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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

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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

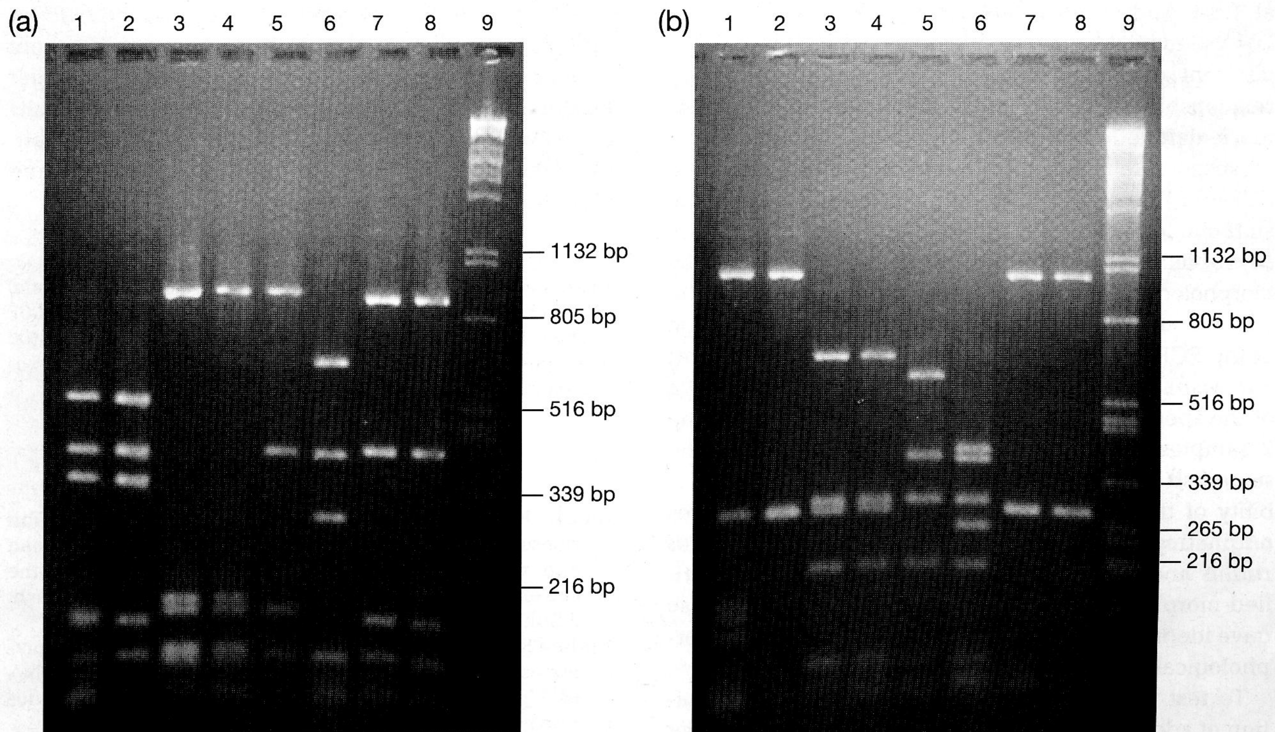

Fig. 1. RFLP patterns of the amplicons digested with (a) *Hin*fI and (b) *Msp*I enzymes. Lane 1, *Myxobolus cyprini*; lane 2, *M. ergensi*; lanes 3 and 4, *M. bramae*; lane 5, *M. macrocapsularis*; lane 6, *M. impressus*; lanes 7 and 8, *M. pseudodispar*; and lane 9, molecular weight marker

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*fI 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.

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LITERATURE CITED

Andree KB, Gresoviac SJ, Hedrick RP (1997) Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* the causative agent of whirling disease in salmonid fish. J Eukaryot Microbiol 44:208–215

Andree KB, MacConnell E, Hedrick RP (1998) A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 34:145–154

Andree KB, El-Matbouli M, Hoffman RW, Hedrick RP (1999a) Comparison of 18S and ITS-1 rDNA sequences of selected geographic isolates of *Myxobolus cerebralis*. Int J Parasitol 29(5):771–775

Andree KB, Székely Cs, Molnár K, Gresoviac SJ, Hedrick RP (1999b) Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvidae) based on small subunit ribosomal DNA sequences. J Parasitol 85(1):68–74

Bartholomew JL, Whipple MJ, Stevens DG, Fryer JL (1997) The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaete as an alternate host. J Parasitol 83:859–868

Clark C (1997) Riboprinting: a tool for the study of genetic diversity in microorganisms. J Eukaryot Microbiol 44: 277–283

El-Mansy A, Molnár K (1997a) Extrapiscine development of *Myxobolus drjagini* Akhmerov, 1954 (Myxosporea, Myxobolidae) in oligochaete alternative hosts. Acta Vet Hung 45:427–438

El-Mansy A, Molnár K (1997b) Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Dis Aquat Org 31:227–232

El-Mansy A, Molnár K, Székely C (1998) Development of *Myxobolus portucalensis* Saraiva & Molnár, 1990 (Myxosporea: Myxobolidae) in the oligochaete *Tubifex tubifex* (Müller). Syst Parasitol 41:95–103

El-Matbouli M, Hoffmann RW (1989) Experimental transmission of two *Myxobolus* spp. developing by sporogeny via tubificid worms. Parasitol Res 75:461–464

El-Matbouli M, Hoffmann RW (1993) *Myxobolus carassii* Klokacheva, 1914 also requires an aquatic oligochaete, *Tubifex tubifex* as intermediate host in its life cycle. Bull Eur Assoc Fish Pathol 13:189–192

Eszterbauer E, Székely C, Molnár K, Baska F (2000) Development of *Myxobolus bramae* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. J Fish Dis 23:19–25

Kent ML, Whitaker DJ, Margolis L (1993) Transmission of *Myxobolus arcticus* Pugachev and Khokhlov, 1979, a myxosporean parasite of Pacific salmon, via a triactinomyxon from the aquatic oligochaete *Stylodrilus heringianus* (Lumbriculidae). Can J Zool 71:1207–1211

Kent ML, Margolis L, Corliss JO (1995) The demise of a class of protists: taxonomic and nomenclatural revisions proposed for the protist phylum Myxozoa Grassé, 1970. Can J Zool 72:932–937

Lom J, Dyková I (1992) Protozoan parasites of fishes. Elsevier Science Publishers, New York

Longshaw M, Feist SW, Canning EU, Okamura B (1999) First identification of PKX in bryozoans from the United Kingdom—molecular evidence. Bull Eur Assoc Fish Pathol 19(4):146–148

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

Molnár K, Székely Cs (1999) *Myxobolus* infection of the gills of common bream (*Abramis brama* L.) in lake Balaton and in the Kis-Balaton reservoir, Hungary. Acta Vet Hung 47(4):419–432

Molnár K, El-Mansy A, Székely Cs, Baska F (1999) Development of *Myxobolus dispar* Thelohan, 1895 (Myxosporea: Myxobolidae) in an oligochaete alternate host *Tubifex tubifex* (Müller). Folia Parasitol 46:15–21

Pomport-Castillon C, Romestand B, De Jonckheere JF (1997) Identification and phylogenetic relationship of Microsporidia by riboprinting. J Eukaryot Microbiol 44:540–544

Ruidisch S, El-Matbouli M, Hoffmann RW (1991) The role of tubificid worms as an intermediate host in the life cycle of *Myxobolus pavlovskii* (Akhmerov, 1954). Parasitol Res 77: 663–667

Schlegel M, Lom J, Stechmann A, Bernhard D, Leipe D, Dykova I, Sogin ML (1996) Phylogenetic analysis of complete small subunit ribosomal RNA coding region of *Myxidium lieberkuehni*: evidence that Myxozoa are Metazoa and related to Bilateralia. Arch Protistenkd 147:1–9

Sidall ME, Martin DS, Bridge D, Desser SS, Cone DK (1995) The demise of a phylum of protists: phylogeny of Myxozoa and other parasitic Cnidaria. J Parasitol 81(6):961–967

Smothers JF, von Dohlen CD, Smith LH, Spall RD (1994) Molecular evidence that the myxozoan protists are metazoans. Science 265:1719–1721

Székely C, Molnár K, Eszterbauer E, Baska F (1999) Experimantal detection of the actinospores of *Myxobolus pseudodispar* (Myxosporea:Myxobolidae) in oligochaete alternate hosts. Dis Aquat Org 38:219–224

Wolf K, Markiw ME (1984) Biology contravenes taxonomy in the myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452

Xiao C, Desser SS (2000) Molecular characterization of myxozoan parasites from Lake Sasajewun, Algonquin Park, Ontario, by riboprinting. J Eukaryot Microbiol 47(1):85–89

Yokoyama H, Ogawa K, Wakabayashi H (1995) *Myxobolus cultus* n. sp. (Myxosporea: Myxobolidae) in the goldfish, *Carassius auratus* transformed from the actinosporean stage in the oligochaete *Branchiura sowerbyi*. J Parasitol 81(3):446–451

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Development of *Myxobolus macrocapsularis* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*

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ABSTRACT: The development of *Myxobolus macrocapsularis* Reuss, 1906, a myxosporean parasite of the gills of common bream *Abramis brama* L., was studied in experimentally infected oligochaetes. In 3 experiments uninfected *Tubifex tubifex* Müller and *Limnodrilus hoffmeisteri* (Claparéde) were exposed to mature myxospores of *M. macrocapsularis*. In all experiments, typical triactinospores developed in *T. tubifex* specimens but no infection was found in *L. hoffmeisteri*. Triactinospores were released from oligochaetes 66 to 99 d after initial exposure. At that time pansporocysts containing 8 triactinospores were located in the gut epithelium of experimental oligochaetes, but free actinosporean stages were also found in the gut lumen of the oligochaetes. Each triactinospore had 3 pyriform polar capsules and a barrel-shaped sporoplasm with 32 secondary cells. The spore body joined the 3 caudal projections with a stout style.

KEY WORDS: *Myxobolus macrocapsularis* · Myxospore · Gill parasite · *Abramis brama* · Intraoligochaete development · *Tubifex tubifex* · Triactinospore

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INTRODUCTION

Myxobolus macrocapsularis Reuss, 1906, described from the white bream *Blicca bjoerkna* as type host, seems to be one of the most commonly occurring myxosporeans on the gills of the common bream *Abramis brama* L. in Lake Balaton (Molnár & Székely 1999). Some aspects of its location and intrapiscine development were studied also by the above authors. Nothing was known, however, about its extrapiscine developmental stages.

Since the intraoligochaete developmental phase of *Myxobolus cerebralis* Hofer was first described by Wolf & Markiw (1984), several other authors have supported the theory that the development of other myxosporeans was also accomplished through oligochaete and fish alternate hosts. They (El-Matbouli & Hoffmann 1989, 1993, Ruidisch et al. 1991, Styer et al. 1991, El-Matbouli et al. 1992, Grossheider & Körting 1992, Benajiba & Marquès 1993, Kent et al. 1993, Yokoyama et al. 1993, 1995, Uspenskaya 1995, Trouillier et al. 1996, Yokoyama 1997, El-Mansy & Molnár 1997a,b, El-Mansy et al. 1998, Székely et al. 1998, Molnár et al. 1999a,b, Székely et al. 1999, Eszterbauer et al. 2000) reported that in each case various oligochaetes proved to be alternate hosts in the development of various myxosporean species. Recent studies have suggested, however, that besides oligochaetes some polychaetes (Bartholomew et al. 1997, Køie 2000) and bryozoans (Longshaw et al. 1999, Kent et al. 2000) can also serve as alternate hosts for fish myxosporeans.

The work presented in this paper forms part of continuing experimental life cycle studies conducted on the most common myxosporeans of Hungarian fish species. In the experiments reported here, the intraoligochaete development of *Myxobolus macrocapsularis* was followed in *Tubifex tubifex*.

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MATERIALS AND METHODS

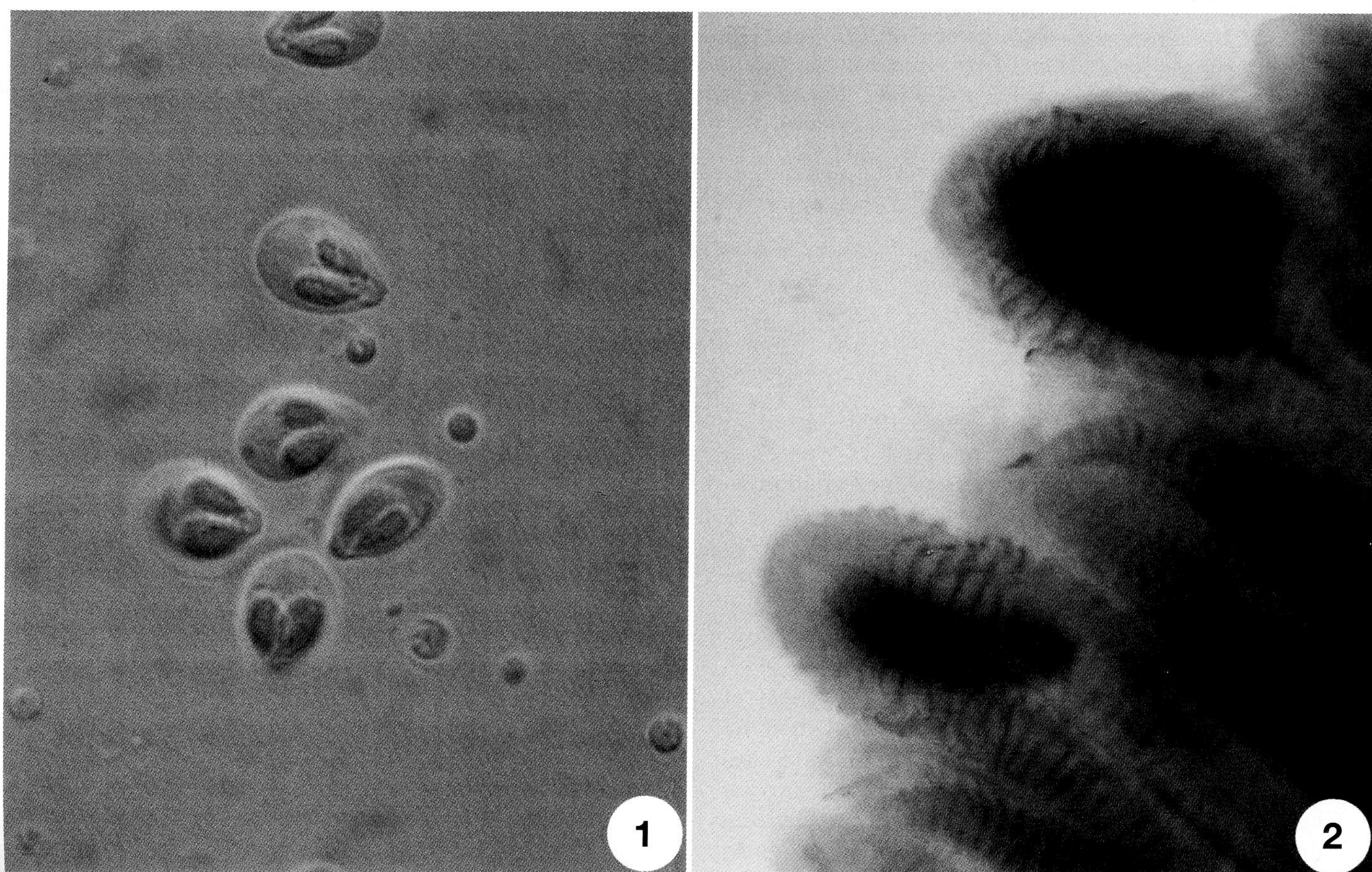
Spores of *Myxobolus macrocapsularis* (Fig. 1) were collected from mature plasmodia of the gills of 3- to 5-yr-old common bream *Abramis brama* seined in Lake Balaton and in the Kis-Balaton Water Reservoir, Hungary. Plasmodia containing about 800 000 to 1 200 000 spores were located at the tip of the gill filaments, which extended characteristically over the tip of the uninfected ones (Fig. 2). Plasmodia were removed mechanically from the gill filaments. For exposure of oligochaetes, myxospores released from plasmodia were identified under a coverslip with a compound microscope.

The method of collection and laboratory culture of oligochaetes free from actinosporean infection were the same as those described by Székely et al. (1999) and Eszterbauer et al. (2000). The oligochaete stock was composed of an about equal number of *Tubifex tubifex* and *Limnodrilus hoffmeisteri* cultured in our laboratory for 5 yr continuously.

Three experiments were performed. In Expt 1, 100 specimens of *Tubifex tubifex* and *Limnodrilus hoffmeisteri* were placed into a small plastic cup of 500 ml volume. About 100 000 spores of *Myxobolus macrocapsularis* were added at the same time so that the spore:worm ratio was 1000:1. In Expt 2 an undetermined number of spores from 3 mature *M. macrocapsularis* plasmodia was added to a plastic cup containing 100 specimens of *T. tubifex* and *L. hoffmeisteri*. In Expt 3, 100 oligochaete specimens (*T. tubifex* and *L. hoffmeisteri*) were placed into a small plastic cup of 500 ml volume. About 3 million *M. macrocapsularis* myxospores were added to oligochaetes. On Day 30 post infection an additional infection was made with 800 000 myxospores. *T. tubifex* and *L. hoffmeisteri* specimens of the original stocks served as controls for the experiments.

All dishes (containing mud in their bottom) were permanently aerated and regularly supplied with fresh water to prevent evaporation and to refresh the water for the oligochaetes. Oligochaetes were fed granulated fish food weekly.

Starting from Day 30 post infection, water from the dishes was filtered through a fine mesh of 10 µm pore size every week. The filtrates were taken up in a drop



Figs. 1 & 2. *Myxobolus macrocapsularis*. Fig. 1. *M. macrocapsularis* myxospores. Fresh preparation. ×1000. Fig. 2. Typical location of *M. macrocapsularis* plasmodia in the afferent artery at the tip of the gill filaments. Fresh preparation. ×20

of water and examined for the presence of actinospores. In the first experiment, we only studied released actinospores filtered from the water of the cup. In Expts 2 and 3, after the first release of triactinospores, all oligochaetes were placed into 2 ml tissue culture plates according to Yokoyama et al. (1991), and after 1 d of incubation they were examined for the release of actinospores under a stereomicroscope. Triactinospores released by the oligochaetes were examined under a coverslip by compound microscope. They were recorded on videotapes and pictures were digitalised by the IMAGO® program as described by Székely (1997). Photographs were taken with an Olympus DP-10 digital camera and drawings were made. Measurements of 52 triactinospores from the 3 experiments were recorded. In the description, all measurements are given in micrometres. The actinosporean stage of *Myxobolus macrocapsularis* was described using the terminology of Janiszewska (1957) as modified by Lom et al. (1997). The intensity of infection of worms releasing actinospores was checked in live conditions under a light microscope and specimens bearing high numbers of actinospores were fixed for histological and semithin sections.

The infected *Tubifex tubifex* specimens were fixed in Bouin's solution, embedded in Paraplast® (Monoject Sci) wax, cut into 4 to 8 µm thick sections, and stained with haematoxylin and eosin. For the semithin section technique, infected *T. tubifex* specimens were fixed in 5% glutaraldehyde in cacodylate buffer for 1.5 h, postfixed in 2% osmium tetroxide for 2 h, washed several times with cacodylate buffer, dehydrated and embedded in Durcupan ACM resin. Semithin sections (0.5 to 1 µm) were stained with 0.1% toluidine blue solution.

RESULTS

Light microscopy

Actinospores of *Myxobolus macrocapsularis* were released only from experimentally infected *Tubifex tubifex* specimens. No infection was found in *Limnodrilus hoffmeisteri*, nor in the control *T. tubifex* specimens. In Expts 1, 2 and 3, actinospores floating in the water of the cups and showing a typical triactinomyxon morphology (Fig. 3) were first filtered on Days 69, 85 and 66, respectively, after initial exposure to *M. macrocapsularis* myxospores. In Expt 1, actinospores were seen only in the filtered water of the cup. In Expts 2 and 3, however, the release of actinospores was taking place continuously from individually controlled oligochaete specimens placed in cell-well plates. When oligochaetes in Expt 2 were placed in cell-well plates, 58 of the surviving specimens proved to be *T. tubifex* and 33 were identified as *L. hoffmeisteri*. Actinospores in plates were released only from 6 *T. tubifex* specimens (10%). No release of actinospores from *L. hoffmeisteri* was observed. In Expt 3, when the exposure of 61 *T. tubifex* and 27 *L. hoffmeisteri* to *M. macrocapsularis* spores was studied in cell-well plates, 7 specimens of *T. tubifex* (11%) released actinospores. In the latter plates a new release of triactinospores was observed 98 d after the first introduction and 68 d after the second supplementation with myxospores. At that time 15 specimens (18%) of the remaining *T. tubifex* started to release actinospores. No release of spores was observed from *L. hoffmeisteri* in this experiment either. In each experiment typical triactinomyxon-type actinospores were obtained. The release of triactinospores lasted about 26 d in Expt 1, 14 d in Expt 2, and 9 and 16 d in Expt 3. In heavily infected live *T. tubifex* specimens placed under a coverslip, pansporocysts were seen in the gut epithelium and free spores were observed in the lumen of the worm's intestine by means of a compound microscope (Fig. 4). From crushed *T. tubifex* specimens pansporocysts were obtained, each of which contained 8 developing triactinospores.

Description of triactinospores

Triactinospores (on the basis of 52 actinospores) (Table 1, Fig. 5) released from the worms' bodies and floating in the water were characterised by 3 pyriform polar capsules, a sporoplasm, a stout style and 3 relatively short caudal processes. Polar capsules were pyriform, 5.14 µm (range 4 to 6 µm) in length and 3.8 µm (3 to 4 µm) in width (Fig. 6a). Sporoplasm was barrel shaped, 20.6 µm (16 to 24 µm) long and 12 µm (10 to 14 µm) wide, and contained 32 spherical secondary cells (Fig. 6b). The style was moderately long (60.2 µm, range 53 to 69 µm). Its width, which was equal in its

Table 1. Measurements of the live triactinospores of *Myxobolus macrocapsularis* (n = 52 actinospores)

Dimension (µm)		Mean ± SD	Min.	Max.
Polar capsules	Length	5.14 ± 0.59	4	6
	Width	3.8 ± 0.2	3	4
Sporoplasm	Length	20.57 ± 1.5	16	24
	Width	12 ± 0.86	10	14
Spore body	Length	27.15 ± 1.895	23	31
	Width	12 ± 0.86	10	14
Style	Length	60.15 ± 3.27	53	69
	Width	9.47 ± 0.5	9	10
Caudal processes	Length	116.61 ± 11.17	94	141
	Width	8.5 ± 0.623	8	10

Figs. 3 & 4. Fig. 3. Floating triactinospores of *Myxobolus macrocapsularis* released from *Tubifex tubifex*. Fresh preparation. ×350. Fig. 4. Squash preparation of a *T. tubifex* infected with pansporocysts of *M. macrocapsularis*. Each pansporocyst contains 8 triactinospores. Fresh preparation. ×500

whole length, measured 9.5 µm (9 to 10 µm). Caudal processes were slightly bent or straight, tapering towards the end and terminating in a sharp point. The length of the caudal processes was 116.6 µm (94 to 141 µm), and their width at the origin was 8.5 µm (8 to 10 µm). The length from the apical point of polar capsules to the end of sporoplasm (spore body) measured 20.6 µm (16 to 24 µm).

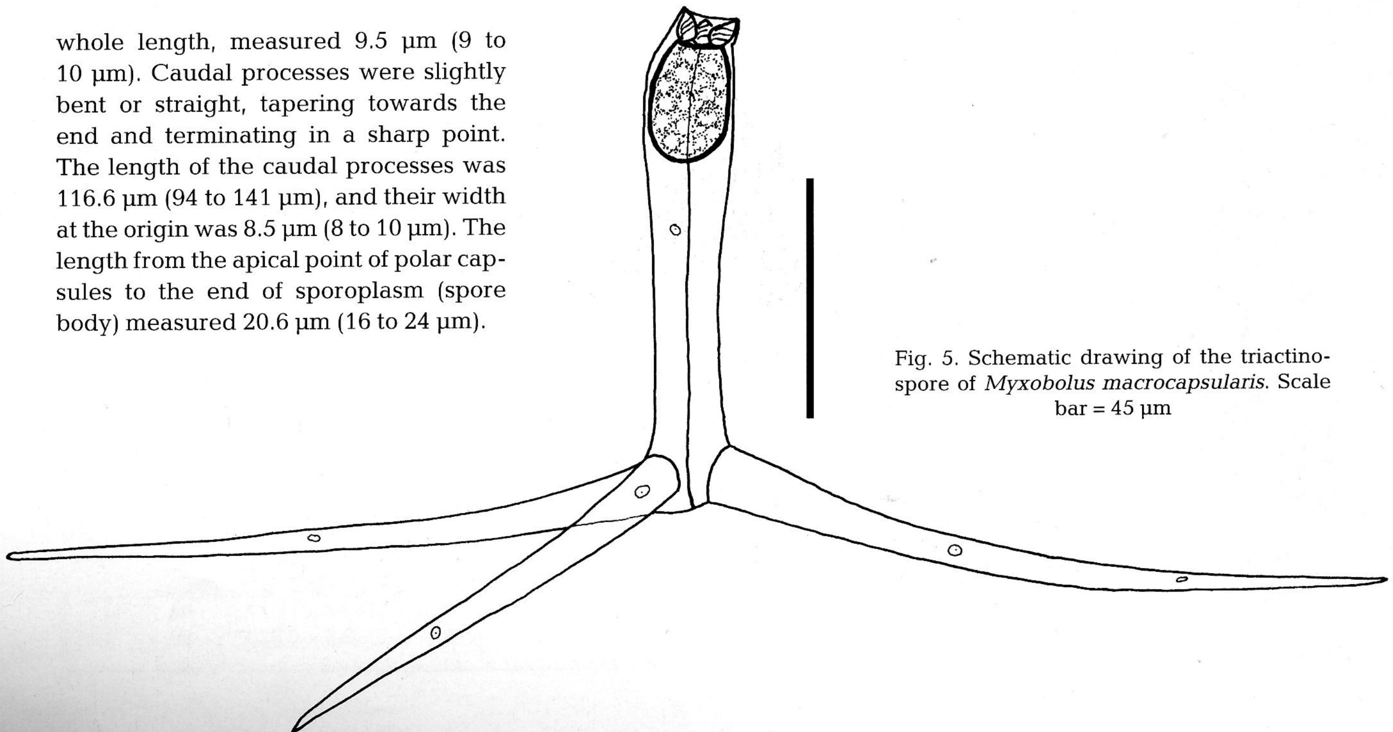


Fig. 5. Schematic drawing of the triactinospore of *Myxobolus macrocapsularis*. Scale bar = 45 µm

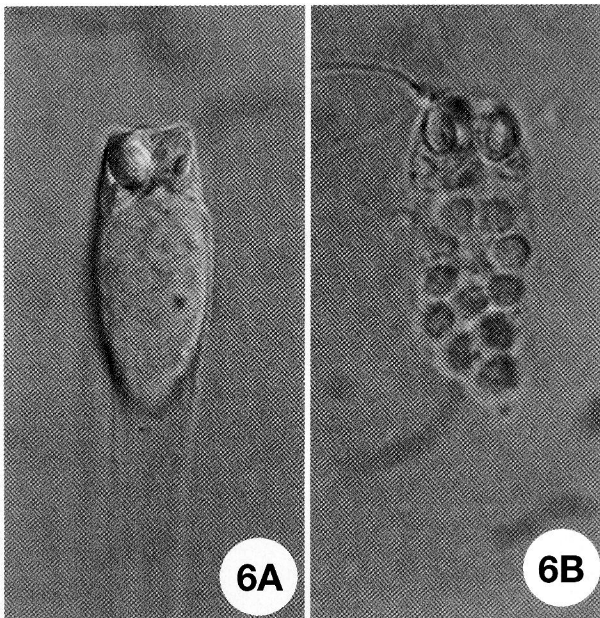


Fig. 6. Spore body of *Myxobolus macrocapsularis* triactinospores. (A) In the uncompressed triactinospore a barrel-shaped sporoplasm with less contrasted secondary cells and 2 of the 3 polar capsules at the tip are seen. (B) In a more compressed triactinospore polar capsules with released polar filaments and some of the 32 secondary cells are seen. Fresh preparation. ×1500

Differential diagnosis

The triactinospore of *Myxobolus macrocapsularis* differs from most of the known species by its small size and its barrel-shaped sporoplasm. The total length of the spore body and the style is much shorter than that of *M. arcticus*, *M. bramae*, *M. cerebralis*, *M. hungaricus*, *M. portucalensis* and *M. pseudodispar*. In their small size, triactinospores of *M. macrocapsularis* resemble those of *M. cotti* but their barrel-shaped sporoplasm differs significantly from the elongated sporoplasm of the latter species.

Histology

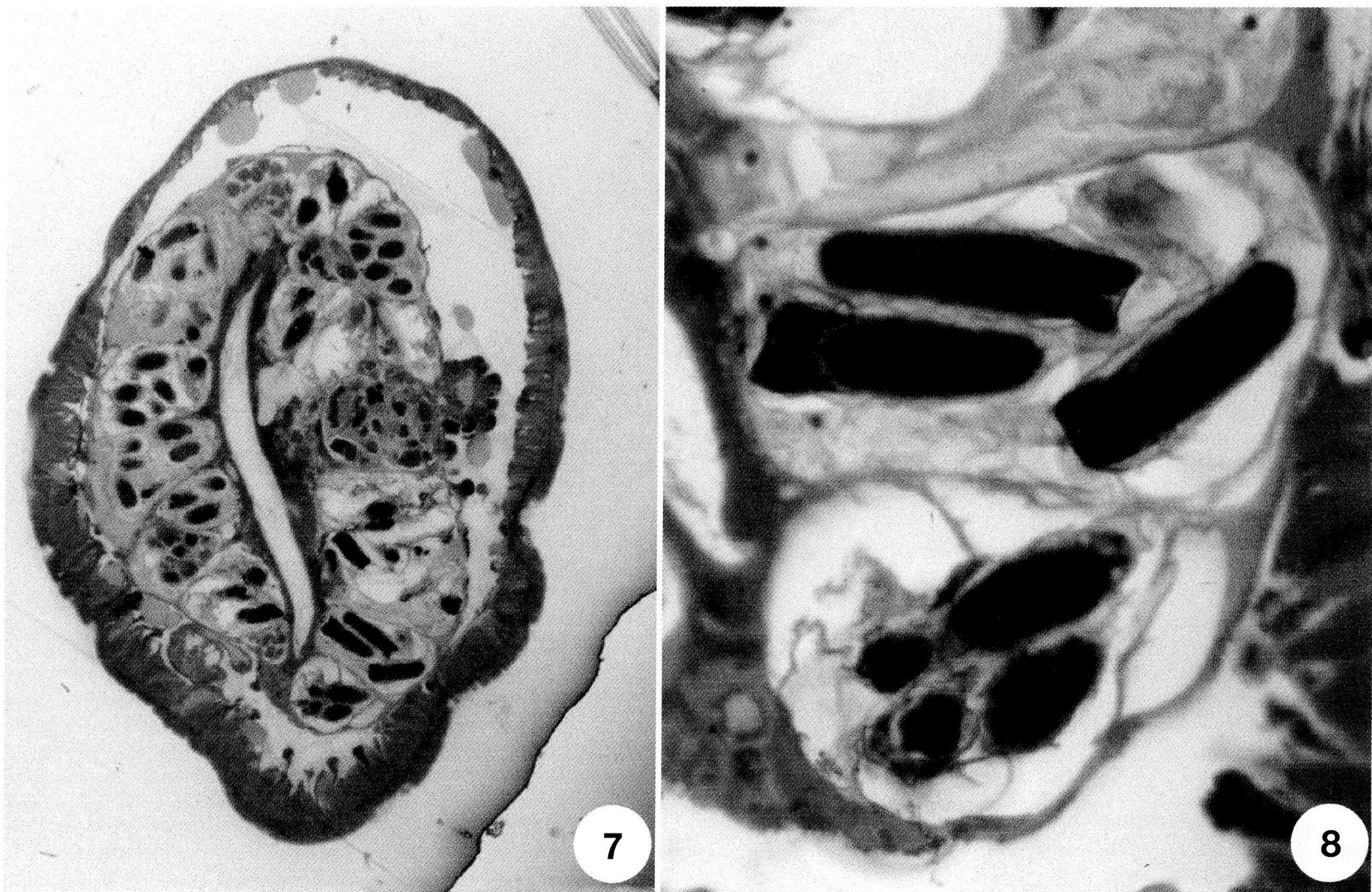
In histological sections and in semithin sections fixed in the period of actinospore release, heavy infection with mature pansporocysts was found in the gut epithelium of the worms. At that time most of the segments of the worms were infected with round or elliptical pansporocysts, each harbouring 8 triactinospores (Fig. 7). In one of the specimens fixed from Expt 3 both early developmental stages and pansporocysts with fully matured spores were found. In pansporocysts located intracellularly in epithelial cells, the polar capsules, the secondary cells of the sporoplasm and the folded caudal processes of the triactinospores were easily distinguishable (Fig. 8). Pansporocysts were segregated from the lumen of the worm's intestine by only a thin layer of the ectoplasm of the infected epithelial cells.

DISCUSSION

Myxobolus macrocapsularis is a relatively little studied parasite of the common bream. In spite of its common occurrence, little is known about its pathogenicity although the intrapiscine development and site preference on the gills were studied by Molnár & Székely (1999). Data obtained in this study show for the first time that the intraoligochaete development of this parasite follows the same pattern as that described by Wolf & Markiw (1984) for *M. cerebralis*. In our experiments the development of *M. macrocapsularis* was successfully completed in *Tubifex tubifex*, in which typical triactinomyxon-type spores developed. From the experiments, it can be concluded that, of the 2 oligochaete species used, only *T. tubifex* is a suitable alternate host for *M. macrocapsularis*, since no development took place in *Limnodrilus hoffmeisteri*.

The prevalence of infection varied in experiments and no measurable correlation between the infection rate of oligochaetes and the number of myxospores introduced into the cups could be determined. The prevalence of infection did not reach the level obtained earlier in this laboratory with *Myxobolus drjagini*, *M. portucalensis*, *M. hungaricus* and *M. dispar* (El-Mansy & Molnár 1997a,b, El-Mansy et al. 1998, Molnár et al. 1999a), but on a lower level *M. macrocapsularis* infected the oligochaetes consistently in all the experiments.

As all 3 experiments were done within a relatively narrow temperature range (room temperature), only scarce information was obtained on the temperature that is optimal for the development of the species. Nevertheless, from the data obtained we could conclude that the period of prepatent development was relatively short compared with that of other myxosporean species, and actinospores were formed in less than 3 mo. A certain synchronism was found in actinospore release, as in the majority of cases triactinospores were released within 2 wk. Expt 3 also showed that reinfection of the already challenged oligochaete stock with further myxospores was successful, and in 1 *Tubifex tubifex* specimen there was evidence of the presence of 2 different actinospore generations, indicating a possible reinfection of an already infected specimen.



Figs. 7 & 8. Fig. 7. Cross-section of an infected *Tubifex tubifex* fixed 69 d after initial exposure to *Myxobolus macrocapsularis* spores. Pansporocysts of *M. macrocapsularis* are located in the epithelial cells of the intestine. Each pansporocyst contains 8 well-developed triactinospores. The pansporocyst is segregated from the lumen of the gut only by a thin layer of the ectoplasm. Semithin section. ×300. Fig. 8. Cross-section of the gut of an infected *T. tubifex* fixed 69 d after initial exposure. In 2 pansporocysts mature triactinospores with dark-staining polar capsules and sporoplasms are seen. The space of the pansporocyst is filled by the folded caudal processes of the spores. Semithin section. ×1500

Histological data suggest that early development of the species takes place in the intestinal epithelium of *Tubifex tubifex* from where mature pansporocysts are released into the intestinal lumen. The intact body wall of oligochaetes infers that actinospores reach the water through the anal opening of the worm.

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LITERATURE CITED

Bartholomew JL, Whipple MJ, Stevens DG, Fryer JL (1997) The life cycle of *Ceratomyxa shasta* a myxosporean parasite of salmonids requires a freshwater polychaete as an alternate host. J Parasitol 83:859–868

Benajiba MH, Marquès A (1993) The alternation of actinomyxidian and myxosporidian sporal forms in the development of *Myxidium giardi* (parasite of *Anguilla anguilla*) through oligochaetes. Bull Eur Assoc Fish Pathol 13:100–103

El-Mansy A, Molnár K (1997a) Extrapiscine development of *Myxobolus drjagini* Akhmerov, 1954 (Myxosporea, Myxobolidae) in oligochaete alternative hosts. Acta Vet Hung 45:427–438

El-Mansy A, Molnár K (1997b) Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Dis Aquat Org 31:227–232

El-Mansy A, Molnár K, Székely Cs (1998) Development of *Myxobolus portucalensis* Saraiva & Molnár, 1990 (Myxosporea: Myxobolidae) in the oligochaete *Tubifex tubifex* (Müller). Syst Parasitol 41:95–103

El-Matbouli M, Hoffmann RW (1989) Experimental transmission of two *Myxobolus* spp. developing by sporogeny via tubificid worms. Parasitol Res 75:461–464

El-Matbouli M, Hoffmann RW (1993) *Myxobolus carassii* Klokacheva, 1914 also requires an aquatic oligochaete, *Tubifex*

tubifex as intermediate host in its life cycle. Bull Eur Assoc Fish Pathol 13:189–192

El-Matbouli M, Fischer-Scherl T, Hoffmann RW (1992) Transmission of *Hoferellus carassii* Achmerov, 1960 to goldfish *Carassius auratus* via an aquatic oligochaete. Bull Eur Assoc Fish Pathol 12:54–56

Eszterbauer E, Székely Cs, Molnár K, Baska F (2000) Development of *Myxobolus bramae* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. J Fish Dis 23:19–25

Grossheider G, Körting W (1992) First evidence that *Hoferellus cyprini* (Doflein, 1898) is transmitted by *Nais* sp. Bull Eur Assoc Fish Pathol 12:17–20

Janiszewska J (1957) Actinomyxidia II. New systematics, sexual cycles, description of new genera and species. Zool Pol 8:3–34

Kent ML, Whitaker DJ, Margolis L (1993) Transmission of *Myxobolus arcticus* Pugachev and Khokhlov, 1979, a myxosporean parasite of Pacific salmon, via a triactinomyxon from the aquatic oligochaete *Stylodrilus heringianus* (Lumbriculidae). Can J Zool 71:1207–1211

Kent ML, Khattra J, Hedrick RP, Devlin RH (2000) *Tetracapsula renicola* n. sp. (Myxozoa: Saccosporidae); the PKX myxozoan: the cause of proliferative kidney disease of salmonid fishes. J Parasitol 86:103–111

Køie M (2000) First record of an actinosporean (Myxozoa) in a marine polichaete Annelid. J Parasitol. 86:871–872

Lom J, McGeorge J, Feist SW, Morris D, Adams A (1997) Guidelines for the uniform characterisation of the actinosporean stages of parasites of the phylum Myxozoa. Dis Aquat Org 30:1–9

Longshaw M, Feist SW, Canning EU, Okamura B (1999) First identification of PKX in bryozoans from the United Kingdom: molecular evidence. Bull Eur Assoc Fish Pathol 19: 146–148

Molnár K, Székely Cs (1999) Survey on *Myxobolus* infection of the gills of the common bream (*Abramis brama* L.) in Lake Balaton and Kis-Balaton reservoirs. Acta Vet Hung 47:419–432

Molnár K, El-Mansy A, Székely Cs, Baska F (1999a) Development of *Myxobolus dispar* Thelohan, 1895 (Myxosporea: Myxobolidae) in an oligochaete alternate host *Tubifex tubifex* (Müller). Folia Parasitol 46:15–21

Molnár K, El-Mansy A, Székely Cs, Baska F (1999b) Development of *Sphaerospora renicola* Dykova and Lom, 1982 (Myxosporea: Sphaerosporidae) in oligochaete alternate hosts. J Fish Dis 22:1–11

Ruidisch S, El-Matbouli M, Hoffmann RW (1991) The role of tubificid worms as an intermediate host in the life cycle of *Myxobolus pavlovskii* (Akhmerov, 1954). Parasitol Res 77:663–667

Styer EL, Harrison LR, Burtle GJ (1991) Experimental production of proliferative gill disease in channel catfish exposed to a myxozoan-infected oligochaete, *Dero digitata*. J Aquat Anim Health 3:288–291

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. Bull Eur Assoc Fish Pathol 17:81–82

Székely Cs, El-Mansy A, Molnár K, Baska F (1998) Development of *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Myxosporea: Myxozoa) in oligochaete alternate hosts. Fish Pathol 33:107–114

Székely Cs, Molnár K, Eszterbauer E, Baska F (1999) Experimental detection of the actinospores of *Myxobolus pseudodispar* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. Dis Aquat Org 38:219–224

Trouillier A, El-Matbouli M, Hoffmann RW (1996) A new look at the life-cycle of *Hoferellus carassii* in the goldfish (*Carassius auratus auratus*) and its relation to 'kidney enlargement disease' (KED). Folia Parasitol 43:173–187

Uspenskaya AV (1995) Alternation of actinosporean and myxosporean phases in the life cycle of *Zschokella nova* (Myxozoa). J Eukaryot Microbiol 42:665–668

Wolf K, Markiw ME (1984) Biology contravenes taxonomy in the myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452

Yokoyama H (1997) Transmission of *Thelohanellus hovorkai* Achmerov, 1960 (Myxosporea: Myxozoa) to common carp *Cyprinus carpio* through the alternate oligochaete host. Syst Parasitol 36:79–84

Yokoyama H, Ogawa K, Wakabayashi H (1991) A new collection method of actinosporeans. A probable infective stage of myxosporeans to fishes from tubificids and experimental infection of goldfish with the actinosporean, *Raabeia* sp. Fish Pathol 26:133–138

Yokoyama H, Ogawa K, Wakabayashi H (1993) Involvement of *Branchiura sowerbyi* (Oligochaeta: Annelida) in the transmission of *Hoferellus carassii* (Myxosporea: Myxozoa), the causative agent of kidney enlargement disease (KED) of goldfish *Carassius auratus*. Fish Pathol 28: 135–139

Yokoyama H, Ogawa K, Wakabayashi H (1995) *Myxobolus cultus* n. sp. (Myxosporea: Myxobolidae) in the goldfish *Carassius auratus* transformed from the actinosporean stage in the oligochaete *Branchiura sowerbyi*. J Parasitol 81:446–451

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