) regarded *M. pseudodispar* as a valid species. On the other hand, Lom & Dyková (1992) stated that *M. cyprini* spores may show morphological variability and during maturation can assume an asymmetrical shape similar to that of *M. pseudodispar*. These authors supposed that most plasmodia developing intracellularly in muscle cells of different cyprinid species belong to *M. cyprini*, and *M. pseudodispar* should merely be regarded as its synonym. The validity of *M. musculi* has never been questioned, and up to this time no studies have examined its relationship to *M. cyprini*. The taxonomic classification of Myxosporea, earlier based only on the morphology of myxospores (Lom & Noble 1984; Lom & Arthur 1989), has been refined with the application of molecular biological methods (Andree, Gresoviac & Hedrick 1997). Kent, Khattra, Hedrick & Devlin (2000) stated that phylogenetic trees constructed by the comparison of 18S rDNA sequences of different myxosporean species agreed with Shulman's (1966) phylogenetic hypothesis in most respects, and that this sensitive method may open new possibilities for the examination of detailed relationships, such as the phylogenetic distance between closely related species. Studying the 18S rDNA of five members of the genus *Kudoa*, Hervio, Kent, Khattra, Sakanari, Yokoyama & Devlin (1997) found that these species were related more by their hosts and geographic origin than by spore morphology. Using 18S rDNA sequences of 10 species, Andree, Székely, Molnár, Gresoviac & Hedrick (1999) came to the conclusion that members of the genus *Myxobolus* tend to cluster according to their tissue location. In contrast, Salim & Desser (2000) using partial 18S rDNA sequences of seven different *Myxobolus* species from cyprinid fish, found that these parasites segregate by spore morphology.

The present paper reports on morphological and molecular biological investigations on the validity and phylogenetic distance of closely related *Myxobolus* species infecting the skeletal muscle of six cyprinid fish species.

Materials and methods

Collection of spores

Myxobolus spp. spores were collected from 1990 during a long-term national research programme on the parasite fauna of fish from natural waters and fish ponds of Hungary. Muscle samples containing plasmodia with thousands of spores were obtained from barbel, roach and bleak, *Alburnus alburnus* (L.), from the River Danube, from common bream, *Abramis brama* (L.), white bream, *Blicca bjoerkna* (L.), roach, rudd, *Scardinius erythrophthalmus* (L.), bleak and bitterling, *Rhodeus sericeus amarus* (Pallas), in Lake Balaton and from common carp from different farm ponds. At least five, several week old fingerlings, and the same number of 2–4-year-old specimens of each infected fish species were dissected. Because of the low number of spores found, infections of the bleak and bitterling were not studied in detail. Fish were killed by an overdose of MS 222 (Sandoz, Basle, Switzerland), followed by severance of the spinal cord.

Small pieces of trunk muscle were compressed between two glass plates. To study the occurrence of larger plasmodia in muscles, samples were examined under a stereomicroscope at 10×, while small plasmodia containing between 100 and 500 spores, found in fingerlings, were compressed under a coverslip at 100×. Compressed tissues from kidney and the rete mirabile of the choroid were similarly examined for the presence of spores disseminated from ruptured plasmodia.

Size measurement of subsamples of live spores isolated from plasmodia were made using a calibrated microscope. Digitized images of spores were obtained using video equipment attached to the microscope (Székely 1997).

The majority of the samples were stored for molecular studies, but spores from each sample were also fixed in glycerol-gelatin as a slide preparation. In most cases, the plasmodia were freed from host cells or from a host connective tissue capsule. They were deep frozen in 1.5-mL centrifuge tubes until further use. The samples contained different numbers of spores (10^3–10^6). At least two samples of myxospores collected from different specimens of the same fish host species at different times were used for molecular biological studies. In the case of *M. pseudodispar* from common bream, four samples of spores were collected from different specimens at different times. The two *M. pseudodispar* samples from roach originated from two different areas of Hungary (Lake Balaton and River Danube).

© 2002
Blackwell Science Ltd

DNA extraction

After defrosting the spores, the DNA was extracted as previously described (Eszterbauer, Benkö, Dán & Molnár 2001). Briefly, the spores were suspended in 500 μL lysis buffer [100 mM NaCl, 10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.2% sodium dodecyl sulphate (SDS), and 0.4 $mgmL^{-1}$ proteinase K] and incubated at 55 °C overnight. The DNA was then extracted with phenol and chloroform and precipitated in ethanol. After centrifugation and washing with 70% ethanol the DNA content was estimated by viewing in a 1.0% agarose gel.

Polymerase chain reaction amplification

The polymerase chain reaction (PCR) was carried out as described by Eszterbauer *et al.* (2001). Primers (MX5 and MX3) specific for the family Myxobolidae (Andree *et al.* 1999) were used for amplification of an approximately 1600 base pair (bp) fragment of the 18S rRNA gene.

The total volume of the PCR reactions was 50 μL, which contained 10–50 ng extracted DNA, 1× REDTaq PCR Reaction Buffer (Sigma, St Louis, MO, USA), 0.2 mmol dNTP (MBI Fermentas, Vilnius, Lithuania), 40 pmol of each primer, and 2.5 U REDTaq DNA polymerase (Sigma, USA) in MilliQ purified water. A PDR 91 DNA Reproducer (BLS Ltd, Budapest, Hungary) was used for amplification. Amplification conditions were: 95 °C for 30 s, 46 °C for 30 s and 72 °C for 60 s for 35 cycles, with a terminal extension at 72 °C for 10 min.

MC5 and MC3, the other primer pair used for PCR amplification, were designed in our laboratory. Their sequences are:

MC5 (forward) 5′-CCTGAGAAACGGCTAC CACATCCA-3′

MC3 (reverse) 5′-GATTAGCCTGACAGATC ACTCCACGA-3′.

The contents of the PCR reactions and the equipment used for amplification were identical with those used for the MX5–MX3 primer pair. The PCR programme was 95 °C for 300 s, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 60 s at 72 °C, and was terminated with an extension at 72 °C for 300 s.

The PCR products were electrophoresed in 1.0% agarose gels (Sigma, St Louis, MO, USA) in TBE buffer. λ phage DNA cut with *Pst*I was used as the molecular weight standard.

Table 1 The location of the 5′ end of the primers on the 18S rRNA gene of *M. cerebralis*

Forward primer	Position	Reverse primer	Position
MX5	66	MX3	1767
MC5	388	MC3	1446
MB5	980	MB3	1004

Sequencing

For DNA sequencing, six primers were used: the MX5–MX3 and MC5–MC3 primer pairs and also the MB5 and MB3 primers designed for sequencing in our laboratory and based on the 18S rDNA sequences of *Myxobolus* species, available in GenBank. The location of the primers compared with the *M. cerebralis* sequence are shown in Table 1. The sequences of the MB5 and MB3 oligonucleotides are:

MB5 (forward) 5′-GGTGATGATTAACAGG AGCGGT-3′

MB3 (reverse) 5′-CCAACCGCTCCTGTTAA TCATC-3′.

The PCR products were sequenced using the PRISM Ready Reaction Dye Deoxy Cycle Sequencing Protocol (Perkin-Elmer, Norwalk, CN, USA) with an ABI 373 A automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were read using the Applied Biosystems 373A DNA Sequencer Data Analysis Program and assembled by the program package Lasergene (DNASTAR).

In the case of the replicates originating from different specimens of the same fish host species, only the MX5 and MX3 primers were used for sequencing.

Phylogenetic analysis

Multiple alignment of the nucleotide sequences was performed with the MultAlin computer program (Corpet 1998). The highly variable regions were removed from the sequences as described by Harrach & Benkö (1998), therefore only 972 nucleotides were used in the phylogenetic calculations from the approximately 1000 bp long 18S rDNA fragments. The 972 bp long, aligned sequences contained two fragments: a 511 bp long fragment from the 5′-end of the 18S rRNA gene and a 461 bp long fragment from the 3′-end.

Phylogenetic calculations were performed by the programs of the PHYLIP version 3.573c package

© 2002 Blackwell Science Ltd

(Felsenstein 1989). The data were analysed with parsimony (DNAPARS) and distance matrix analyses (DNADIST using Kimura-2 distance parameter followed by FITCH with global rearrangements). Gaps of up to three bases were treated as special (vs. missing) characters. For bootstrap analysis, the mentioned programs were preceded by SEQBOOT (molecular sequences; 100 data sets) and followed by CONSENSE.

Results

Morphology

Plasmodia containing developmental stages or spores were found in the muscle of all the eight fish species examined. Young plasmodia were detected intracellularly in muscle cells of the trunk musculature in all cases. More mature plasmodia, in addition to the residue of the host cell, were surrounded by a connective tissue capsule. In some cases, free spores from disrupted plasmodia were detected in the extracellular space between muscle cells. At a more chronic stage scattered spores from ruptured plasmodia could be demonstrated in the gut lumen, in the capillaries of the choroid and the gills, and particularly in the melano-macrophage centres of the kidney. The most severe and common infection of skeletal muscle was in the roach where encapsulated plasmodia were always detectable in older fish. Spores found in common carp, barbel, roach, rudd, common bream and white bream were about the same size (10–13 μm) and showed a very similar morphology. The common feature of the spores was the small, indistinct intercapsular process and the polar filaments, which were only loosely wound in the polar capsule with not more than four coils. In each fish, even in a single plasmodium, three different morphological types of spores were found. Some spores had a typical elliptical shape and approximately equal polar capsules at the apex, while other spores had a less regular shape, and polar capsules of unequal size were lateral to the apex. In the third spore type, the opening of the polar capsules was typically located at the lateral side of the highly deformed spore (Fig. 1). Although morphologically variable spores were found in all fish species studied, in roach, rudd, white bream and common bream the number of asymmetric spores was much higher than in common carp. In common carp, deformed spores were found in encysted plasmodia and in spores engulfed by macrophages in different organs. Deformed spores were found only occasionally in barbel (Fig. 2). Morphologically similar spores to those described above were also found in the muscle of bleak and bitterling, but these were not characterized further because the quantity of spores was not sufficient for molecular studies.

The shape and measurements of the spores in every fish species agreed with the original descriptions and therefore in molecular studies we used the most widely accepted terminology and systematics. Based on original descriptions and typical hosts, spores from the common carp were tentatively identified as *M. cyprini* Doflein, spores from barbel as *M. musculi* Keysselitz, while spores from the

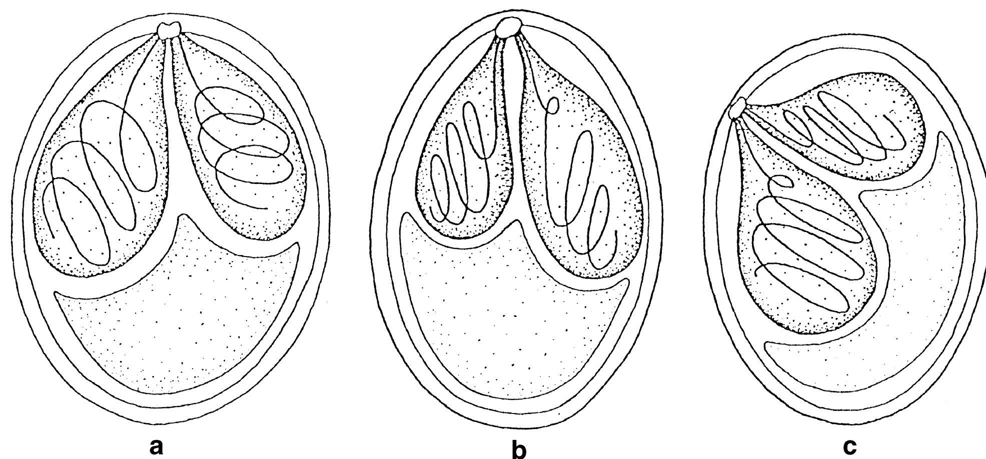

Figure 1 Schematic illustrations of spores of intracellular *Myxobolus* spp. from the muscle of cyprinid fish. (a) Spore with relatively regular shape and with only slightly differing polar capsules. (b) Spore with relatively regular shape but with polar capsules of different size. (c) Spore with irregular shape and polar capsules of different size.

© 2002
Blackwell Science Ltd

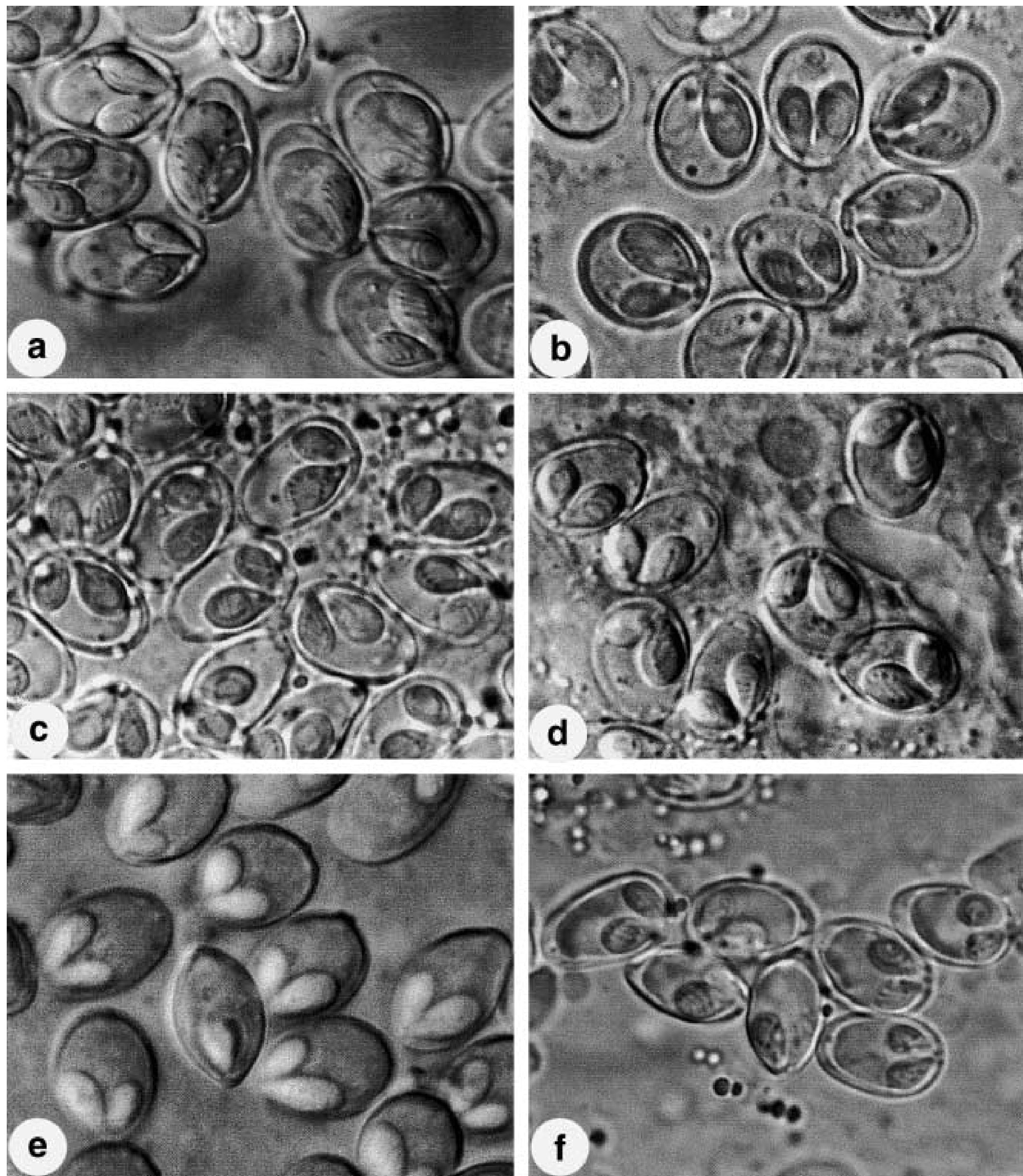

Figure 2 Myxospores of intracellular *Myxobolus* spp. collected from the muscle of cyprinid fish. (a) *M. cyprini* from common carp, (b) *M. musculi* from barbel, (c) *M. pseudodispar* from roach, (d) *M. pseudodispar* from white bream, (e) *M. pseudodispar* from rudd and (f) *M. pseudodispar* from common bream (fresh preparations, ×2000).

roach, rudd, white bream and common bream were identified as *M. pseudodispar* Gorbunova.

PCR and phylogenetic analysis

The specific primer pairs MX5–MX3 and MC5–MC3 successfully amplified approximately 1600 and 1000 bp fragments, respectively, of the 18S rRNA gene from every sample of *Myxobolus* examined. The replicates of myxospores isolated from different specimens of the same fish host were successfully amplified with the MX5–MX3 primer pair and were sequenced with these two primers. Thus, approximately 500 bp DNA sequences from the 5′ and 3′ end of the 1600 bp DNA fragment were obtained. The DNA sequences of the PCR products have been deposited in GenBank and accession numbers are

© 2002
Blackwell Science Ltd

Table 2 *Myxobolus* species sequenced from different host species and localities

Species	Host	Locality	GenBank number	Length of sequence (bp)
M. pseudodispar RO1	Roach	Lake Balaton	AF380145	1554
M. pseudodispar RO2	Roach	River Danube	AF466651	5′ end: 560
			AF466656	3′ end: 500
M. pseudodispar RD1	Rudd	Lake Balaton	AF380142	1556
M. pseudodispar RD2	Rudd	Lake Balaton	AF466650	5′ end: 520
			AF466655	3′ end: 500
M. pseudodispar WB1	White bream	Lake Balaton	AF380143	1551
M. pseudodispar WB2	White bream	Lake Balaton	Same as AF380143[a]	5′ end: 520
			AF466654	3′ end: 500
M. pseudodispar CB1	Common bream	Lake Balaton	AF380144	1551
M. pseudodispar CB2	Common bream	Lake Balaton	AF466648	5′ end: 570
			AF466652	3′ end: 500
M. pseudodispar CB3	Common bream	Lake Balaton	AF466649	5′ end: 570
		(Keszthely area)	AF466653	3′ end: 500
M. pseudodispar CB4	Common bream	Lake Balaton	Same as AF466649[a]	5′ end: 520
		(Siófok area)	Same as AF466653[a]	3′ end: 500
M. cyprini 1	Common carp	Fish farm	AF380140	1554
M. cyprini 2	Common carp	Fish farm	Same as AF380140[a]	5′ end: 520
			AF466657	3′ end: 500
M. musculi 1	Barbel	River Danube	AF380141	1556
M. musculi 2	Barbel	River Danube	Same as AF380141[a]	5′ end: 520
			AF466658	3′ end: 500

[a] The partial sequence of the sample was 100% identical with a previously sequenced sample of the given parasite species collected from the given fish-host species.

Figure 3 Phylogenetic tree generated by distance matrix analysis and showing relationships between *Myxobolus* spp. collected from the muscle of cyprinid species. The length of the edited alignment sequences was 972 bp, containing a 511-bp fragment from the 5′-end of the 18S rRNA gene and a 461-bp fragment from the 3′-end. *Myxobolus cerebralis* (U96492) was chosen as an outgroup. Sequences published in this study are listed in Table 2. Numbers at nodes indicate bootstrap confidence levels obtained by distance matrix analysis. Scale bar is equal to 10% difference. *CB: common bream, WB: white bream, RO: roach, RD: rudd.

listed in Table 2. In several cases, the partial sequences of the replicates were completely identical with a previously sequenced sample of the given parasite species (Table 2).

Distance matrix analysis (Fig. 3) distinguished six groups, with very high (99–100) bootstrap confidence levels. *Myxobolus cerebralis*, the only non-muscular *Myxobolus* species, was found to be

© 2002 Blackwell Science Ltd

very different, and was a suitable outgroup. *Myxobolus musculi* and *M. cyprini* clustered, but with a very low confidence level.

Similarly, all sequences from the putative *M. pseudodispar* collected from four different fish species clustered, but with a low bootstrap value. The cluster of the 10 *M. pseudodispar* sequences diverged into two subclusters with high bootstrap confidence levels. The first subcluster contained the four samples originating from common bream and the two from white bream. The other subcluster consisted of spores collected from roach and rudd. Genetic distances obtained from the distance matrix outfile are shown in Table 3.

Parsimony analysis confirmed the clustering according to fish species, but not to spore morphology. The subcluster containing the *M. pseudodispar* samples originating from roach and rudd joined to the cluster of samples of *M. cyprini* and *M. musculi,* even if only with a low bootstrap value (60). The other parts of the phylogenetic tree (Fig. 4) were congruent to the tree generated by the distance matrix analysis.

Discussion

Until recently, only spore morphology served as a means of identification of the more than 500 known *Myxobolus* species. Because of the difficulties of experimental studies, little was known about host specificity, and morphologically similar spores from genetically different hosts were often described as the same species. Thus, Shulman (1966) recorded as many as 40 hosts for certain *Myxobolus* species. To enlarge the criteria for species identification, Molnár (1994) suggested that the host, organ and tissue specificity should also be considered. Molecular biological methods offer great scope for the correct identification of species.

Myxobolus spp. infecting the skeletal muscles of cyprinids serve as examples of the difficulties associated with methods of identification based on spore morphology. It is not surprising that *M. pseudodispar* differing from the original description of *M. cyprini* in its asymmetric spore shape and different sized polar capsules was described by Gorbunova (1936) as a new species. Similarly, it is understandable that Lom & Dyková (1992), who observed great morphological variability in the spores of *M. cyprini,* considered *M. pseudodispar* a junior synonym. During the present survey

Table 3 Genetic distances obtained from the distance matrix outfile based on 972 bp fragments of the 18S rRNA gene of 14 *Myxobolus* samples isolated from the muscle of cyprinids and the outgroup, *M. cerebralis*

	M.p.RO1	M.p.RO2	M.p.RD1	M.p.RD2	M.p.WB1	M.p.WB2	M.p.CB1	M.p.CB2	M.p.CB3	M.p.CB4	M.m.2	M.m.1	M.c.2	M.c.1	M.cer.
M.p.RO1	0.0000	0.0031	0.0295	0.0252	0.0470	0.0469	0.0447	0.0436	0.0425	0.0425	0.0361	0.0372	0.0562	0.0573	0.2018
M.p.RO2		0.0000	0.0306	0.0284	0.0481	0.0481	0.0469	0.0458	0.0447	0.0447	0.0394	0.0405	0.0596	0.0608	0.2033
M.p.RD1			0.0000	0.0062	0.0479	0.0502	0.0480	0.0491	0.0480	0.0480	0.0493	0.0482	0.0642	0.0630	0.2133
M.p.RD2				0.0000	0.0512	0.0490	0.0502	0.0490	0.0479	0.0479	0.0448	0.0459	0.0595	0.0607	0.2160
M.p.WB1					0.0000	0.0021	0.0114	0.0125	0.0114	0.0114	0.0525	0.0514	0.0629	0.0618	0.1974
M.p.WB2						0.0000	0.0135	0.0125	0.0114	0.0114	0.0525	0.0536	0.0629	0.0641	0.2003
M.p.CB1							0.0000	0.0031	0.0021	0.0021	0.0492	0.0481	0.0584	0.0573	0.1916
M.p.CB2								0.0000	0.0010	0.0010	0.0481	0.0492	0.0550	0.0561	0.1931
M.p.CB3									0.0000	0.0000	0.0469	0.0481	0.0561	0.0573	0.1933
M.p.CB4										0.0000	0.0469	0.0481	0.0561	0.0573	0.1933
M.m.2											0.0000	0.0010	0.0460	0.0471	0.1887
M.m.1												0.0000	0.0471	0.0460	0.1872
M.c.2													0.0000	0.0010	0.2051
M.c.1														0.0000	0.2036
M.cer.															0.0000

M.p.: *Myxobolus pseudodispar*, M.c.: *Myxobolus cyprini*, M.m.: *Myxobolus musculi*, M.cer.: *Myxobolus cerebralis*, CB: common bream, WB: white bream, RO: roach, RD: rudd.

© 2002
Blackwell Science Ltd

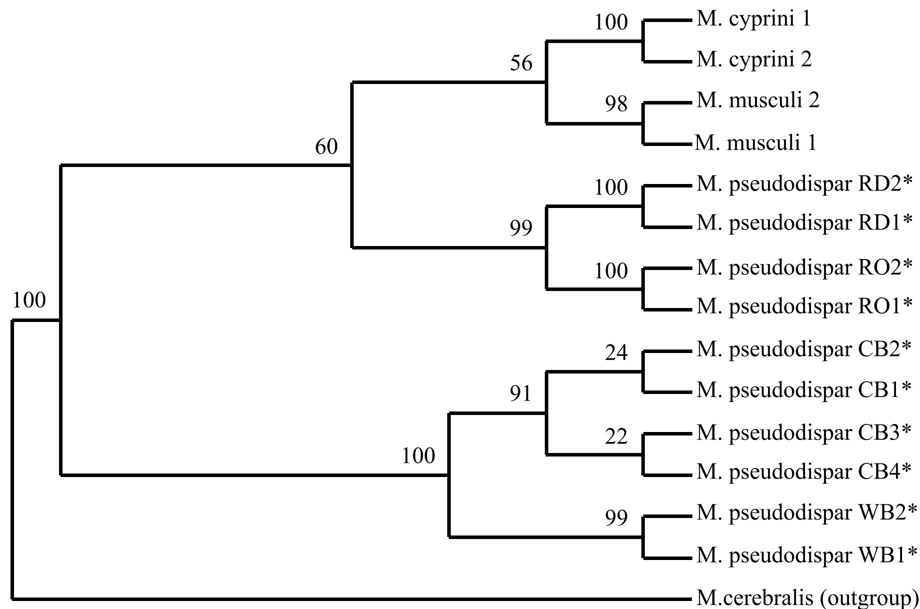

Figure 4 Phylogenetic tree created by parsimony analysis showing the relationships among the muscle-infecting *Myxobolus* species examined. The length of the edited alignment sequences was 972 bp, containing a 511-bp fragment from the 5′-end of the 18S rRNA gene and a 461-bp fragment from the 3′-end. *Myxobolus cerebralis* (U96492) was chosen as an outgroup. Sequences published in this study are listed in Table 2. *CB: common bream, WB: white bream, RO: roach, RD: rudd.

M. pfeifferi, the best known parasite of the muscle of barbel, was not found, but another species identified as *M. musculi,* commonly infecting the muscle cells of barbel, did occur. Based on spore morphology, particularly the structure of the polar capsules, this latter species was erroneously identified as *M. ergensi* Lom in a previous study (Eszterbauer *et al.* 2001). The spores found in the muscle of the barbel corresponded to the published diagrams of *M. ergensi* spores in all respects; however, considering the specific location of plasmodia, we consider that this species should have been determined as *M. musculi,* which was described earlier but depicted less accurately.

Among the genus *Myxobolus,* strictly host specific parasites and those with a relatively wide host range are found with equal frequency. According to Molnár (1994), *M. drjagini* and *M. pavlovskii,* infecting Chinese carp introduced to Europe, represent the strictly host specific species. *Myxobolus drjagini* infects only silver carp, *Hypophthalmichthys molitrix* (Valenciennes), while *M. pavlovskii* develops in both silver carp and bighead carp, *Aristichthys nobilis* (Richardson). *Myxobolus cerebralis,* the best known *Myxobolus* species, is an example of a species with a wider host range, infecting a number of salmonid species (Hedrick, El-Matbouli, Adkinson & MacConnell 1998; Thomson, Nehring, Bowden & Wygant 1999).

Using classical zoological methods it is very difficult to determine the validity of morphologically similar myxosporeans with identical tissue affinity and developing in taxonomically closely related host species. In such cases, cross-infection experiments and molecular biological methods can help. In the case of the muscle parasites *M. cyprini*, *M. musculi* and *M. pseudodispar*, the cyprinid fish hosts can be considered relatively closely related species, although Zardoya & Doadrio (1999) suggest that *Cyprinus* and *Barbus* species are more closely related to each other than to members of the other four cyprinid genera included in this study, i.e. *Rutilus, Scardinius, Abramis* and *Blicca*. Eszterbauer *et al.* (2001), using a combined PCR-restriction fragment length polymorphism (RFLP) method, studied muscle infecting – *M. pseudodispar* from roach and rudd, *M. cyprini* from common carp and *M. musculi* (previously identified as *M. ergensi*) from barbel. The results for the *M. pseudodispar* samples from different fish species were identical. The results for *M. cyprini* and *M. musculi* were similar to each other, but were markedly different from *M. pseudodispar*.

In the present study, 14 *Myxobolus* samples originating from the muscle of six cyprinid species

© 2002
Blackwell Science Ltd

were compared based on their 18S rDNA sequences. The DNA sequences of *Myxobolus* samples collected from different specimens of a given fish species were consistently more similar to each other than to those of spore samples originating from other fish hosts. The great similarity of DNA sequences indicates that the *Myxobolus* spore samples originating from different fish species are closely related to each other, and suggests that these parasites could represent populations of the same species. If we accept this 'one-species' concept this species should be designated *M. cyprini* based on the priority of its description.

However, the differences in the DNA sequences of spores collected from different fish species seem to reflect the phylogenetic relationship of the fish hosts (i.e. there is a similar topology of the phylogenetic tree of the parasites and cyprinid hosts) (Zardoya & Doadrio 1999). Therefore it is possible that *M. cyprini*, *M. musculi* and *M. pseudodispar* are distinct species. In addition, a further subdivision of the species *M. pseudodispar* according to the host species may be considered. However, this suggestion is difficult to explain in ecological terms, i.e. that muscle-parasitic myxosporeans (species or populations) spread vertically within a given biotope. In this case, parasites originating from a given fish species would infect, even after establishing infection in alternate hosts, only that fish species from which they originated. This possible route of development would indicate that the morphologically similar parasites found in cyprinids do not belong to the same species. Although this hypothesis seems extremely unlikely, it should not be ruled out until refuted by cross-infection experiments. Molecular biological methods appear to be excellent tools for studying this question and providing very robust answers; however, only infection experiments and sequence-level phylogenetic analyses of further spore samples will definitively determine whether muscle-parasitic myxosporeans of cyprinids represent valid species or only synonyms. Until further studies give a satisfactory answer to this question, we suggest that *M. cyprini, M. musculi* and *M. pseudodispar* are valid species in accordance with the original descriptions.

Acknowledgements

This work was supported by the Hungarian Scientific Research Fund (OTKA) grants no. T 29200, T 31755 and A312. Special thanks are due to Zsuzsa Kis for making the histological sections, Dr Ferenc Baska, Dr György Csaba, Dr Mária Láng and Dr Gábor Majoros for kindly providing Danube and pond fish, and Dr Mária Benkö for critically reading the manuscript.

References

Andree K.B., Gresoviac S.J. & Hedrick R.P. (1997) Small subunit ribosomal sequences unite actinosporean and myxosporean states of *Myxobolus cerebralis*, the causative agent of whirling disease in salmonid fishes. *Journal of Eukaryotic Microbiology* **44,** 208–215.

Andree K.B., Székely C., Molnár K., Gresoviac S.J. & Hedrick R.P. (1999) Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvidae) based on small subunit ribosomal DNA sequences. *Journal of Parasitology* **85,** 68–74.

Baska F. (1986) Histological studies on the development of *Myxobolus pseudodispar* Gorbunova, 1936 in the roach (*Rutilus rutilus*). *Acta Veterinaria Hungarica* **35,** 251–257.

Bykhovski B.E. (1962) *Key to the Parasites of Freshwater Fish of the USSR.* Izdatelst'stvo Akademii Nauk USSR, Moscow-Leningrad (In Russian).

Corpet F. (1998) Multiple sequence alignment with hierarchical clustering. *Nucleid Acids Research* **16,** 10881–10890.

Eszterbauer E., Benkö M., Dán Á. & Molnár K. (2001) Identification of fish parasitic *Myxobolus* (Myxosporea) species using a combined PCR-RFLP method. *Diseases of Aquatic Organisms* **44,** 35–39.

Felsenstein J. (1989) PHYLIP – Phylogeny inference package (version 3.2). *Cladistics* **5,** 164–166.

Gorbunova M.N. (1936) Changes in the parasitic fauna of pike and roach according to their age. *Uchenye Zapiski Leningradskogo Ordena Lenina Gosudarskogo Universiteta. No. 7* (Biology Series Fascicule 3), pp. 5–30. Problems of Economic Parasitology, Leningrad (In Russian).

Harrach B. & Benkö M. (1998) Phylogenetic analysis of adenovirus sequences. In: *Methods in Molecular Medicine*, Vol. 21: *Adenovirus Methods and Protocols* (ed. by W.S.M. Wold), pp. 309–339. Humana Press Inc., Totowa, NJ, USA.

Hedrick R.P., El-Matbouli M., Adkinson M.A. & MacConnell E. (1998) Whirling disease: re-emergence among wild trout. *Immunological Reviews* **166,** 365–376.

Hervio D.M.L., Kent M.L., Khattra J., Sakanari J., Yokoyama H. & Devlin R.H. (1997) Taxonomy of *Kudoa* species (Myxosporea) using small subunit ribosomal DNA sequence. *Canadian Journal of Zoology* **75,** 2112–2119.

Kent M.L., Khattra J., Hedrick R.P. & Devlin R.H. (2000) *Tetracapsula renicola* n. sp. (Myxozoa: Saccosporidae); the PKX myxozoan – the cause of proliferative kidney disease of salmonid fishes. *Journal of Parasitology* **86,** 103–111.

Keysselitz G. (1908) Über durch Sporozoen (Myxosporidien) hervorgerufene pathologische Veränderungen. *Verhandlungen der Gesellschaft Deutscher Naturforscher und Ärzte* **2,** 542–543.

Kocylowski B. & Myaczynski T. (1960) *Diseases of Fish and Crayfish.* PWRiL, Warszawa (In Polish).

© 2002
Blackwell Science Ltd

Lom J. & Arthur J.R. (1989) A guideline for the preparation of species descriptions in Myxosporea. *Journal of Fish Diseases* **12,** 151–156.

Lom J. & Dyková I. (1992) *Protozoan Parasites of Fishes*. Elsevier, New York.

Lom J. & Noble E.R. (1984) Revised classification of the Myxosporea Bütschli, 1881. *Folia Parasitologica* **31,** 193–205.

Markevich A.P. (1951) *Parasite Fauna of Fish of the Ukraine SSR*. Kiev, Ukraine (in Russian).

Molnár K. (1994) Comments on the host, organ and tissue specificity of fish myxosporeans and types of their intrapiscine development. *Parasitologica Hungarica* **27,** 5–20.

Molnár K. & Kovács-Gayer É. (1985) The pathogenicity and development within the host fish o*f Myxobolus cypri*ni Doflein, 1898. *Parasitology* **90,** 549–555.

Ogawa K., Delgahapytiya K.P., Furuta T. & Wakabayashi H. (1992) Histological studies on the host response to *Myxobolus artus* Akhmerov, 1960 (Myxozoa: Myxobolidae) infection in the skeletal muscle of carp, *Cyprinus carpio* L. *Journal of Fish Biology* **41,** 363–371.

Salim K.Y. & Desser S.S. (2000) Descriptions and phylogenetic systematics of *Myxobolus* spp. from cyprinids in Algonquin Park, Ontario. *Journal of Eukaryotic Microbiology* **47,** 309–318.

Schäperclaus W. (1954) *Fischkrankheiten*. Akademie Verlag, Berlin.

Shulman S.S. (1966) *Myxosporidia of the Fauna of USSR*. Izdatel'stvo Nauka, Moscow (In Russian).

Székely Cs. (1997) Possible applications of video technology and digital image processing in fish parasitology: morphological examination of the groups Apicomplexa and Myxosporea-Actinosporea by video technology. *Bulletin of the European Association of Fish Pathologists* **17,** 81–82.

Thomson K.G., Nehring R.B., Bowden C. & Wygant T. (1999) Field exposure of seven species or subspecies of salmonid to *Myxobolus cerebralis* in the Colorado River, Middle Park, Colorado. *Journal of Aquatic Animal Health* **11,** 312–329.

Zardoya R. & Doadrio I. (1999) Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *Journal of Molecular Evolution* **9,** 227–237.

Received: 2 July 2001
Accepted: 25 June 2002

© 2002
Blackwell Science Ltd