Vol. 66: 233–243, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published September 23

# Life cycle studies of *Myxobolus parviformis* sp. n. (Myxozoa: Myxobolidae) from bream

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ABSTRACT: We experimentally followed the life cycle of *Myxobolus parviformis* sp. n., a myxozoan parasite from the gills of common bream *Abramis brama* L. Establishing the development of both stages (myxospore and actinospore) in 2 consecutive, full transmission trials, we were able to separate plasmodia of a common genotype from sympatric *Myxobolus* spp. occurring in naturally infected gill lamellae. Therefore, isolated gill plasmodia representing individual myxosporean 'clones' were used for subsequent infection of oligochaetes after molecular and morphological identification. The plasmodia of this species are located in median to distal regions of the primary gill filaments, forming small spherical pseudocysts. The comparatively small myxospores share some uniform characteristics: they taper posteriorly, have 2 inward inclining polar capsules that occupy half of the spores' interior space, and usually show 4 posterior sutural edge markings. The corresponding actinosporean has already been described morphologically and molecularly. The 18S rDNA sequence of the actinosporean stage was identical in all our samples, including myxospores. The triactinomyxon had a stout style, 32 sporozoites and short tapering caudal processes, and was shed by the tubificid oligochaete *Limnodrilus hoffmeisteri*. The ellipsoid sporoplasm was covered by a soft sheath which was emitted after valve shell opening upon stimulation by agitation or fish mucus. The molecular data (unique restriction fragment length polymorphism pattern and a 1586 bp 18S rDNA sequence) clearly differ from those for similar species and, together with the morphological data, justify the description of this parasite as a new species.

KEY WORDS: Myxozoa · *Myxobolus parviformis* · *Abramis brama* · Life cycle · Actinospore · Taxonomy · Sporoplasm sheath



## INTRODUCTION

The gills are the most affected target tissue for piscine infections by myxozoans. Many members of the Myxozoa have been described to specifically develop in common bream *Abramis brama* L. gill tissue (Shulman 1966), and most belong to the family Myxobolidae. The most prominent are *Myxobolus bramae* Reuss, 1906, *M. macrocapsularis* Reuss, 1906, *M. hungaricus* Jaczó, 1940, *M. impressus* Miroshnichenko, 1980, *M. exiguus* Thélohan, 1895, and *M. muelleri* Bütschli, 1882 (Lom & Dyková 1995, Molnár & Székely 1999). The life cycles of 3 of these have been fully described: *M. hungaricus* (El-Mansy & Molnár 1997), *M. bramae* (Eszterbauer et al. 2000) and *M. macrocapsularis* (Székely et al. 2002), and all involved triactinomyxon type stages from an oligochaete host. Studies demonstrated that all these species are valid taxa according to 18S rDNA sequence data, and this was also reflected by the characteristic affinities of their sporogonic stages with a specific location along the gill filaments of the fish host (Molnár 2002). Unfortunately, the development of sporogonic stages of different species in proximal locations of gill filaments can lead

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to confusion through post-infectional merging of sporogonic stages or mature plasmodia. This can be misinterpreted as intraspecific morphological variation and may weaken epidemiological data and hamper taxonomical research.

The pathology of most gill-infecting myxozoans is largely unclear, but severe effects such as increased mortality have been described from aquaculture, e.g. in infections of carp by *Myxobolus koi* Kudo, 1919 (Hoshina 1952) and for *Henneguya psorospermica* Thélohan, 1895, impairing branchial oxygen uptake in perch *Perca fluviatilis* (Lom & Dyková 1995). Thus, a negative impact on respiratory function, which causes stress, can generally be assumed.

In this study, we present a myxozoan parasite, *Myxobolus parviformis* sp. n., infecting the gills of bream in Germany. Its 18S rDNA sequence has been previously detected in a myxospore isolate from bream gills in Hungary, although the myxospore's morphology has not been analysed (Eszterbauer unpubl. data).

The detection of myxozoan species in host tissue isolates has been greatly improved by PCR techniques specifically amplifying parasite 18S rDNA genes (Andree et al. 1999), enabling differentiation between very similar parasites, including gill-infecting species, by (e.g.) restriction fragment length polymorphism, RFLP (riboprinting analysis: Xiao & Desser 2000; PCR-RFLP: Eszterbauer et al. 2001, 2002). The present study describes a possible modus operandi for connecting experimental and molecular techniques in life cycle studies with *a priori* contaminated hosts from the field.

## MATERIALS AND METHODS

**Parasites and hosts.** Naturally infected adult common bream were caught by line in the river Aisch near Höchstadt, Bavaria, Germany. They harboured at least 3 *Myxobolus* morphotypes in their gills, but these morphotypes were not further determined. Large fused cysts (up to 7 mm) from gill lamellae were excised and ruptured. The isolated myxospores were kept in 5 ml copper-free well water that was exchanged every 2 d. We collected 2 yr old specimens (7 to 10 cm) of common bream for infection from a carp-rearing pond, and kept them at 18°C in 50 l plastic tanks with no flow-through system; 4 of these were dissected before the experiment began to check for possible enzootic gill infections by myxozoans; all were negative.

Mixed tubificid oligochaetes (obtained from a pet shop) were kept in aerated 4 l water tanks with a bottom substrate of washed, coarse grained sand at 18°C, and constantly monitored for myxozoan infections for >1 yr. A mixture of minced horse dung, frozen *Artemia* sp., spray-dried *Spirulina* spp. and frozen lettuce was provided weekly as food. To obtain naïve oligochaetes, offspring from these cultures were collected by manually sorting out cocoons and freshly hatched oligochaetes, which were then raised in a separate container (500 ml) with sterile sand as bottom substrate.

Triactinomyxon spores (TAMs) were filtered from the containers through 20 µm nylon mesh or obtained from single oligochaetes isolated in cell well plates. To harvest myxospores, infected fish were anaesthetised by a sharp blow on the cranium and killed by spinal severance. The gill arches and lamellae of 5 specimens were homogenised after the addition of small amounts of tap water. Myxospores were obtained from tissue by homogenisation for 2 to 5 min at 11 500 rpm using an Ultra Turrax (IKA Labortechnik). Tissue remnants were then removed by passing the homogenate through 100 µm mesh filters and the suspension was allowed to settle at 4°C. Alternatively, single cysts for individual screening were thoroughly dissected from gill lamellae under a stereomicroscope using sterile needles. They were transferred to Eppendorf tubes with 30 µl of tap water and could be stored for several days at 6°C.

Crude bream mucus homogenate was prepared from wild bream from the same collection site. Fish were killed and mucus was scraped off immediately with a blunt knife while rubbing small amounts of deionised water onto the fish surface. The collected mucus was homogenised for 20 min on ice at 13 500 rpm using an Ultra Turrax (IKA Labortechnik), and dissolved for 10 min by an ultrasonic processor (VP 50H, Dr. Hielscher GmbH; amplitude 80 %, cycle 0.8). Insoluble components were removed by centrifugation at 2200 × *g* and 4°C for 10 min. The supernatant served as a chemical stimulant for sheath release.

**Experimental infections.** Myxospores obtained from naturally infected bream gills were used for initial oligochaete infection. The culture was divided into 2 equal parts (~2500 oligochaetes each) one of which was exposed to a pool of myxospores (>4 million), while the other served as control. The water of all oligochaete cultures was not exchanged for 3 wk after exposure. Actinospore producing oligochaetes were sorted by the cell well method (Yokoyama et al. 1991) based on actinospore morphological features (proportions, number of sporozoites). Triactinomyxon spores with the herein specified morphological features were used for infection of 5 young bream (pure fish infection). To obtain higher infection intensity for further parasite cultivation (bulk infection), 10 bream specimens were exposed by repeated addition of an actinospore suspension from whole culture filtrates containing >3000 TAMs to the aquaria every third day for 3 wk (cohabitation), and a further 10 fish were exposed by individual incubation for 3 h in 3 l filtrate containing 1500 to 2000 actinospores; 10 fish served as controls.

For the second trial, myxospores from selected cysts of the first trial were screened for the morphological features examined in this study. Matching isolates were partially used for PCR-RFLP detection and compared to the actinosporean counterpart. Their remnants were pooled and used for experimental infection of a naïve oligochaete culture ('pure worm infection') at an estimated dose of 1000 spores per oligochaete. For bulk infection of further oligochaetes (derived from the former control group), 1 culture (~2000 oligochaetes) was inoculated with total gill homogenate. In this trial, only actinospores from the 'pure worm infection' were used for further infection of 3 bream specimens (mode of infection was cohabitation). The basic steps for the isolation of a myxozoan species from sympatric species as performed in this study are shown in Fig. 1.

**DNA extraction and PCR.** From all stages that were used for infection, PCR samples from filtrates or tissue homogenates were prepared by centrifugation (10 000 × *g*, 15 min) and mixing of the pellet with ethanol (>70% vol). A myxospore sample was prepared by pooling 4 individually excised cysts from the first trial bream, which revealed mature myxospores with identical *Myxobolus parviformis* morphometrics. For amplification, samples were centrifuged at 5000 × *g* for 5 min. Spore pellets were suspended in 500 µl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, and 0.4 mg $ml^{-1}$ Proteinase K) and incubated at 55°C for 3 to 4 h. DNA was then purified using the Miniprep Express Matrix (BIO 101) as described by Eszterbauer (2004).

A nested PCR system was used for amplification. DNA was amplified with the 18e-18g′ universal primer pair (Hillis & Dixon 1991, modified by Andree et al. 1999). This was followed by a second round PCR with the MX5-MX3 primer pair (Andree et al. 1999). In both steps of nested PCR, the total volume of the PCR reactions was 50 ml, which contained approximately 10 to 50 ng DNA, 1× *Taq* PCR reaction buffer (MBI Fermentas), 1.25 mM $MgCl_2$, 0.2 mM dNTP mix (Sigma), 50 pmol of each primer and 2 units of *Taq* DNA polymerase (MBI Fermentas). MJ Research PTC-200 and

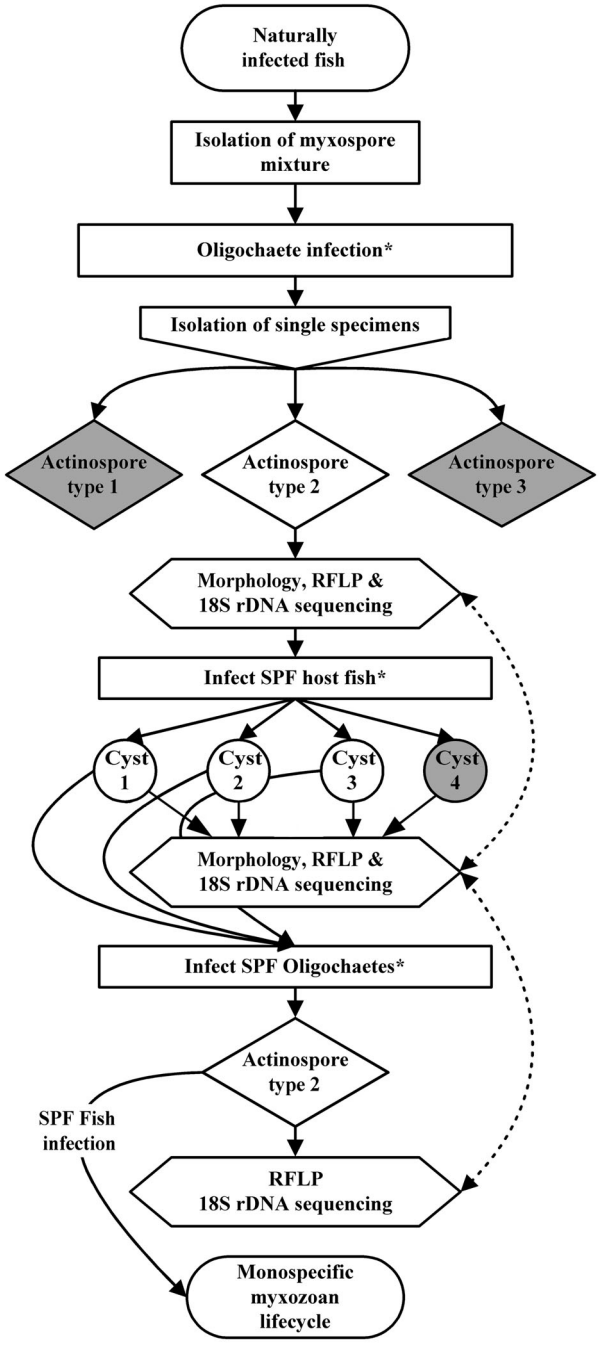


Fig. 1. Procedure for isolation of contamination-free myxozoan lifecycle from naturally infected fish harbouring sympatric species of myxozoans. Myxospore isolates collected from host tissue are added to a mixture of long-term monitored oligochaetes. Actinospores produced are obtained from single infected oligochaetes and classified by morphology and PCR-RFLP/18S rDNA sequencing. Infection of specific pathogen-free (SPF) host fish specimens is done with actinospores of desired morphotype/genotype (Actinospore Type 2 in this example). Myxosporean cysts from these fish are carefully isolated and individual morphology and PCR-RFLP pattern/18S rDNA sequence are recorded. If the data are consistent with those obtained from the actinospore morphotype, contents of matching cysts are pooled and used for infection of an SPF oligochaete culture yielding a monospecific myxozoan infection. RFLP of 18S rDNA with multiple restriction enzymes is often sufficient to monitor possible contaminations with sympatric species. Together with morphological congruity, repeated passage and comparison of 18S rDNA sequences of parasite stages at each step provide adequate and reproducible results to establish laboratory cycle. Parallel to these basic steps, infection of oligochaetes and fishes using mixed parasite isolates from initial transmission trials can be performed to increase total yield of parasite stages. These mixed infections are then purified stepwise. Dotted lines: comparisons; shading: contaminative myxozoan stages not used for further infection; *: similarly sized aliquot retained as control

Biometra T1 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, 50°C for 30 s and 72°C for 60 s for 35 cycles, and terminating with an extension at 72°C for 7 min. The PCR products were electrophoresed in 1.0% agarose gels (Sigma) in TBE (Tris-borate-EDTA) buffer and then purified with Geneclean III Kit (BIO 101).

**PCR-RFLP.** We applied 3 frequent-cutter restriction endonucleases, *Hin*fI, *Msp*I and *Taq*I, as described previously by Eszterbauer et al. (2001), and 1 µl of the PCR products was digested in a 20 µl reaction mixture containing 10 U of enzymes (MBI Fermentas). Following 2 h incubation at 37°C with *Hin*fI and *Msp*I, or at 65°C in the case of *Taq*I, the digested products were electrophoresed on a 1.5% agarose gel.

**Cloning and sequencing.** Purified PCR fragments of a pooled TAM sample from the first infection trial were cloned into pGEM-T Vector System I (Promega) following the manufacturer's manual. Positive clones were selected using the blue-white colour screening method. We grew 7 individual positive clones in LB containing 100 µg $ml^{-1}$ ampicillin. Inserts were further confirmed by digestion with *Msp*I restriction enzyme. Positive clones were sequenced with the universal forward primer pUC/M13 (Promega). The PCR products and positive clones were then sequenced in both directions with primers listed in Table 1 (except universal primers 18e and 18g′), using the ABI BigDye Terminator Version 3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). For sequence assembling, the STADEN Sequence Analysis Package, Version 2001.0 (Staden 1996) was used.

**Identification of oligochaete hosts.** We fixed 3 infected tubificid host specimens in Bouin's fluid; 2 other worms were preserved directly in 80% ethanol. The posterior end of 1 ethanol-preserved individual was set aside for DNA extraction (see below), while the remainder of the material, including the anterior end of the partitioned worm, was mounted on microscope slides and examined under a light microscope.

DNA was then extracted from the selected worm, using the DNAeasy Tissue Kit (Qiagen). A fragment of the mitochondrial 16S rDNA gene, about 520 bp long, was amplified with the universal primers 16S ar-L and 16S br-H (Palumbi et al. 1991), and the Ready-To-Go PCR beads (Amersham Pharmacia Biotech) as 25 µl reactions, using a DNA Thermal Cycler 480 (Perkin Elmer). The amplification profile was 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 90 s, with an initial single denaturing step at 95°C for 5 min, and a final single extension step at 72°C for 8 min.

The PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were then done with ABI PRISM BigDye Terminator Version 1.1 Cycle Sequencing Kits (Applied Biosystems) on a GeneAmp PCR System 9700 (PE Applied Biosystems), and sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Both strands were sequenced and then assembled to a complete sequence using the Staden Package (Staden et al. 1998). The sequence obtained (GenBank Accession No. AY836151) was aligned with those of 14 other representatives of the subfamily Tubificinae (covering 7 different genera), using Clustal X, Version 1.8 (Thompson et al. 1997), with default settings; the other worms were previously sequenced by C. Erséus (unpubl. data).

## RESULTS

### Description

***Myxobolus parviformis* sp. n. (Figs. 2 to 5)**

Triactinomyxon Type 1; Hallett et al. (2005).

**Type material:** Syntype myxospores mounted in glycerine-gelatine (and a photo series) deposited in the protozoan collection of the Zoological Department, Hungarian Natural History Museum. Coll. No. HNHM-69902/1-2.

**Actinospores:** Detailed measurements in Table 2. Triactinomyxon-type spores (Fig. 2; 111.0 to 142.5 µm total length; mean 127 ± 6 SD µm, n = 31) with stout styles (100.3 µm long, 10.3 µm wide) and sharply pointed processes with acuminate, often tapering tips (101.7 µm long); 3 pyriform polar capsules (7.3 µm long) with 5 filament coils (n = 6) and a dense, vacuole-like inclusion. Valve cell nuclei always located in median or caudal process positions, not at process bases or along style. Sporozoite count generally 32 (>80%, n = 10 from different oligochaete hosts), sometimes less,

Table 1. Primers used for PCR and/or sequencing of parasites

| Primer | Sequence | Source |
|---|---|---|
| 18e | 5′-CTG GTT GAT TCT GCC AGT-3′ | Hillis & Dixon (1991) |
| 18g′ | 5′-CGG TAC TAG CGA CGG GCG GTG TG-3′ | Hillis & Dixon (1991) |
| MX5 | 5′- CTG CGG ACG GCT CAG TAA ATC AGT-3′ | Andree et al. (1999) |
| MX3 | 5′- CCA GGA CAT CTT AGG GCA TCA CAG A-3′ | Andree et al. (1999) |
| MB5r | 5′-ACC GCT CCT GTT AAT CAT CAC C-3′ | Eszterbauer (2004) |
| MB3f | 5′-GAT GAT TAA CAG GAG CGG TTG G-3′ | Eszterbauer (2004) |
| MC5 | 5′-CCT GAG AAA CGG CTA CCA CAT CCA-3′ | Molnár et al. (2002) |
| MC3 | 5′-GAT TAG CCT GAC AGA TCA CTC CAC GA-3′ | Molnár et al. (2002) |

Table 2. *Myxobolus parviformis* sp. n. Mean measurements (µm) of triactinomyxon spores (n = 31 individuals) from various oligochaete hosts

| Measurements | Mean ± SD | Min. | Max. |
|---|---|---|---|
| Total length | 127.0 ± 6 | 111.0 | 142.5 |
| Polar capsules | | | |
| Length | 7.3 ± 0.9 | 5.8 | 9.5 |
| Width | 3.89 ± 0.4 | 3.7 | 4.7 |
| Sporoplasm | | | |
| Length | 26.7 ± 3.1 | 20.0 | 34.2 |
| Width | 11.8 ± 1.4 | 9.5 | 14.7 |
| Spore body | | | |
| Length | 33.9 ± 3.1 | 28.9 | 42.1 |
| Width | 11.8 ± 1.4 | 9.5 | 14.7 |
| Style | | | |
| Length | 100.3 ± 6.3 | 84.7 | 112.6 |
| Width | 10.3 ± 1.0 | 6.3 | 12.1 |
| Caudal processes | | | |
| Length | 101.7 ± 6.8 | 86.2 | 118.3 |
| Width | 9.3 ± 1.1 | 6.3 | 12.1 |

embedded in an ellipsoid to barrel-shaped sporoplasm, visible in uncompressed fresh mounts (Fig. 3A, inset). Sporoplasm (26.7 µm long, 11.8 µm wide), enclosed in secondary detachable sheath (Fig. 3B).

**Myxospores:** Detailed measurements in Table 3. Development in subspherical plasmodia (<300 µm), presumably of vascular type (Fig. 3C), in regions of secondary gill lamellae. Lens-like spores (11.2 µm long, 9.6 µm wide) with 2 valves, biconvex in sutural view, reverse ovoid proportions in valvular view, tapering posteriorly (Fig. 3D). Around posterior sutural ridge usually 4 (maximum 6) sutural edge markings; 2 pyriform polar capsules of equal size (5.1 µm long), slight inward inclination of about 25°, 5 filament coils (n = 8; Fig. 4), occupy about half the interior space. Triangular intercapsular process present between capsules. No mucus coat, surface ridges or iodinophilous vacuole observed.

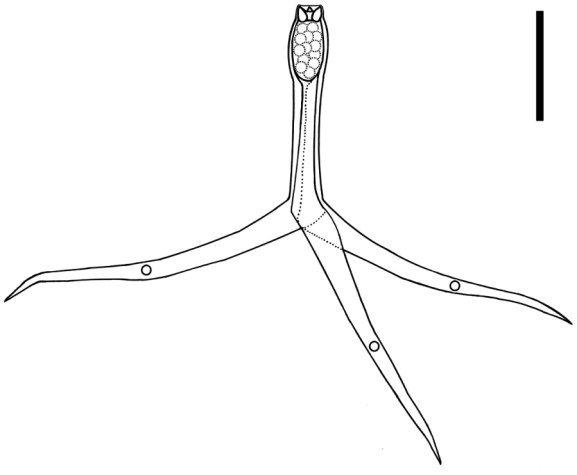

Fig. 2. *Myxobolus parviformis* sp. n. Triactinomyxon spore. Scale bar = 50 µm

Table 3. Mean measurements (µm) of *Myxobolus parviformis* sp. n. myxospores (n = 33) from *Abramis brama* gill lamellae

| Measurements | Mean ± SD | Min. | Max. |
|---|---|---|---|
| Total length | 11.2 ± 0.5 | 9.9 | 12.1 |
| Polar capsules | | | |
| Length | 5.1 ± 0.2 | 4.6 | 5.8 |
| Width | 3.3 ± 0.3 | 2.7 | 3.8 |
| Spore body | | | |
| Length | 5.1 ± 0.6 | 4.2 | 6.7 |
| Width | 9.6 ± 0.3 | 8.7 | 10.4 |
| Thickness (n = 20) | 7.2 ± 0.7 | 6.1 | 8.5 |

**Geographic source:** River Aisch near Erlangen, Bavaria, Germany.

**Vertebrate host (type host):** *Abramis brama* L.

**Invertebrate hosts:** *Limnodrilus hoffmeisteri* Claparéde, 1862, and most probably also *Tubifex tubifex* Müller, 1774.

**Site of infection:** Myxospore stages in respiratory plates (lamellae) of gills, in upper region of gill filaments up to the tip, forming spherical pseudocysts (plasmodia). Actinospore stages formed in the gut epithelium.

**Differential diagnosis:** The species differs from hitherto known myxosporeans found in bream gills in its myxospore morphology. It most closely resembles the myxospores of *Myxobolus exiguus*, *M. encephalicus* Landsberg & Lom, 1991, and *M. muelleri*. However, the 18S rDNA sequences of all these other species differ considerably from that of the present species. Furthermore, *M. exiguus* and *M. muelleri* also infect other tissues and hosts. The actinospores of our species are very similar to those of *M. macrocapsularis* (Székely et al. 2002), which also develop in bream gills but then form large clubbed plasmodia at the filament tips. Finally, the myxospores of *M. macrocapsularis* differ in size, shape and number of sutural markings.

**Etymology:** The epithet 'parviformis' refers to the comparatively small size of all life cycle stages that were produced in our experimental infections. This includes gill plasmodia, triactinomyxon spores and myxospores.

### Sporoplasm sheath

The sporoplasm sheath is a soft, pouch-like structure that carries the polar capsules apically. It is expelled after spore valves are opened along deep (up to half of the style length), hyphenation ridges emanating from the sutural lines of the valves. After 5 s of vortexing a spore suspension, the opening of the actinosporean valves (Fig. 5A) could be observed in several speci-

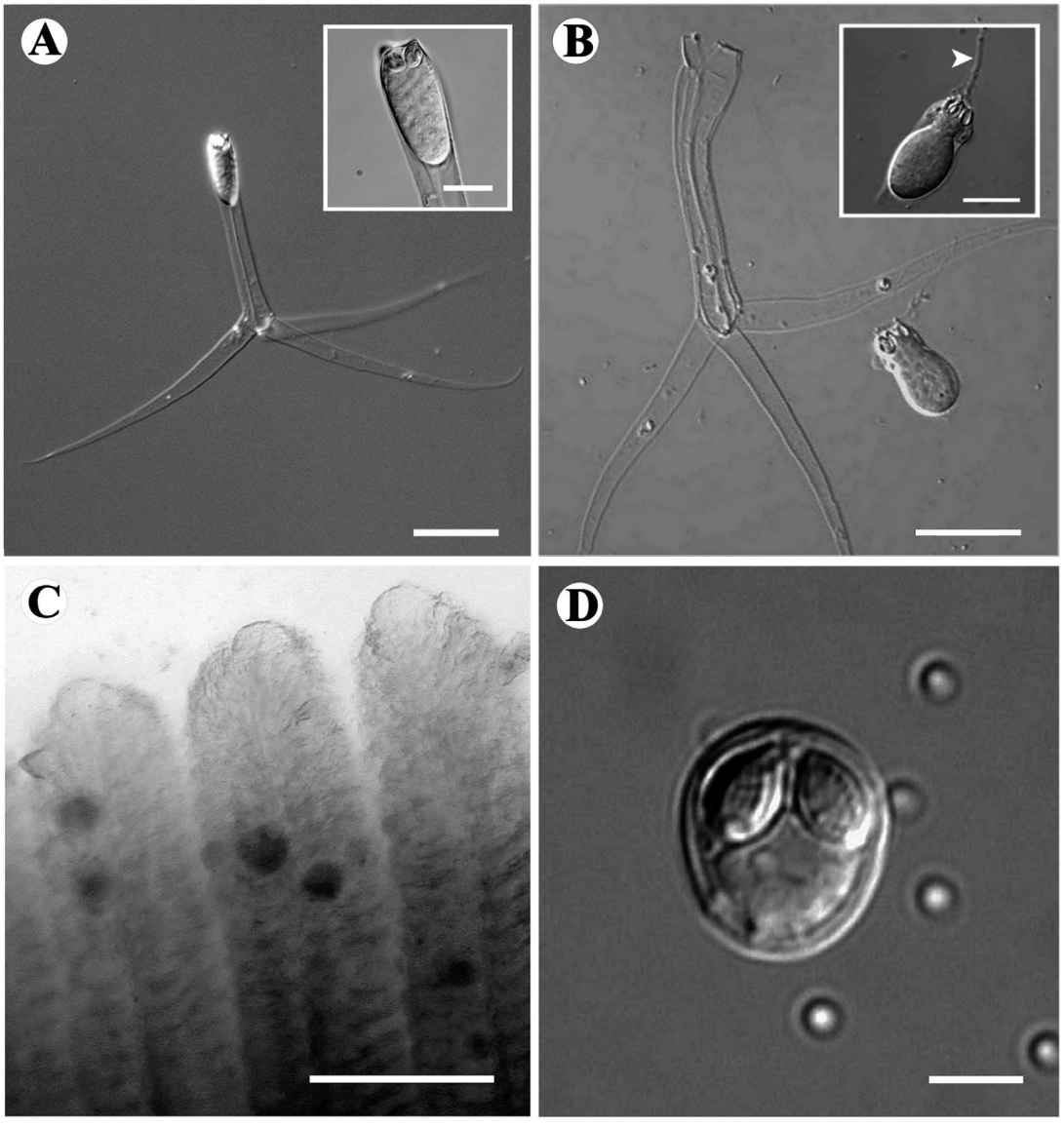


Fig. 3. *Myxobolus parviformis* sp. n. (A) Uncompressed fresh mount of triactinomyxon spore; note location of valve cell nuclei in caudal process regions, scale bar = 40 µm; inset: apical region of style, scale bar = 10 µm. (B) Apically opened triactinomyxon with released sheath unit, scale bar = 30 µm; inset: uncompressed fresh mount of intact sheath unit containing sporoplasm, polar filaments are extruded (arrowhead), scale bar = 10 µm. (C) Mature plasmodia (pseudocysts) in *Abramis brama* gill filaments, scale bar = 500 µm. (D) Myxospore from dissected pseudocyst from gill tissue of experimentally infected *A. brama*, scale bar = 5 µm

mens, which released the sheath unit (Fig. 5B). This reaction plus the emergence of the amoeboid germ could also be generated by incubation of the spores in homogenised bream skin mucus. The sheath unit bore the attached polar capsules and was immobile; the sporoplasm germ had to emerge from the detachable sheath to become freely motile (Fig. 5C). The emerging mass could be identified as the amoeboid primary cell by fluorescein-diacetate staining (according to Markiw 1992) of its demarcating membrane (data not shown).

**Infection experiments**

Oligochaetes produced actinospores (~300 000 per week) 136 d after the first exposure (post-exposure, p.e.) in the first trial. The worms produced 2 actinosporean types in the initial trial, but *Myxobolus parviformis* TAMs could clearly be distinguished by their diminutive size, and constituted over 80 % in TAM number throughout actinospore production. The control worms did not produce any actinosporeans. Bream specimens infected with whole filtrate actinospore suspensions initially car-

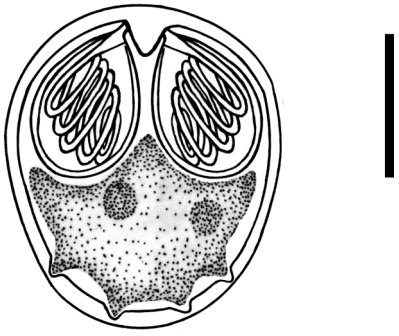

Fig. 4. *Myxobolus parviformis* sp. n. Myxospore. Scale bar = 5 µm

ried pseudocysts containing mature myxospores in their secondary gill lamellae 147 d p.e. The pseudocysts were not visible to the naked eye, being <300 µm. Specimens that were infected by cohabitation yielded a higher infection intensity (mean 3.2 identifiable cysts per primary gill filament, n = 10) than individually infected speciemens (mean 0.54 identifiable cysts per primary gill filament, n = 9).

In the second trial, actinospore production by naïve parasite-free oligochaetes began after 114 d, and lasted over 3 mo at 18°C; it could be prolonged in selected host specimens by keeping the oligochaetes at 4 to 6°C for over 300 d. Bream that were exposed to actinospores from 'pure infections' were positive after 67 d, and developed only *Myxobolus parviformis* myxospores in numerous pseudocysts; no other spore type was found in gill homogenates.

### Oligochaete host identification

Although none of the tubificids was sexually mature, they matched the morphological characteristics of either young *Tubifex tubifex* Müller, 1774 (3 specimens) or young *Limnodrilus hoffmeisteri* Claparede, 1862 (2 specimens, including the partitioned specimen: last subsection of 'Materials and methods'). The single oligochaete host sequence obtained was most similar to that of a positively identified *L. hoffmeisteri* specimen (with a 10.4% sequence divergence), and second-most similar to that of an *L. udekemianus* (with a 14.4% sequence divergence). There are great differences in the 16S sequences of ubiquitous, cosmopolitan tubificid taxa; e.g. an average divergence of 12.2% was noted between various *Limnodrilus* spp. (Beauchamp et al. 2001). Therefore, it is likely that our sequenced host worm was indeed *L. hoffmeisteri*.

### Parasite identification

Sequence data of the myxospore samples obtained from the first trial were compared with actinospores shed by various isolated worms and a pool of actinospores from 'pure infection' (second trial) culture filtrates. The universal 18e-18g' primers and the specific primer pair MX5-MX3 successfully amplified approximately 1900 and 1600 bp fragments of the 18S rDNA from every sample examined, respectively. In the case of the myxospore sample, PCR with MX5-MX3 produced a weak unspecific fragment as well. For sequencing, the DNA fragment of the expected size was isolated from the agarose gel and purified with Geneclean III Kit as previously mentioned (subsection 'PCR-RFLP'). The unspecific band, about 1400 bp in size, was also isolated, purified and the 5'-end of the fragment was sequenced directly. With BLAST search, this DNA fragment determined as virtually identical to the 18S rDNA of a planktonic alga, *Spumella elongata* (Chrysophyceae) (AJ236859); only a 1 nucleotide difference was observed in a 690 bp long overlapping DNA sequence.

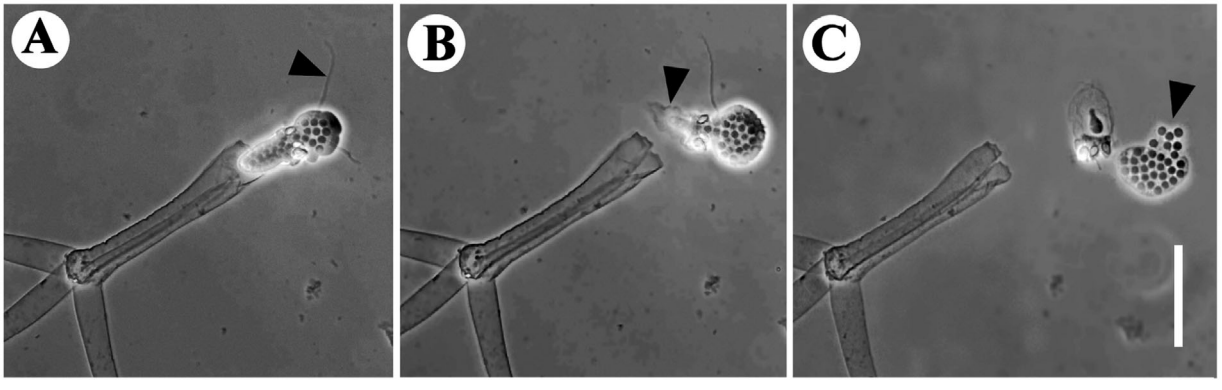


Fig. 5. *Myxobolus parviformis* sp. n. Wet mount of triactinomyxon spore, showing emergence of sporoplasm after spore valve opening and sheath unit emission upon incubation in crude homogenised bream skin mucus. (A) Inner sheath opening after polar filament discharge (arrowhead). (B) Sporoplasm release from inner sheath (arrowhead). (C) Empty inner sheath bearing the 3 polar capsules; note sporozoites (arrowhead) emerging from primary cell. Scale bar = 50 µm

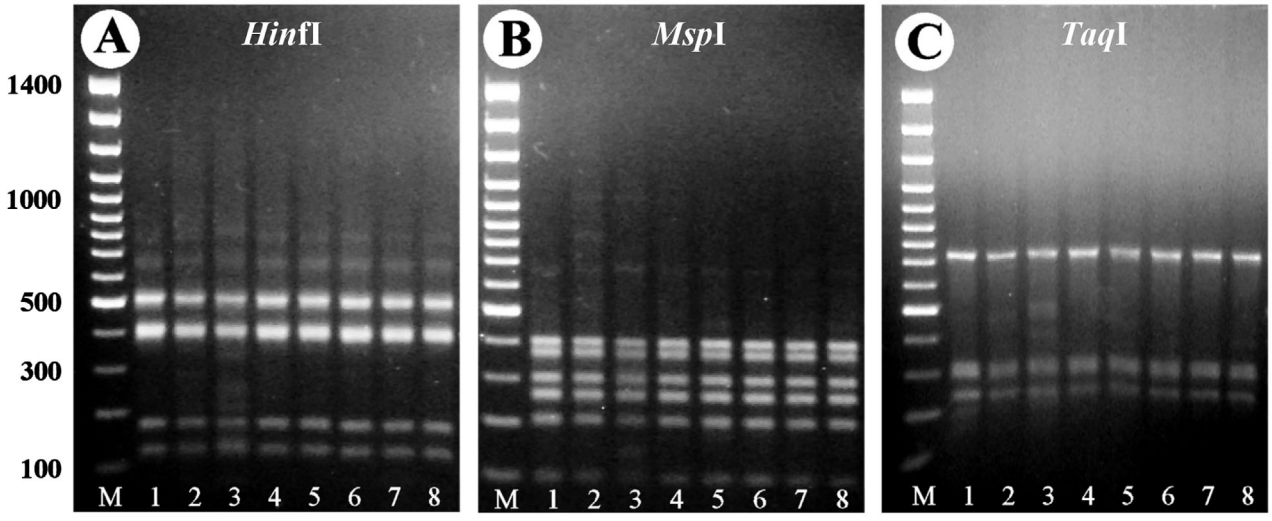


Fig. 6. *Myxobolus parviformis* sp. n. RFLP patterns of 18S rDNA PCR products digested with (A) *Hin*fI, (B) *Msp*I and (C) *Taq*I enzymes. Lane M: 100 bp DNA ladder; Lane 1: triactinomyxon spores (TAMs from first infection cycle (pooled filtrate); Lane 2: TAMs from first infection cycle (from isolated positive *Tubifex tubifex*); Lane 3: collected cysts from bream gills; Lanes 4 to 8: TAMs from second infection cycle (from single, isolated, infected *T. tubifex*)

The restriction fragment patterns of all samples examined were identical using the 3 restriction enzymes (Fig. 6). Assembled sequences of the myxosporean and triactinomyxon (TAM) developmental stages of *Myxobolus parviformis* were also 100% identical. A 1586 bp DNA sequence from the myxosporean stage was deposited in the GenBank under Accession No. AY836151. We found that 2 sequences of cloned fragments of TAM samples from the first infection trial were 100% identical with pooled actinosporean samples obtained from selected worms from the second trial and the myxospore sample, while 1 to 3 nucleotide differences (0.06 to 0.19%) at different positions were observed among sequences of the other 4 clones. Using a BLAST search, 1 of the 7 clones was determined as the partial 18S rDNA of a planktonic alga belonging to the genus *Chrysosaccus* (Chrysophyceae).

In comparisons with sequences of other myxozoans available in GenBank, that of triactinomyxon 'Type 1' (AY495704), produced by Hallett et al. (2005) from similar TAMs released by commercially purchased *Tubifex tubifex* specimens, was found to be 99.94% similar to the *Myxobolus parviformis* sequence; there is only a single nucleotide difference within 1586 bp. Contaminating myxozoan DNA fragments were not amplified by the nested PCR in any of our samples.

## DISCUSSION

Our study describes a myxozoan species whose actinospore had already been described (Hallett et al. 2005) and whose 18S rDNA sequence was already in GenBank (Hallett et al. 2005), but for which there was no knowledge of its myxospore counterpart. Hallett et al. (2005) identified *Tubifex tubifex* as the oligochaete host, supporting our observation that the parasite is not restricted to *Limnodrilus hoffmeisteri*.

We followed 2 full developmental cycles of this parasite from field material, plus 1 ancillary transmission step with myxozoan-free oligochaetes. A molecular analysis of both actinosporean and myxosporean stages derived from the experiments supported our morphological findings. RFLP patterns were consistently different from those of other myxosporean species studied previously, including some common parasites of bream, such as *Myxobolus bramae*, *M. macrocapsularis*, *M. hungaricus* and *M. impressus* (Eszterbauer et al. 2002). There are yet no accepted guidelines (such as the number of base pair differences in certain genes) that define how a particular parasite species should be demarcated from other species. Nevertheless, great similarities or differences provide important information, upon which the status and validity of a stipulated species can be to some extent evaluated.

It was found advantageous (with morphological analysis) to collect non-fused, single cysts to isolate a genotypically homogenous lineage throughout the subsequent transmission procedures. By separating the desired parasite morphotypes, other myxozoan species contained in the initial infection material could be successfully suppressed by the selective use of phenotypically identical 'clones' for the next transmission step, which also were proved to be identical by PCR-RFLP. A species that would produce similar acti-

nospores is *Myxobolus macrocapsularis*. In addition to the fact that no contamination was detected either by PCR-RFLP or by DNA sequencing, the myxospore mixture used for the first trial and all examined plasmodia from the transmission experiments contained no *M. macrocapsularis* myxospores, so contamination with this species can be excluded.

The actinospore description presented largely follows to the guidelines of Lom & Arthur (1989), but we also adopted the suggestion of Paperna (1973) and Molnár (2002) to provide more exact data on the location of developing plasmodia within the gill arches. It has become clear that tissue specificity is an important factor in myxozoan speciation (Eszterbauer 2004), and there is reason to assume a great diagnostic relevance of target sites (i.e. certain types of cells) within an affected tissue. Therefore, additional histological studies are required to reveal such specificity, especially among questionable species in a comparative approach. Nevertheless, it is not known whether *Myxobolus parviformis* is polyxenous and restricted to the gill tissue, where plasmodia were found in the present study. It is possible that Leuciscinae other than *Abramis brama* also may serve as suitable hosts, and even tissues other than gills could be microhabitats for this parasite. These possibilities can only be clarified by transmission experiments. However, in the face of the extraordinarily high number of *Myxobolus* spp. already described, we cannot exclude the possibility of a senior synonym of our species. No myxozoan taxonomist can ignore the fact that these parasites vary in shape and size (the main species-defining characteristics beside that of host species) according to their position in the host, and perhaps also depending on host condition or parasite strain. Morphological variation is a common phenomenon in other myxozoan species (e.g. *M. pseudodispar*) and even in their actinosporean stages (Hallett et al. 2002, 2004, Molnár et al. 2002). In most cases, transmission experiments to ascertain the full host range, which would give a valuable basis for a new species description, have not been performed.

Janiszewska (1955) noted an 'inner envelope' as a principle structural component of the 'Actinomyxidia', and designated this the 'endospore' enclosed in a 'sheath', in contrast to the 'epispore' derived by the valve cells. According to her observations, the envelope is formed by 1 or 2 cells, but she did not consider this sheath as a functional entity in transmission. The term envelope was later considered obsolete by Lom & Arthur (1989). The actinosporean sheath unit, once expelled, displays a striking phenotype, resembling an actinosporean genus referred to as '*Neoactinomyxum* Granata, 1922' by some authors (Jirovec 1940, Oumouna et al. 2003), although in the original genus description (Granata 1922), *N. globosum* displayed the unique flower-like shape described by later authors (Marquès 1984, El-Mansy et al. 1998, Xiao & Desser 1998). Specimens without such flattened floating appendices were either described from histological material, or were gathered from field material filtrates, not from isolated hosts. The taxonomic validity of similarly shaped neoactinomyxum type actinospores, e.g. *N. globosum* Granata, 1925 as described by Özer et al. (2002), is unquestionable. Nevertheless, the present study points out the possibility that some rarely found actinospores might in be fact sheath units lacking their original valve structure. One reason why this sheath structure has so rarely been mentioned by previous authors might be that their studies focused on morphological descriptions rather than on the behaviour and physiological functions involved in the transmission of actinospore stages.

The function of the sheath in transmission is unclear. The polar capsules are widely counter-sunk and covered by the valve cells, implying a somewhat different invasion strategy than that used by actinospores with a spore architecture including acute protruding polar capsule tips. For instance, polar capsules of some aurantiactinomyxon-type spores are almost fully covered by the valve cells. According to our observations, the polar capsules of *Myxobolus cerebralis* and *Henneguya nuesslini* TAMs are much more protruding than those of *M. parviformis*, and a similarly prominent sheath has not yet been observed (Kallert et al. 2004, 2005). In these species, the sporoplasm's ability to migrate down the style to the origin of the caudal processes while leaving the polar capsules in an apical position is frequently observed. We never saw this behaviour in *M. parviformis*, and put this down to the fact that the sheath is more tightly entrenched in its apical position. However, in electron microscopical studies, El-Matbouli et al. (1999) described a filamentous structure on the host's epidermis after penetration of *M. cerebralis* sporoplasms into rainbow trout. This observation appears to parallel our observations for *M. parviformis*. However, the sheath unit could comprise the actual infective substage of the actinosporean form of *M. parviformis*. If in this species attachment does not occur through contact with the fish surface, the valve cells could open passively (maybe upon stimulation by host cues) and release the sheath unit. This would then presumably sink to the bottom, where it could readily be taken up by bream, which feed by filtering sediment through their gills. Alternatively, the sporoplasm is protected from deleterious outer conditions (e.g. osmolarity changes, bacterial activity or mechanical impairment). The inner sheath could comprise an adaptation to habitats with stagnant water, or perhaps another mode of infection (e.g. oral), or be just an additional device for protection in freshwater during the

critical moments following attachment to the host. Detailed ultrastructural data are needed to better understand the function of the inner sheath. For instance, there is no information on whether the gills themselves are the portal of entry for gill-infecting myxozoans. Indeed, we know little about the invasion strategy and developmental pathways of any gill-infecting myxozoan species. The sporozoites may reach the gill lamellae via the blood, whereby invasion would be possible over the whole fish surface, or the actinospores may attach directly to the gills from the surrounding water and the sporozoites directly reach the site of development.

With the improvement of methods to elucidate myxozoan life cycles and the consequent increase in available molecular data (Kent et al. 2001), increased attention must be given to reproducibility. Mixtures of parasites from infected oligochaetes often corrupt the results of transmission experiments. Depletion and the preliminary exclusion of contamination by selective use of well-separated transmission stages are imperative for obtaining comprehensive life cycle descriptions. To avoid invalid results through confusion of myxosporean stages with possible actinosporean counterparts, the whole development of the relevant parasite should be followed in replicate experiments, thus decreasing the likelihood of an error generated by (e.g.) a single molecular comparison of 2 life cycle stages without considering the possibility of DNA contamination. As both developmental stages were found in 2 consecutive transmission trials in the present study, and this was supported by molecular identification, the developmental cycle of our myxozoan species would appear to be valid.

To generally improve research on myxozoan parasites in future life cycle studies, it would be advantageous to include invertebrate host molecular identification methods. As in trematodes, the strict specificity of myxozoan parasites for certain host taxa could provide a powerful tool for distinguishing morphologically indistinguishable transmission stages and even strains. Furthermore, it is recommended that functional morphological characteristics of their life cycle stages be examined, and critical assessments of the ecological impacts of the parasites and their phenotypic characteristics be made.

*Acknowledgements.* We wish to thank Dr. Sascha Hallet and Dr. Stephen Atkinson for their much appreciated advice and comments. The work on the identification of the oligochaete hosts was supported by the Swedish Research Council, grant no. 621-2001-2788 (to C.E., while at the Swedish Museum of Natural History, Stockholm); oligochaete sequencing was done by Ms. Bodil Cronholm. All other molecular biological work was supported by the Hungarian Scientific Research Fund (OTKA) grants No. F045908 and T042464.

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*Editorial responsibility: Wolfgang Körting, Hannover, Germany*

*Submitted: January 19, 2005; Accepted: May 24, 2005*
*Proofs received from author(s): September 13, 2005*