Vol. 44: 35–39, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published January 26

Identification of fish-parasitic *Myxobolus* (Myxosporea) species using a combined PCR-RFLP method

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ABSTRACT: Polymerase chain reaction (PCR) with primers specific for the family Myxobolidae was used to amplify a part of the 18S ribosomal RNA gene of *Myxobolus* species. The length of the amplified fragments was approximately 1600 base pairs. Six *Myxobolus* species identified on the basis of morphological features were compared using a combined PCR-RFLP method. The cleavage patterns generated by 2 frequent cutter restriction enzymes (*Hin*fI and *Msp*I) were suitable for the differentiation of the examined *Myxobolus* species.

KEY WORDS: *Myxobolus* spp. · *Myxosporea* · Fish parasite · PCR · RFLP

INTRODUCTION

Myxosporea are common endoparasites of fishes. For a long time they were considered to be a phylum of Protozoa, but recently an increasing number of authors have challenged the taxonomy (Smothers et al. 1994, Kent et al. 1995, Sidall et al. 1995, Schlegel et al. 1996) and related this group to metazoans.

One of the richest genera of the Myxosporea is the genus *Myxobolus*. To date as many as 500 *Myxobolus* species infecting fish are known, of which 444 valid species were recorded by Lom & Dyková (1992). Until recently, differentiation of the species was based on a morphological characterisation of the spores, although attempts to consider the organ and tissue specificity of the individual intrapiscine developmental stages were also made (Molnár 1994). The main difficulty in identification of the species is caused by the high degree of similarity in morphology even though there are differences in the size or shape of the spore structure. The differences, however, are often minimal between the spores of *Myxobolus* species living in taxonomically distant host species and in different locations. Identification is further hampered by a lack of information concerning the host specificity of myxosporeans. Infection experiments aiming at the clarification of host specificity are difficult because of the complicated reproduction of Myxosporea in 2 alternate hosts: fish and invertebrate hosts (oligochaete worms [Wolf & Markiw 1984], polychaetes [Bartholomew et al. 1997] or bryozoans [Longshaw et al. 1999]) as alternate hosts. At present, the development of only 12 *Myxobolus* species has been investigated in the oligochaete host (Wolf & Markiw 1984, El-Matbouli & Hoffman 1989, 1993, Ruidish et al. 1991, Kent et al. 1993, Yokoyama et al. 1995, El-Mansy & Molnár 1997a,b, El-Mansy et al. 1998, Molnár et al. 1999, Székely et al. 1999, Eszterbauer et al. 2000). The full cycle including both (intraoligochaete and intrapiscine) stages could be reproduced in the case of 3 species only: *Myxobolus cerebralis* (Wolf & Markiw 1984) *M. cultus* (Yokoyama et al. 1995) and *M. pseudodispar* (Székely et al. unpubl.). The reasons for the very limited number of successful infection experiments are the labour-intensive and technically difficult nature of such experi-

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ments, and complications related to the establishment of parasite-free fish and oligochaete stocks.

The use of molecular biological methods seems to be a more straightforward approach which may replace the use of infection experiments in the differentiation of the myxosporean species. Numerous protistan parasites have been studied by PCR and restriction fragment length polymorphism (RFLP) of the 18S ribosomal RNA (rRNA) gene; this technique is often referred to as 'riboprinting' (Clark 1997, Pomport-Castillon et al. 1997). There have been several reports on the study of the 18S rRNA gene of different *Myxobolus* species at the DNA sequence level (Andree et al. 1998). Phylogenetic analysis (Andree et al. 1999b) and genetic comparisons of developmental stages of Myxosporea species (Andree et al. 1997) were also described.

In the present work, the feasibility of using a simple method of polymerase chain reaction (PCR) and subsequent RFLP for the purpose of differenting of several *Myxobolus* species was tested.

MATERIAL AND METHODS

Sources of spores. Myxospores of 6 *Myxobolus* species were collected from different fishes in Hungary, as shown in Table 1.

The plasmodia which were filled with mature spores were ruptured by use of a needle and the contents were collected carefully in 1.5 ml microfuge tubes. The spores (approx. 2×10^6) were then centrifuged at low speed ($1000 \times g$).

Table 1. Sources of myxospores. Six *Myxobolus* species were collected from cyprinid fishes in Hungary

Sample	Myxosporean species	Host species	Locality of collection	Organ specificity
1	*Myxobolus cyprini* Doflein, 1898	Common carp *Cyprinus carpio*	Lake Balaton	Muscle
2	*M. ergensi* Lom, 1969	Barbel *Barbus barbus*	River Danube	Muscle
3	*M. bramae* Reuss, 1906	Common bream *Abramis brama*	Lake Balaton	Gills
4	*M. bramae* Reuss, 1906	Common bream *A. brama*	Lake Balaton	Gills
5	*M. macrocapsularis* Reuss, 1906	Common bream *A. brama*	Lake Balaton	Gills
6	*M. impressus* Miroshnichenko, 1980	Common bream *A. brama*	Lake Balaton	Gills
7	*M. pseudodispar* Gorbunova, 1936	Roach *Rutilus rutilus*	Lake Balaton	Muscle
8	*M. pseudodispar* Gorbunova, 1936	Rudd *Scardinius erythrophthalmus*	Lake Balaton	Muscle

DNA extraction. The DNA was extracted by suspending the spores in 500 µl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, 0.4 mg ml^{-1} Proteinase K) and incubating them at 55°C overnight. Then, 500 µl of phenol:chloroform (1:1) was added to the digested spores and mixed gently. After centrifugation at $5200 \times g$ for 10 min the upper phase was transferred into a new tube. If necessary, the extraction step was repeated and was followed by a chloroform treatment. After the last centrifugation, the upper phase was transferred into a fresh tube and mixed with 0.1 volume of sodium acetate (3 M, pH 5.2) with 2 volumes of 96% ethanol. The DNA was precipitated at –70°C for 10 min and then pelleted by centrifugation at $17\,900 \times g$ for 10 min. The pellet was washed once with 70% ethanol, air-dried for several minutes and resuspended in distilled water. The DNA content was estimated by agarose gel electrophoresis in comparison with a known amount of λ phage DNA (0.1 mg).

PCR amplification. Oligonucleotide primers specific for the family Myxobolidae (Andree et al. 1999b) were described and kindly supplied by Karl Andree. The sequence of the forward primer (MX5) was: 5′-CTG-CGGACGGCTCAGTAA ATCAGT-3′; the sequence of the reverse primer (MX3) was: 5′-CCAGGACATCT-TAGGG CATCACAGA-3′. These primers amplify an approximately 1600 bp long fragment from the 18S rRNA gene.

The PCR was carried out in 50 µl volumes. Reactions contained approx. 10 to 50 ng extracted DNA, 1× RED-Taq PCR Reaction Buffer (Sigma, USA), 0.2 mmol dNTP (MBI Fermentas, Lituania), 40 pmol of each primer, 2.5 U REDTaq DNA polymerase (Sigma, USA) and distilled water. A PDR 91 DNA Reproducer manufactured by the BLS Ltd., Hungary, was used for amplification.

Two different programs were applied for amplification. Both of them contained 35 cycles, were proceeded by a denaturation step at 95°C for 5 min and finished with an extended elongation step at 72°C for 5 min. The 3 different temperatures and durations (for DNA denaturation, primer annealing and primer elongation) of the cycles were in the first program: 95°C for 60 s, 47°C for 60 s, 72°C for 120 s; and in the second program: 95°C for 30 s, 46°C for 30 s and 72°C for 60 s. The first program was used for the amplification of *Myxobolus bramae* and *M. macrocapsularis* while the samples of other *Myxobolus* species were amplified using the second program.

Detection of PCR products. The PCR products were electrophoresed on 1.0% agarose gels (Sigma, USA) in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0) and photographed using Kodak digital camera with the Kodak Digital Science 1D v. 3.0.2 software. λ phage DNA digested with *Pst*I was used as molecular weight standard.

RFLP analysis. Restriction enzymes for the RFLP of the PCR products were selected by analysing those 18S rRNA gene sequences of myxosporean species which were available from the GenBank (accession numbers: AF115255, AF085182, AF085181, AF085180, AF085179, AF085178, AF085177, AF085176, U96495, U96494, and U96492) (Smothers et al. 1994, Andree et al. 1997, 1999a,b). Using the software MapDraw, the restriction site analysis program of the package Lasergene, DNASTAR, 2 frequent cutter restriction endonucleases, *Hin*fI and *Msp*I were chosen. With these enzymes 2 or 3 µl of the PCR products were digested in a 20 µl reaction mixture containing 15 U of *Hin*fI or *Msp*I enzymes (MBI Fermentase, Lituania). Following incubation at 37°C for 2 h, the digested products were electrophoresed on a 1.5% agarose gel containing 0.1% ethidium bromide. The sizes of restriction fragments were estimated graphically (using the MS Excel program) by comparing migration distance of fragments with that of known sizes of marker DNA.

RESULTS

PCR

The primers MX5 and MX3 specific for the family Myxobolidae successfully amplified approx. 1600 bp fragments of the 18S rRNA gene from every sample of the *Myxobolus* species examined. For 2 species, namely *M. bramae* and *M. macrocapsularis*, an elevated annealing temperature was needed to eliminate non-specific bands. The identity of the PCR products was confirmed by partial DNA sequencing (authors' unpubl. manuscript).

RFLP

The restriction fragment patterns of the PCR products generated by *Hin*fI or *Msp*I enzymes are presented in Fig. 1. For the unambiguous differentiation of the majority of the examined species, 2 enzymes were necessary. In the cases of *Myxobolus bramae* and *M. impressus*, however, each pattern was characteristic and easily distinguishable from the other examined species. The number and calculated size of the DNA fragments resulting from the restriction enzyme digestions are shown in Table 2. Since the resolution of the

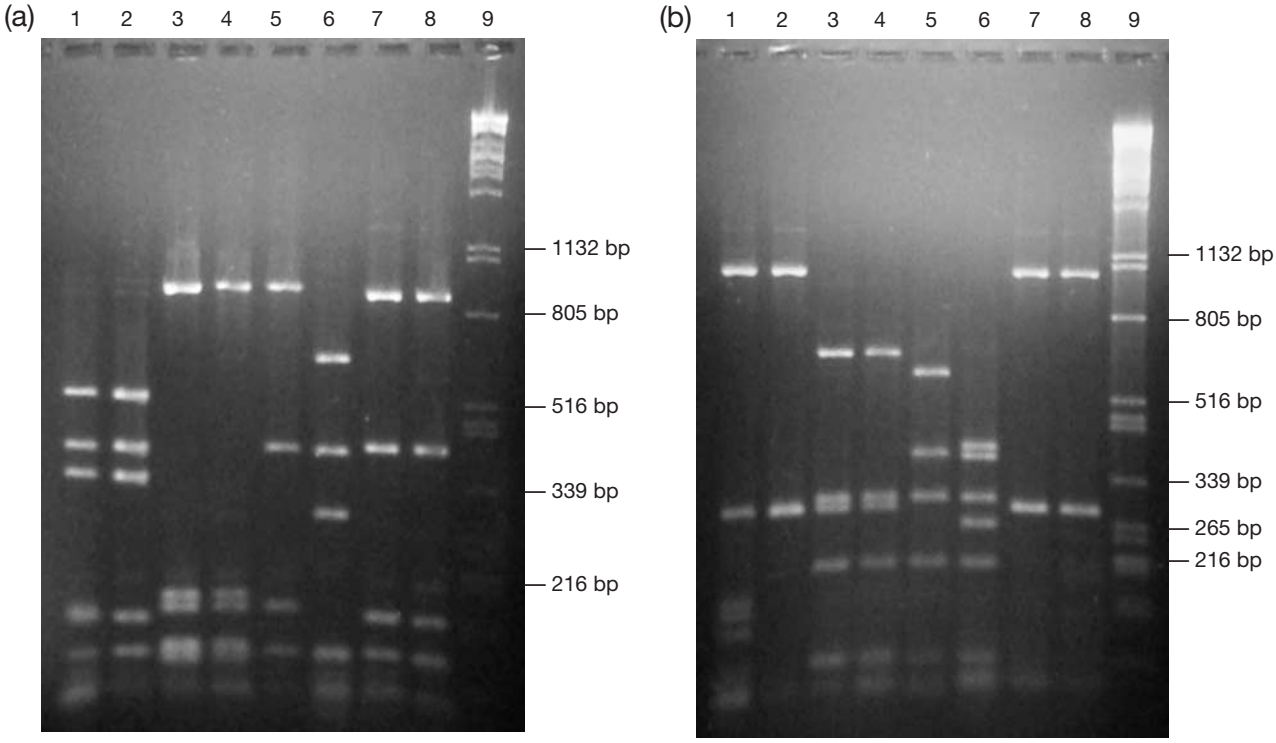

Fig. 1. RFLP patterns of the amplicons digested with (a) *Hin*fI and (b) *Msp*I enzymes. Lane 1, *Myxobolus cyprini*; lane 2, *M. ergensi*; lanes 3 and 4, *M. bramae*; lane 5, *M. macrocapsularis*; lane 6, *M. impressus*; lanes 7 and 8, *M. pseudodispar*; and lane 9, molecular weight marker

Table 2. Approximate sizes of fragments of approx. 1600 bp PCR products digested with *Hin*fI and *Msp*I restriction enzymes (in base pairs)

Sample	Species	*Hin*fI		*Msp*I	
		No. of fragments	Approximate sizes of fragments in base pairs (bp)	No. of fragments	Approximate sizes of fragments in base pairs (bp)
1	*Myxobolus cyprini*	5	555 / 420 / 365 / 175 / 130	5	1055 / 290 / 170 / 150 / 135
2	*M. ergensi*	5	555 / 420 / 365 / 175 / 130	3	1055 / 290[a]
3	*M. bramae* 1	5	945 / 195 / 185 / 145 / 130	4	675 / 315 / 290 / 215
4	*M. bramae* 2	5	945 / 195 / 185 / 145 / 130	4	675 / 315 / 290 / 215
5	*M. macrocapsularis*	4	945 / 420 / 185 / 130	4	615 / 400 / 315 / 215
6	*M. impressus*	4	665 / 420 / 310 / 130	5	410 / 390 / 315 / 275 / 215
7	*M. pseudodispar* 1	4	900 / 420 / 175 / 130	3	1055 / 290[a]
8	*M. pseudodispar* 2	4	900 / 420 / 175 / 130	3	1055 / 290[a]

[a]Double bands

gel does not allow exact detection of fragments less than 100 bp in size, the fragments listed in Table 2 do not always add up to the full size of the PCR products.

DISCUSSION

Recently, DNA sequence data from different members of the genus *Myxobolus* have become available. Most studies were done on the 18S rRNA gene. In the GenBank approximately 20 sequences originating from different *Myxobolus* species are deposited (Smothers et al. 1994, Andree et al. 1997, 1999a,b). The availability of DNA sequences allows a phylogenetic comparison of the different species and an investigation of the evolutionary relationships between them. While sequencing provides more detailed information on the differentiation of myxosporeans and can be used for phylogenetic studies, PCR-RFLP analysis represents a quick and easy method, suitable for routine diagnostic purposes, to identify *Myxobolus* species that are difficult to distinguish by morphology and tissue tropism (Molnár & Székely 1999).

In the case of the 6 species examined, the cleavage of the PCR product with the restriction enzymes *Hin*fI and *Msp*I produced patterns which were characteristic of the species. The identical restriction patterns of the 2 samples of *Myxobolus bramae* collected from the same fish stock at different times also show the reliability of this method. Moreover, 2 samples of spores originating from closely related fish species (*Rutilus rutilus* and *Scardinius erythrophthalmus*) and identified morphologically as *Myxobolus pseudodispar* also gave identical patterns, proving the conformity of morphological and molecular genetic methods.

To test the reliability of our method, an examination of additional myxosporean species is planned. For the ultimate confirmation of our results, we also intend to determine the full DNA sequence of the PCR products.

Since the preparation of the present manuscript, a paper reporting very similar work has been published. Xiao & Desser (2000) also described a combined PCR restriction analysis method (so-called riboprinting) for the comparison of 18 myxosporeans, including 7 *Myxobolus* species which were different from the species in our investigation. They have used 9 restriction enzymes and, based on the resultant patterns, a phylogenetic tree was constructed. With the exception of *M. pendula* and *M. pellicides*, the other myxosporeans examined by Xiao & Desser (2000) could be well differentiated using, among others, the *Hin*fl enzyme. In their study, the PCR primers were also specific for the 18S rRNA gene but complementary to different regions of the gene and capable of amplifying a longer product (approx. 2100 bp). Nevertheless, their results correspond with our observations, proving the feasibility of the PCR-RFLP method in the differentiation and identification of myxosporeans.

Acknowledgements. This work was supported by the National Research Fund of Hungary (OTKA, project No. T 029200). Special thanks are due to Dr Csaba Székely for his essential help, particularly in collection and identification of myxosporeans.

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Editorial responsibility: Carey Cunningham, Aberdeen, Scotland, UK

Submitted: July 11, 2000; Accepted: October 25, 2000
Proofs received from author(s): December 20, 2000