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# *Ellipsomyxa gobii* (Myxozoa: Ceratomyxidae) in the common goby *Pomatoschistus microps* (Teleostei: Gobiidae) uses *Nereis* spp. (Annelida: Polychaeta) as invertebrate hosts

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**Abstract.** *Nereis diversicolor* O.F. Müller and *N. succinea* Frey et Leuckart (Polychaeta, Nereidae) living in brackish shallow areas in Denmark are naturally infected with tetractinomyxon actinospores. Infected *Nereis* spp. were experimentally fed to various potential fish hosts, and the actinosporean stages developed into myxosporean stages of *Ellipsomyxa gobii* Køie, 2003 (Ceratomyxidae) in the gallbladder of the common goby *Pomatoschistus microps* (Krøyer) (Gobiidae). The European eel *Anguilla anguilla* (L.), three-spined stickleback *Gasterosteus aculeatus* L., small sand eel *Ammodytes tobianus* L., flounder *Platichthys flesus* (L.), European plaice *Pleuronectes platessa* L. and common sole *Solea solea* (L.) did not become experimentally infected. In Danish shallow brackish areas *P. microps* is naturally infected with *E. gobii*, in some areas with a prevalence >90%. We compared small subunit ribosomal DNA sequences of the actinosporean with *E. gobii* from *P. microps*. Sequences were identical, which further verifies that both forms belong to the same organism. This is the first myxozoan two-host life cycle in the marine environment.

The life cycles of some 30 freshwater myxosporeans have been experimentally elucidated (see review by Kent et al. 2001). Actinosporean stages develop in oligochaetes in all of these life cycles, except for *Ceratomyxa shasta*, which uses a polychaete alternate host (Bartholomew et al. 1997). Actinospores have been recorded in marine oligochaete and polychaete annelids and in a sipunculid worm (Ikeda 1912, Hallett et al. 1998, 1999, Hallett and Lester 1999, Køie 2002). In Denmark, the polychaete *Nereis diversicolor* O.F. Müller has been recorded as host for actinosporeans (Køie 2000). Identical actinospores have more recently been found in *N. succinea* Frey et Leuckart from the Øresund, Denmark. Although several actinosporeans have been described from marine invertebrates, none have been linked to myxozoan stages found in fishes. The aim, therefore, of the present study was to identify the fish host and myxosporean forms corresponding to the actinosporean stages in *Nereis* spp. using both transmission studies and molecular comparisons.

## MATERIALS AND METHODS

*Nereis diversicolor* and *N. succinea* (Annelida, Polychaeta, Nereidae) were collected at 0.2 to 0.5 m depth in the North harbour of Helsingør and in the Nivå Bay, the Øresund, between Helsingør and Copenhagen. Some specimens were examined and used for experimental infections immediately upon capture, others were kept in aquaria with a thick layer of sand and fed on defrosted boiled mussels and krill. They were kept in filtered, recirculated seawater at 10°C and 30‰ salinity.

The fish species mentioned below and in Table 1 occur in the Nivå Bay and are therefore potential hosts for the actinospores in *Nereis* spp. The common goby *Pomatoschistus microps* (Krøyer) were caught in the Nivå Bay, the North harbour of Helsingør, the Roskilde Fjord off Frederikssund and the Norsminde Fjord, eastern Jutland. The remaining fish were caught in the Øresund at depths less than 10 m. The European eel *Anguilla anguilla* (L.) (15 specimens), three-spined stickleback *Gasterosteus aculeatus* L. (20 specimens), *P. microps* (228 specimens), small sand eel *Ammodytes tobianus* L. (20) and 0- and 1-groups of flounder *Platichthys flesus* (L.) (14), European plaice *Pleuronectes platessa* L. (8) and common sole *Solea solea* (L.) (8) were examined for natural infection with myxozoans immediately upon capture. Fresh squash preparations were made of the urine, the urinary bladder wall, various parts of the kidney, the bile, the gallbladder wall, the liver, the musculature and the gill filaments.

The above mentioned fish species were additionally used for experimental infections and as controls (Table 1). They were kept in aquaria provided with filtered, recirculated seawater (10°C, 30‰ salinity) for four to eight months before experimental infection. Before and after the four-week period of experimental infection the fish were fed on boiled mussels and defrosted krill and mysids. The sand eels were fed on pieces of chopped infected *Nereis* spp. (*N. diversicolor* and *N. succinea*) mixed with chopped defrosted mysids and live laboratory-bred *Artemia salina*. The remaining fish species were exposed to pieces of or to whole infected *Nereis* spp. checked for the presence of actinospores. The fish exposed to infected *Nereis* spp. were examined three to four months after the last exposure. Twenty-two to 55 specimens of each fish species were controls (Table 1). The control specimens were fed on defrosted food

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**Table 1.** Records of myxosporeans in control and exposed marine fish. The fish were caught in the northern Øresund at a depth of less than 10 m, apart from *Pomatoschistus microps* which were caught at 0.5 m in the Nivå Bay.

| Fish species | Length (cm) | Control no. | Naturally infected | Exposed no. | Exposed infected |
|---|---|---|---|---|---|
| *Anguilla anguilla* | 20–35 | 30 | – | 15 | – |
| *Gasterosteus aculeatus* | 5–7 | 50 | – | 12 | – |
| *Pomatoschistus microps* | 3.0–5.5 | 20 | 2 (10%) | 11 | 10 (91%) |
| *Ammodytes tobianus* | 10–16 | 37 | – | 22 | – |
| *Platichthys flesus* | 6–14 | 55 | – | 20 | – |
| *Pleuronectes platessa* | 6–13 | 20 | – | 15 | – |
| *Solea solea* | 6–16 | 22 | – | 12 | – |

during the whole period and examined using the same procedure as for the exposed fish.

The actinosporidian stages in *Nereis* spp. and the myxosporidian stages in naturally infected *P. microps* were fixed in 100% ethanol for molecular analysis. Nucleic acid was extracted from preserved specimens using the QIAgen DNeasy Kit (QIAGEN, Valencia, California). The polymerase chain reaction (PCR) was used to amplify small subunit (SSU) ribosomal DNA (rDNA) sequences from these samples using 4 sets of myxozoan PCR primers. Forward primer 18E (5'-CTG GTT GAT CCT GCC AGT) was used with reverse primer Kud4R (5'- GTG CTT TAT TCA AGG CAC). Forward primers Kud3F (5'-CAG ATA CCG TCC TAG TTC), Kud4F (5'- AGC GAG ACC ACG ATC TTT) and Kud6F (5'-TCA CTA TCG GAA TGA ACG) were all used with reverse primer 18R (5'- CTA CGG AAA CCT TGT TAC G). Polymerase chain reactions were run with the following conditions: 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 × reaction buffer (QIAGEN, Valencia, California), 1.25 units of *Taq* DNA polymerase, 25 pmol of each primer, and 50–600 ng of genomic DNA. Thermocycling was performed in an MJ Research DNA Engine 200 (MJ Research, Watertown, Massachusetts) for 35 cycles consisting of 94ºC for 30 sec, 56ºC for 45 sec, 72ºC for 60 sec, preceded by an initial denaturation at 95ºC for 3 min, and followed by a final extension at 72°C for 7 min. PCR products were visualized on a 1.5% agarose gel run at 100 V for 1 hr containing 0.1 µg/ml ethidium bromide. PCR products were excised from the gel and purified using the QIAgen Gel Extraction Kit (QIAGEN, Valencia, California). Sequencing reactions were performed in both directions with PCR primers using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 and sequence determined on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, California).

## RESULTS

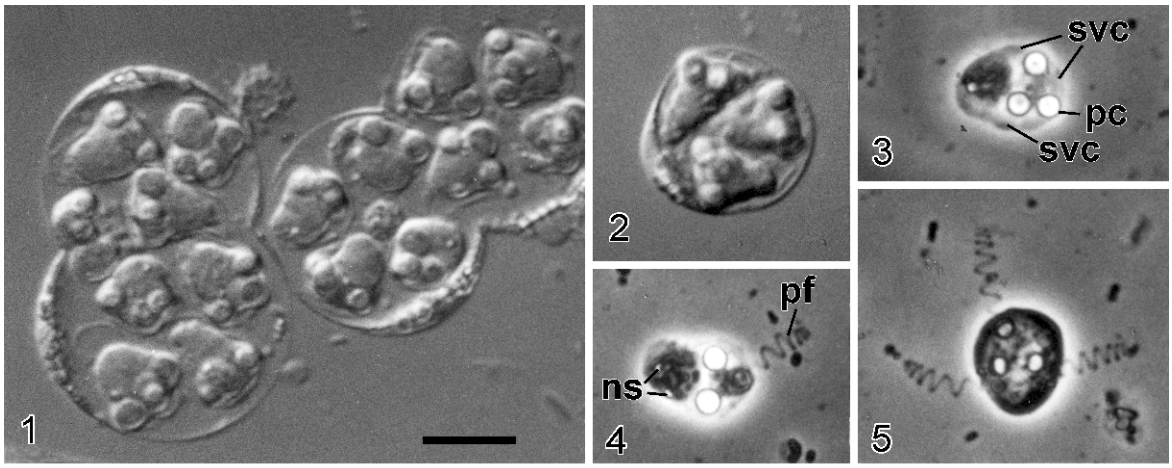
Only *Pomatoschistus microps* harboured a natural infection when examined immediately upon capture. Myxosporean stages were found in the gallbladder, hepatic and bile ducts (Køie 2003). The gallbladder myxozoan *Ellipsomyxa gobii* Køie, 2003 (Ceratomyxidae) was found in 6 of 100 (6%) and 45 of 100 (45%) of 3.0–5.5 cm long specimens of *P. microps* from the Nivå Bay and the North harbour of Helsingør, respectively (May–August, 2001 and 2003, respectively) (Køie 2003). Twenty-eight of 30 (93%) specimens from the Roskilde Fjord (September 2003) and a single specimen of *P. microps* from Norsminde Fjord (October 2003) were infected with mature actinospores of *E. gobii*.

Only *P. microps* showed a difference in infection when comparing the exposed fish with those from the control group (Table 1). Two of 20 (10%) control specimens of *P. microps* were naturally infected with *E. gobii*. The gallbladders of 10 of 11 (91%) exposed specimens of *P. microps* were infected with *E. gobii*. No other myxozoan species were found in the control or exposed specimens of *P. microps*. None of the remaining exposed fish became infected. Gobies examined four months post-exposure contained plasmodia and mature myxospores in the gallbladder wall, bile and hepatic ducts.

One percent and 3% of *Nereis* spp. (1–6 cm long) from Nivå Bay and North harbour of Helsingør respectively, were infected with tetractinomyxon actinosporean stages. Most infected specimens were 2–4 cm long. One of 5, 2 of 18, and 6 of 20 *Nereis* spp., 3–5 cm long from 0.5 m depth, from Vellerup Vig, the Isefjord (September 2002), the Roskilde Fjord (September 2003) and the Norsminde Fjord (October 2003), respectively, were infected.

The actinosporean stages were described by Køie (2000). However, this description was based on one small infected *N. diversicolor*. From May to October 2000–2002 more than 20,000 *Nereis* spp. (*Nereis diversicolor* and *N. succinea*, about half/half) from the Nivå Bay and the North harbour of Helsingør, were examined. *Nereis diversicolor* and *N. succinea* showed similar prevalence of infection, but because an exact identification of each specimen is time consuming only some infected specimens were identified to species.

In heavily infected *Nereis* spp., pansporocysts and free actinospores were found in most tissues, but especially in and between the musculature. The actinosporean stages were not found in the blood vessels, the external epithelia, the intestinal wall or the lumen. Most free pansporocysts have a more or less central constriction (Fig. 1). This shape is concealed in flattened specimens in squash preparations of host tissue. Most pansporocysts contain eight actinospores. As observed in fresh mounts, the wall of most pansporocysts is composed of two enveloping cells with flat nuclei (Fig. 1). These nuclei are apparently decomposed when the actinospores are fully developed.




**Figs. 1–5.** Different developmental stages of live *Ellipsomyxa gobii* in naturally infected *Nereis* sp. Figs. 1, 2 interference contrast, Figs. 3–5 phase contrast. **Fig. 1.** Two pansporocysts, each with eight actinospores. **Fig. 2.** Pansporocyst with three actinospores. **Fig. 3.** Actinospore with degenerated nucleus of shell valve cells and three polar capsules. **Fig. 4.** Actinospore with one extruded polar filament. **Fig. 5.** Actinospore with three extruded filaments. ns – nucleus of sporoplasm; pc – polar capsule or nucleus of polar capsule cell; pf – polar filament; svc – degenerated nucleus of shell valve cell. All to same scale; scale bar = 10 µm.

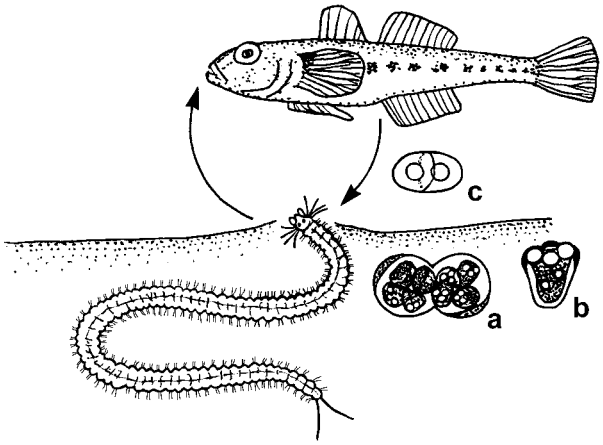



**Fig. 6.** Schematic illustration of the life cycle of *Ellipsomyxa gobii*. The polychaetes *Nereis diversicolor* and *N. succinea* act as the invertebrate hosts and the common goby *Pomatoschistus microps* acts as the fish host. a – pansporocyst with eight actinospores; b – actinospore; c – myxospore. Not to scale.

A few percent of the pansporocysts contain less than eight actinospores, from one to seven. The wall of the pansporocysts with few actinospores is apparently composed of only one enveloping cell with one flat nucleus (Fig. 2).

The eight cells of the actinospore are distinguished in flattened live specimens (Figs. 3, 4). In mature actinospores the three nuclei of the shell valve cells appear to be dead and disinte grating, and are often indistinct. The nuclei of the binucleate sporoplasm are also often indistinct. In addition, one nucleus surrounds each of the three spherical polar capsules.

Extrusion of the polar filaments was provoked by addition of e.g. a droplet of 80% lactic acid to a squash preparation in seawater and by cover glass pressure following evaporation of the surrounded seawater. Actinospores isolated in seawater at 5°C for two weeks did not change morphology, but extrusion of the polar filaments was more easily provoked by cover glass pressure than in actinospores recently removed from the fish host. The coiled polar filament is most often only partly stretched out, revealing 6–7 turns (Figs. 4, 5). The maximum length of the fully extended polar filaments is 32 µm. In some naturally infected *Nereis* spp., pansporocysts and free actinospores were encapsulated by annelid cells, and brown granuloma-like aggregates often occurred in infected polychaetes. Some polychaetes contained hundreds of these aggregates of different sizes and no live actinosporean stages, suggesting that the polychaete is able to eliminate the parasite. A schematic illustration of the life cycle is shown in Fig. 6.

Overlapping SSU rDNA sequences obtained from *Ellipsomyxa gobii* and the *Nereis* spp. actinosporean were each aligned by eye and joined to yield single contiguous sequences. Sequences were deposited in GenBank with the following lengths and accession numbers; *Nereis* spp. actinosporean SSU rDNA, 1695 basepairs (bp) (AY 505127) and *E. gobii* from *P. microps* SSU rDNA, 1697 bp (AY505126). Actinosporean and myxosporean sequences were identical to one another as determined by visual alignment and comparison.

## DISCUSSION

This report is the first known complete life cycle of a marine myxozoan. Even though direct transmission from fish to fish without undergoing actinosporean development has been recorded (Diamant 1997, Redondo et al. 2002, Yasuda et al. 2002), it is likely that these myxozoans also undergo actinosporean development within invertebrate hosts. Previously only four polychaete species, one freshwater and three marine, have been recorded to harbour actinosporean stages (Bartholomew et al. 1997, Køie 2000, 2002). In addition, two actinosporean stages were described from a sipunculid worm (Ikeda 1912). Actinosporeans from polychaetes and sipunculids are all tetractinomyxon type. It is unknown whether the actinosporeans assigned to e.g. the genera *Tetraspora* and *Endocapsa* described from Australian marine tubificid oligochaetes (Hallett and Lester 1999, Hallett et al. 1999) require a vertebrate to complete their life cycle (Lester et al. 1998). The triactinomyxons recorded in seawater (e.g. Roubal et al. 1997) probably belong to genera with members in both freshwater and seawater (e.g. *Myxidium* and *Myxobolus*) whereas members of myxozoan families that are strictly marine (Lom and Dyková 1992) (including the Ceratomyxidae with a freshwater species) are expected to have their actinosporean stages in either polychaetes or sipunculids (or oligochaetes if the genera *Tetraspora* and *Endocapsa* have a counterpart in marine fish). Given the number of marine myxozoan species, it is probable that many hundreds of such life cycles exist. It is, therefore, somewhat surprising that tetractinomyxon actinospores have not been more frequently described because polychaetes have been examined for protistan parasites for over one hundred years.

Experimental life cycles with marine myxozoans are often difficult to conduct because verified parasite-free marine fish hosts are usually not available. A low prevalence of infection occurred in unexposed gobies, which might bring some doubt about the authenticity of our results. However, comparisons of SSU rDNA sequences between the myxosporean stage from gobies and the actinosporeans verify that they indeed belong to the same species. This approach has been used successfully for linking life-cycle stages for five freshwater myxozoans (Andree et al. 1997, Bartholomew et al. 1997, Lin et al. 1999, Longshaw et al. 1999, Pote et al. 2000). Given the variable nature of the myxozoan SSU rDNA (Kent et al. 2001) it is highly unlikely that identical SSU sequences would be obtained from two different species. In fact, intraspecific variation in the SSU rDNA has been reported for *Myxidium lieberkuehni* (Schlegel et al. 1996), *Myxobolus cerebralis* (Andree et al. 1999), *Myxobolus pseudodispar* (Molnár et al. 2002) and *Kudoa thyrsites* (Whipps et al. 2003).

*Nereis diversicolor* and *N. succinea* are omnivorous, living on detritus, plants and animals. The former species may in addition form a mucus net (Hartmann-Schröder 1996). The *E. gobii* spores released by the gobies are so small that they probably are suspended in the water. They may be trapped in the mucous net of the suspension-feeding *N. diversicolor*. Both polychaete species may acquire the myxospores by eating dead gobies. It is most likely that the myxospores enter the polychaete body via the epithelium of the alimentary tract, even though penetration through the external surface may not be excluded.

The food of the common goby consists mostly of small crustaceans (Wheeler 1969). However, annelids also compose an important part of their food and when digging for *Nereis* spp. the common gobies shoal around and collect in depressions in the sandy bottom to catch the small liberated worms. Also, the fact that the common goby is infected with the third-stage larva of the parasitic nematode *Dichelyne* (*Cucullanellus*) *minutus* (Rudolphi), which uses *N. diversicolor* as an intermediate host, shows that this annelid constitutes a food item for this fish (Køie 2001). It is unknown whether the actinospores enter the fish host via the alimentary tract or via the external surface.

About two thousand myxozoan species have been described, and for only a fraction of these has the life cycle been elucidated. Furthermore, until now, no two-host life cycle has been demonstrated for any marine myxo-zoan. The experimental infections carried out in this study, together with the SSU rDNA sequence comparisons, clearly demonstrate that the life cycle of *E. gobii* alter-nates between invertebrate (*Nereis* spp.) and vertebrate (*P. microps*) hosts.

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