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Genetic relationship among gill-infecting *Myxobolus* species (Myxosporea) of cyprinids: molecular evidence of importance of tissue-specificity

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ABSTRACT: The importance of tissue-specificity was studied in *Myxobolus* spp. infecting the gills of 7 cyprinid species. The 18S rDNA of 10 *Myxobolus* species was amplified by optimised nested-PCR, resulting in ~1600 bp PCR products. Phylogenetic trees generated by distance matrix and parsimony analyses revealed 4 main groups. Muscle-infecting species all belonged to the same group, while members of 2 gill-infecting groups were clearly distinguishable on the basis of tissue-specificity, and were also recognisable by differences in spore morphology. On the basis of tissue tropism, phylogenetic relationships among the species examined indicate that genetic separation is a more ancient evolutionary feature than host-specificity.

KEY WORDS: *Myxobolus* · Myxosporeans · Phylogeny · 18S rDNA · Tissue tropism · Cyprinids · Gills

INTRODUCTION

The genus *Myxobolus* is one of the richest groups among myxosporeans, containing about 450 valid species (Landsberg & Lom 1991). These species have been identified principally on the basis of spore morphology, although additional features such as organ- and tissue-specificity of intrapiscine development stages and the identity of the host species have also been used for specific assignments (Molnár 1994, Molnár & Székely 1999).

As molecular biological methods have become increasingly important in parasitological studies, the taxonomic classification of myxosporeans has been expanded with phylogenetic analyses. Phylogenetic trees produced by 18S rDNA sequences of myxosporeans available in the GenBank largely agreed with Shulman's (1966) phylogenetic hypotheses, and have revealed the importance of several phenotypic features in species identification (Kent et al. 2000, 2001). Based on the 18S rDNA sequences of 10 myxosporean species, Andree et al. (1999) found that parasite species were related mostly by their location in the tissue of the fish host. Examinations of 5 *Kudoa* species suggested that members of this genus are segregated more by host and geographic origin than by spore morphology (Hervio et al. 1997). On the other hand, Salim & Desser (2000), using the 18S rDNA sequences of 7 *Myxobolus* species from cyprinids, found that the parasites were grouped by spore morphology. Studying the genetic relationships among closely related *Myxobolus* species infecting the skeletal muscle of cyprinids, Molnár et al. (2002) confirmed the validity of these morphologically barely distinguishable species, and suggested that these muscle-infecting species grouped according to fish-host species.

Gill-parasitic *Myxobolus* species are a taxonomically peculiar group within myxosporeans. In most of the cases, several parasite species infect 1 fish host, colonising different gill tissues. Several authors have reported site-selection for different species (Cone & Wiles 1985, Molnár & Székely 1999), but the strictly defined locations of cyprinid-infecting myxosporeans in the gill apparatus were first described by Molnár

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(2002). *Myxobolus* species parasitising the gills were also differentiated using a combined polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method by Xiao & Desser (2000) and Eszterbauer et al. (2001). Using this method, Eszterbauer (2002) confirmed that the morphologically indistinguishable *M. elegans* from orfe and *M. hungaricus* from common bream are indeed valid species. Furthermore, Eszterbauer et al. (2002) proved that *M. pavlovskii* is also present in bighead and silver carp.

The present paper reports the results of phylogenetic analyses of 10 *Myxobolus* species infecting the gills of cyprinids in order to determine their genetic relationship and examine the importance of tissue-specificity in the fish hosts.

MATERIALS AND METHODS

Collection of spores. Myxospores of 10 *Myxobolus* species were collected from 7 fish species in Hungarian natural lakes or rivers and fish farms between 1999 and 2002 (Table 1). After collection and transportation to the laboratory, the 2 to 4 yr old fish were sacrificed using an overdose of the anaesthetic MS 222, followed by severance of the spinal cord.

For studying the occurrence of plasmodia in the gills, samples were examined with a stereomicroscope at 10× magnification. Spores isolated from the plasmodia were carefully examined under coverslip at 100× magnification with a compound microscope to identify the parasites. Digital pictures of the spore samples were also taken.

In most cases, we freed the plasmodia from the host cells or from the capsule formed by the connective tissue of the host under a stereomicroscope, and froze them at –20°C in 1.5 ml centrifuge tubes until further use. The samples contained different numbers of spores (10^2 to 10^6). At least 2 different samples of myxospores collected from different fish specimens at different times were used for molecular biological examinations. In the case of *Myxobolus bramae*, 4 samples of spores were collected from different fish specimens at different times.

DNA extraction. After defrosting the spores, samples were centrifuged at low speed (1000 × *g*). The spores were suspended in 500 µl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% sodiums dodecyl sulphate [SDS], and 0.4 mg ml^{-1} Proteinase-K) and incubated at 55°C for 3 to 4 h. An equal volume of Miniprep Express Matrix (BIO 101) was then added. After centrifugation at 17 900 × *g* for 1 min, the pellet was washed once with 80% ethanol, air-dried and

Table 1. *Myxobolus* sp. Gill-infecting myxosporeans examined. Samples collected from sites in Hungary between 1999 and 2002

Species	Fish host	Locality	Site preference in fish	GenBank No.	Sequence length (bp)
M. bramae Reuss, 1906	Common bream *Abramis brama*	Lake Balaton	Intralamellar/ vascular	AF507968	1580
M. macrocapsularis Reuss, 1906	Common bream	Lake Balaton	Intralamellar/ vascular	AF507969	1577
M. impressus Miroshnichenko, 1980	Common bream	Lake Balaton	Interlamellar/ epithelial	AF507970	1577
M. basilamellaris Lom et Molnár, 1983	Common carp *Cyprinus carpio*	Fish farm	Basifilamental/ epithelial (?)	AF507971	1592
M. dispar Thélohan, 1895	Common carp	Lake Balaton	Intralamellar/ vascular	AF507972	1578
M. pavlovskii Akhmerov, 1954	Bighead *Aristichthys nobilis*	Fish farm	Interlamellar/ epithelial	AF507973	1578
M. pavlovskii Akhmerov, 1954	Silver carp *Hypophthalmichthys molitrix*	Fish farm	Interlamellar/ epithelial	100% identical with AF507973	1578
Myxobolus sp. (*hungaricus*-like spores)	Common bream	Lake Balaton	Intralamellar/ vascular	AY325283	1614
M. muelleri Bütschli, 1882	Chub *Leuciscus cephalus*	River Danube	Intralamellar/ vascular	AY325284	1616
M. intimus Zaika, 1965	Roach *Rutilus rutilus*	Lake Balaton	Intralamellar/ vascular	AY325285	1589
M. obesus Gurley, 1893	Bleak *Alburnus alburnus*	River Danube	Intralamellar/ vascular	AY325286	1596

eluted with MilliQ-purified water, followed by centrifugation at 17 900 × *g* for 2 min; the upper phase was then transferred into a fresh tube. DNA content was estimated by agarose gel electrophoresis against a known amount of DNA.

PCR amplification. DNA was first amplified with the 18e–18g′ universal primer-pair as described by Hillis & Dixon (1991) and modified by Andree et al. (1999), producing a ~1900 bp fragment of the 18S rRNA gene. This was followed by a second PCR amplification using the MX5–MX3 primer-pair specific for the family Myxobolidae (Andree et al. 1999). This primer-pair amplified an approximately 1600 bp fragment from the previously amplified PCR product.

For both steps of the nested PCR, the total volume of the PCR reaction was 50 µl, and comprised approx. 10 to 50 ng extracted DNA in first PCR, 1 × REDTaq PCR reaction buffer (Sigma), 0.2 mmol dNTP (Sigma), 40 pmol of each primer, 2.5 U REDTaq DNA polymerase (Sigma) in Milli-Q-purified water. I used 0.1 to 1 µl of the first PCR product in the nested PCR. A Perkin-Elmer GeneAmp PCR system 2400 and an MJ Research PTC-200 thermocycles were used for amplification. Amplification conditions in the first round were: 95°C for 50 s, 56°C for 50 s and 72°C for 80 s for 35 cycles, with a terminal extension at 72°C for 7 min, followed in the second round with 95°C for 30 s, 46°C for 30 s and 72°C for 60 s for 35 cycles, terminating with an extension at 72°C for 7 min. The PCR products were electrophoresed in 1.0% agarose gels.

Sequencing. For DNA sequencing, 8 primers were used: the MX5 and MX3 described above, and MC5, MC3, MB5, MB3, MB3f and MB5r primers, designed for sequencing in our laboratory, based on the 18S rDNA sequences of *Myxobolus* species available in GenBank (Molnár et al. 2002). The sequences of the oligonucleotides were: MC5 (forward) 5′-CCTGAGAAACGGCTACCACATCCA-3′; MC3 (reverse) 5′-GATTAGCCTGACAGATCACTCCACGA-3′; MB5 (forward) 5′-GGTGATGATTAACAGGAGCGGT-3′; MB3 (reverse) 5′-CCAACCGCTCCTGTTAATCATC-3′; MB3f (forward) 5′-GATGATTAACAGGAGCGGTTGG-3′; MB5r (reverse) 5′-ACCGCTCCTGTTAATCATCACC-3′.

The PCR products were sequenced using the PRISM ready reaction dye deoxy cycle sequencing protocol (Perkin-Elmer) with an ABI 373A automated DNA sequencer (Applied Biosystems). The nucleotide sequences were read using the Applied Biosystems 373A DNA sequencer data analysis programme. For sequence assembly, Lasergene (DNASTAR) and STADEN sequence analysis package, Version 2001.0 (Staden 1996) were used.

Phylogenetic analysis. *Myxobolus* sp. 18S rRNA gene sequences from GenBank were subjected to phylogenetic analysis; they consist of *M. cerebralis* (U96492), *M. pseudodispar* from roach *Rutilus rutilus* (AF380145), *M. pseudodispar* from rudd *Scardinius erythrophthalmus* (AF380142), *M. pseudodispar* from white bream *Blicca bjoerkna* (AF380143), *M. pseudodispar* from common bream *Abramis brama* (AF380144), *M. cyprini* (AF380140), *M. musculi* (AF380141), *M. pendula* (AF378340), *M. pellicides* (AF378339), *M. algonquinensis* (AF378335), *M. bibullatus* (AF378336), *M. bramae* (AF085177), *M. ellipsoides* (AF085178), *M. xiaoi* 1 (AF186843), *M. xiaoi* 2 (AF186842), *M. elegans* (AF448445), *M. hungaricus* (AF448444) and *M. lentisuturalis* (AY119688) as outgroup.

Nucleotide sequences were aligned with the MultAlin computer program (Corpet 1988). Highly variable regions containing more than 5 gaps were removed from the sequences, as described by Harrach & Benkö (1998); therefore, 1593 nucleotides were used in the phylogenetic calculations.

Phylogenetic calculations were performed with PHYLIP Version 3.573c (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 5 bases were treated as special (as opposed to missing) characters. For bootstrap analysis, the mentioned programmes were preceded by SEQBOOT (molecular sequences; 1000 data sets), followed by CONSENSE.

RESULTS

PCR and sequencing

The universal 18e–18g′ primers and specific primer pair MX5–MX3 successfully amplified approx. 1900 and 1600 bp fragments of the 18S rRNA gene from every sample of *Myxobolus* species examined, respectively. The DNA sequences of the PCR products have been deposited in GenBank, and the Accession numbers are listed in Table 1. In each case, the sequences of the replicates were 100% identical with a previously sequenced sample of the relevant parasite species.

Phylogenetic analysis

The edited alignment was 1593 bp long. Distance matrix analysis (Fig. 1) distinguished 4 main groups with high bootstrap confidence levels. *Myxobolus lentisuturalis* was expected to differ most from the other species on the basis of the results of Dykova et al. (2002), and was therefore chosen as the outgroup. For

M. xiaoi, 2 sequences did not cluster with any of the main groups. *M. cerebralis*, the only *Myxobolus* species infecting the head-cartilage, grouped with *M. bramae* (AF 085177) and *M. ellipsoides*.

Muscle-infecting parasites, including *Myxobolus cyprini*, *M. musculi* and *M. pseudodispar* collected from 4 cyprinid species, formed a separate group, as described by Molnár et al. (2002).

Other *Myxobolus* species located on the gills clustered in 2 separate groups. Group 1: *M. hungaricus*, *M. intimus*, *M. obesus*, a *Myxobolus* species unidentifiable on the basis of morphological characters, and *M. pendula* and *M. pellicides* from the gill arches of cyprinids; Group 2: *M. bramae*, *M. macrocapsularis*, *M. impressus*, *M. basilamellaris*, *M. dispar*, *M. pavlovskii*, *M. muelleri*, *M. algonquinensis*, *M. elegans* and *M. bibullatus*, a gill parasite from *Catostomus commersoni*.

Parsimony analysis confirmed this clustering pattern, but with some differences in the bootstrap values (Fig. 1). Muscle parasites segregated as described previously by Molnár et al. (2002).

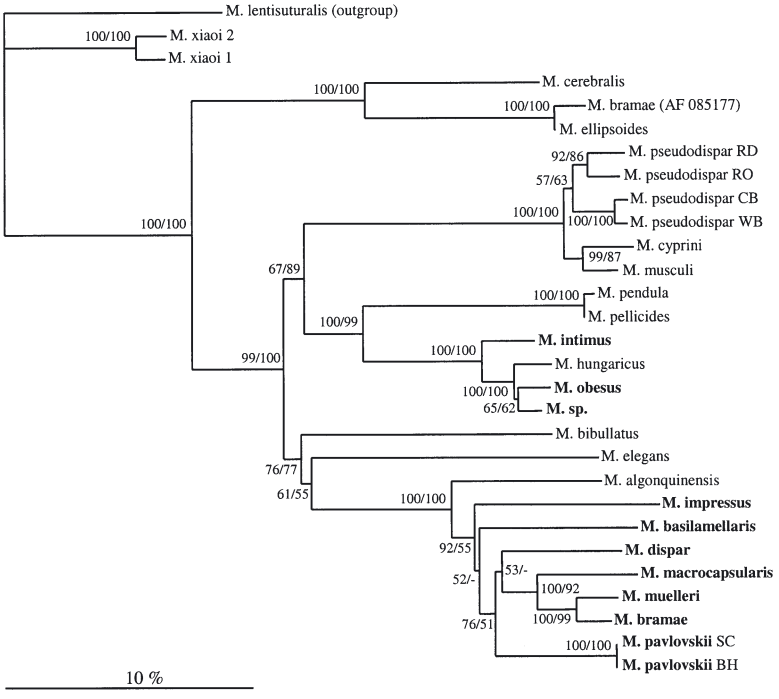

Fig. 1. *Myxobolus* spp. Phylogenetic tree generated by distance matrix analysis, showing relationship among myxosporeans collected from gills and muscle of cyprinids. Numbers above nodes indicate bootstrap confidence levels in percent (based on 1000 repetitions) for distance matrix/parsimony analyses, respectively. RD: rudd *Scardinius erythrophthalmus*, RO: roach *Rutilus rutilus*, CB: common bream *Abramis brama*, WB: white bream *Blicca bjoerkna*, SC: silver carp *Hypophthalmichthys molitrix*, BH: bighead *Aristichthys nobilis*

DISCUSSION

Traditionally spore morphology has been used as a basic feature for the identification of myxosporean species. In the early 1990s, other phenotypic features such as host-, organ- and tissue-specificity were suggested as important characters for specific assignment (Molnár 1994). Since then, several studies have confirmed the importance of these features and, in several cases, found them to correlate with the results of molecular data based on the comparison of 18S rDNA sequences (Kent et al. 2001).

Muscle-infecting species used for phylogenetic analysis provide a good example of the difficulty of morphology-based identification. As Molnár et al. (2002) demonstrated, in the case of species infecting the skeletal muscle of cyprinids, molecular biological methods are essential for proving the validity of the species described on the basis of morphological features. In addition, Molnár et al. (2002) reported that *Myxobolus pseudodispar* samples collected from different fish-host species seemed to reflect the phylogenetic relationships of the fish hosts. This could be helpful in understanding the mechanism of specific divergence among myxosporeans. Using ~1600 bp, aligned DNA sequences of muscle-infecting species, the results of the distance matrix and parsimony analyses in the present study gave the same clustering as described earlier by Molnár et al. (2002), using aligned DNA sequences of only 970 bp.

In the case of gill-infecting parasites, little is known about the exact location of the myxospores in fish. Many *Myxobolus* species develop mature spores in the gills, but in their species descriptions, investigators rarely mention the gill tissue, where the parasites form plasmodia containing mature myxospores. Indicating that, in addition to spore morphology, exact location and tissue tropism is also essential for species identification, Molnár (2002) distinguished 6 types of site preference among gill-infecting myxosporeans: (1) Species belonging to the interlamellar-epithelial type develop in the epithelium between the gill lamellae (secondary lamellae); this type of infection is represented by *M. impressus* and *M. pavlovskii*. (2) Parasites of the interfilamental-epithelial type usually form large plasmodia in the epithelium of filaments. (3) The basifilamental type, representing a special case of interfila-

mental location, is represented by *M. basilamellaris*, which forms plasmodia on the base of the gill filaments (primary lamellae) and may also be associated with the compact connective tissue of the gill arch. (4) In the case of the intralamellar-vascular type, plasmodia develop in the vascular network of the gill lamellae, and plasmodia of different size are recognisable within a single lamella (e.g. *Henneguya creplini*, *M. bramae* and *M. hungaricus*). (5) Intrafilamental-vascular parasites appear in the afferent artery of the gill filaments, forming a large plasmodium at the end of filaments (e.g. *M. bramae*, *M. macrocapsularis*, *M. dispar*). (6) The last type has an intrafilamental–chondroidal location, with plasmodia developing in the chondroid tissue of the gill arch; a typical example is *M. intrachondrealis* from carp.

The present study examined the phylogenetic relations of 10 gill-infecting *Myxobolus* species. The phylogenetic trees generated by distance matrix and parsimony analyses revealed 4 main groups, which were easily distinguishable. *M. lentisuturalis* was chosen as an outgroup: although it forms plasmodia in the skeletal muscle tissue, it is located at some distance from the cluster of muscle-infecting species. Its position corresponded to that indicated by the molecular phylogenetic results of Dykova et al. (2002), who clearly demonstrated the independent position of this species among the *Myxobolus* species available in GenBank (including the muscle-infecting species used for phylogenetic analysis in the present study). *M. xiaoi* from the cartilage of the gill arch of *Notemiconus crysoleucas* and *Notropis cornutus* were located closest to *M. lentisuturalis* on the present phylogenetic tree, as also reported by Dykova et al. (2002).

Gill-infecting *Myxobolus* species formed 2 main groups on the phylogenetic tree. The smaller cluster contained *M. obesus*, *M. intimus*, *Myxobolus* sp. examined in this study and *M. hungaricus*. These species displayed high similarity in spore morphology, i.e. oval-shaped myxospores with pointed apexes, and 2 unique ribs on the surface of the spore valves, running parallel to the suture; furthermore they all belonged to the intralamellar-vascular type of gill-infecting myxosporeans. *M. pendula* (AF 378340) and *M. pellicides* (AF 378339), located in the gill arches, also clustered with these 4 species. Unfortunately, no information is available in the species descriptions in the literature concerning their tissue tropism, which may be either vascular or chondroidal type. The separation of this smaller group from the main gill-parasite cluster, and its relative closeness to the muscle-infecting group suggest that this group has a different evolutionary origin from the other gill parasites. It is possible that they developed parallel to the muscle parasites but, at some point, adapted to gill infection.

The gill parasites belonging to the main group showed similar spore morphology. Their elliptical spore shape and the polar capsules located at the apex diverged markedly from the spore shape of all other species examined. The only exception was the spores of *Myxobolus elegans*, which had an oval shape similar to that of *M. hungaricus* myxospores (Eszterbauer 2002). *M. impressus* and *M. pavlovskii*, which both formed plasmodia in the epithelial tissue, and *M. basilamellaris* (presumably originating from the gill epithelium) were clearly distinguishable from species belonging to the intralamellar-vascular type of gill myxosporeans (*M. bramae*, *M. macrocapsularis*, *M. dispar*, and *M. muelleri*). *M. algonquinensis* (AF378335) from the ovary of *Notropis cornutus* clustered close to the species developing interlamellarly. This phylogenetic position suggests that this species forms plasmodia in epithelial-like tissue or is associated with connective tissue within the ovary.

Myxobolus cerebralis, the parasite infecting the head cartilage of salmonids, clustered with the gill-parasitic *M. bramae* (AF085177) collected from *Abramis brama* and *M. ellipsoides* from *Rutilus rutilus.* The positions of these 2 gill parasites are rather interesting, because they differed in host-, organ- and tissue-specificity from *M. cerebralis*, although the tissue tropism of *M. ellipsoides* is not mentioned in either the GenBank or in the related article by Andree et al. (1999). It is noteworthy that the genetic difference between the sequences of *M. bramae* (AF085177) and *M. ellipsoides* was almost as low as (e.g.) that between *M. pseudodispar* samples from *Abramis brama* and those from *Blicca bjoerkna*, for which morphological differences were not recognisable (the genetic distances obtained from the distance matrix outfile were 0.0133 between *M. bramae* AF085177 and *M. ellipsoides* and 0.0107 for *M. pseudodispar* from *A. brama* and *B. bjoerkna*).

With regard to the *Myxobolus bramae* species originating from common bream, Andree et al. (1999) deposited a partial 18S rDNA sequence in GenBank under the name *M. bramae* (AF085177); however the DNA sequence of *M. bramae* (AF507968) examined in this study differed from the AF085177 sequence, although both myxospores were collected from the gills of common bream originating from Lake Balaton in Hungary. The genetic distances in the distance matrix outfile were rather high (0.3232), showing that these 2 sequences are unlikely to belong to the same parasite species. It is well known that a dozen or so myxosporean species can occur on the gills of common bream, and 8 *Myxobolus* species have already been described from this host fish (Landsberg & Lom 1991). Molnár & Székely (1999) reported 4 *Myxobolus* species collected from different locations in the gills of com-

mon bream in Hungary, and suggested that the simultaneous presence of several species on the same fish specimen could cause difficulties during collection of myxospores and possible contamination. Contamination with other gill-infecting myxosporean species may be a reason for the differences found between the 2 DNA sequences assigned to *M. bramae*. To avoid contamination, it is important to collect samples very carefully and to check all ruptured cysts with a compound microscope. If the amplified PCR product can be sequenced without significant background and the sequences overlapping in 2 directions clearly merge into 1 unambiguous sequence, then the possibility of contamination or chimeric sequences is fairly low.

Since most of the samples used in this study were collected from Lake Balaton or the River Danube in Hungary, the eventual influence of geographic origin on phylogenetic relations could not be unequivocally confirmed or excluded. Nevertheless, several species originating from Ontario, Canada (*Myxobolus pendula*, *M. pellicides*, *M. bibullatus* and *M. algonquinensis*) clustered with the Hungarian species. Further species from different locations should be studied to assess if geographical location has an effect on the phylogeny of *Myxobolus* species as in the case of (e.g.) *Kudoa* species (Kent et al. 2001).

The phylogenetic tree revealed by this study demonstrated that tissue tropism may play an important role in the genetic relationships among myxosporean species. Although examination of further myxosporean species is necessary to confirm these results, they do seem to indicate that genetic separation based on tissue tropism is a more ancient evolutionary feature than (e.g.) host-specificity. The phylogenetic relationships among the *Myxobolus* species examined also suggest that new species evolved when parasite species came into contact with 'new' fish hosts and adapted in order to be able to infect them. After adaptation, they developed as separate species, but in most cases their location within the new fish hosts (organ and/or tissue tropism) remained unchanged.

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