Molecular Detection and Phylogenetic Placement of a Microsporidian from English Sole (Pleuronectes vetulus) Affected by X-Cell Pseudotumors

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ABSTRACT: Flatfish tissue samples exhibiting X-cell pseudotumors were tested with a number of ribosomal DNA (rDNA) general primers in polymerase chain reactions (PCRs). Microsporidian primers resulted in the amplification of a rDNA fragment and molecular phylogenetic analysis indicated that although the organism did not relate closely with any current microsporidian genera, it was most similar to organisms placed in the Enterocytozoonidae (Hillis et al., 1991). A series of polymerase chain reactions (PCRs) (Saiki, 1990) were carried out using a pair of universal small subunit (SSU) rDNA primers, 18e and 18g (Hillis and Dixon, 1991), that amplify a large portion of this relatively conserved gene region (1,300–2,000 bp for many eukaryotic organisms). The reactions were performed in 50-μL volumes using standard PCR buffer (Gibco BRL, Gaithersburg, Maryland), 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 1.5 mM MgCl₂, 1.25 units of Taq DNA polymerase, 20 pmol of each primer, and approximately 100 ng genomic DNA template. Thermal cycling was performed in a PTC-200 DNA Engine (MJ Research, Watertown, Massachusetts), with an initial DNA denaturation step (95 C for 3 min) followed by 30 cycles of amplification (94 C for 45 sec, 53 C for 45 sec, and 72 C for 2 min), and a final 5-min extension at 72 C. Variations of magnesium concentration, annealing temperature, and cycling profile were also performed to check for possible differences in the banding pattern generated between tumor and nontumor samples. The second set of primers, 530f and 580r, are microsporidian general primers (Vossbrinck et al., 1999) that were designed to be specific for LSU rDNA sequence data obtained with the abovementioned second primer set; they amplify a 100-bp fragment within the LSU. These primers were designed to be specific for LSU sequence of the flatfish microsporidian. The primers were used...
to test for amplification with other tumor samples from all 5 fish originally collected. Reactions were similar to the first PCR, except for a 55°C annealing temperature, 45 sec extension, and 35 amplification cycles. The fifth and last primer pair examined is located in the SSU and 5.8S genes and is capable of amplifying *Hematodinium*-type dinoflagellates (Hudson and Adlard, 1996). The PCR reactions were the same as the first primer set except for a 55°C annealing temperature and 1-min extensions.

PCR products were resolved by agarose gel electrophoresis and bands excised and purified with the QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, California) according to the manufacturer’s protocol. Direct sequencing of the purified PCR products was performed with the Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Cleveland, Ohio) according to the manufacturer’s protocol.

Alignment of rDNA sequences for the flatfish microsporidian with other representative species of microsporidia was generated via Clustal W, version 1.6 (Thompson et al., 1994). Phylogenetic analyses were performed with the Molecular Evolutionary Genetics Analysis program (version 1.01, Kumar et al., 1993). Genetic distances were calculated using the method of Jukes and Cantor (1969). Phylogenetic relationships were inferred using the neighbor-joining tree building method (Saitou and Nei, 1987) with statistical estimates of branch point validity presented as bootstrap confidence levels (500 replications) and by the branch-and-bound parsimony method.

Gram stains (Fig. 2) were performed on the tumor samples

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**Figure 1.** Histological section of hematoxylin and eosin stained X-cell pseudotumor (angioepithelial nodule type) from English sole. Note abundant leukocytes intermixed with X-cells (arrows). Bar = 20 μm.
that tested positive by PCR for microsporidia. Tissue smears were prepared from portions of tissue used originally in the genomic DNA extractions.

The universal primers 18e and 18g consistently amplified flatfish rDNA both from tumor and nontumor samples under all PCR conditions tested. The microsporidian general primers 530f and 580r gave a weak amplification from 2 tumor samples (from the same fish) that were originally tested. Initial sequence data (352 bp) from the LSU was moderately conserved (86% similar) to *N. salmonis* from British Columbia (Docker et al., 1997). This led us to pursue sequencing the SSU and the more variable internal transcribed spacer (ITS) region. The microsporidian SSU amplification with primers ES-A, ES-B, ES-C, and ES-D yielded a clear product when tested with 1 of the tumor samples. In this case, 1,250 bp of the SSU was 87% similar to *N. salmonis*, and the ITS region of 248 bp was 69% similar. Both rDNA sequences were examined for similarity with other known *N. salmonis*-like sequences (Docker et al., 1997; Gresoviac et al., 1999) and in databases available through the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Maryland) using the Basic Local Alignment Search Tool (BLAST) routine (Altschul et al., 1990); they were most similar to *N. salmonis* (GenBank U78176).

Both neighbor-joining distance (Fig. 3 and Table I) and parsimony (not shown) methods generated the same phylogenetic trees across the SSU region of 1,250 bp. The flatfish microsporidian clustered with group II microsporidia, as defined by Baker et al. (1997), being closest to *N. salmonis*. It did not cluster with various other fish-infecting microsporidians such as numerous muscle-infecting *Pleistophora* species (Nilsen et al., 1998).

All tumor and nontumor samples of the 5 fishes originally collected were examined with Microlsu-1f and Microlsu-2r. Most of the tumor samples tested positive by PCR. Nine of 11 tumor samples tested positive, whereas 12 of 41 nontumor sam-
Table I. Genetic distances (Jukes-Cantor) for 1,250 by of small subunit rDNA sequence data.*

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* Standard errors are shown below the diagonal. 1, Microsporidian from English sole; 2, Nucleospora salmonis; 3, Enterocytozoon salmonis; 4, Enterocytozoon bieneusi (human); 5, E. bieneusi (macaque); 6, Endoreticulatus schubergi; 7, Pleistophora sp.; 8, Vittaforma cornea; 9, Encephalitozoon hellem; 10, Nosema apis; 11, Loma salmonae; 12, Microsporidium prosopium; 13, Amblyospora californica.

LITERATURE CITED


First Record of an Actinosporean (Myxozoa) in a Marine Polychaete Annelid

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ABSTRACT: The marine polychaete Nereis (Hediste) diversicolor (Annelida) from shallow water in the Øresund, Denmark, was found to be infected with an actinosporean stage of a myxozoan parasite. The body length of the pyriform actinospore is 12–16 μm and its maximum width is 10–12 μm. The spore is triangular in apical view, with the 3 spherical polar capsules distally. The spore is without caudal processes. Eight spores develop in each pansporocyst. Free spores and pansporocysts were not found in the epidermal layer, in the intestinal epithelium, or in the lumen of the intestine. The myxosporean stage in fish is unknown. This is the first record of an actinosporean stage in a marine polychaete, but because marine oligochaetes are rare, compared with polychaetes, the latter are believed to play an important role as invertebrate (alternate) hosts in marine myxozoan life cycles.

Transmission studies, as well as recent molecular data, have indicated that the 2 classes Myxosporea and Actinosporea represent different life-cycle stages of Myxozoa. In freshwater, the actinospores develop in oligochaete annelids. However, apart from shallow brackish water habitats, oligochaetes are not common in the marine environment. Even though actinospores have been recorded in marine oligochaetes and in a sipunculan worm (Caullery and Mesnil, 1905; Ikeda, 1912; Roubal et al., 1997; Hallett et al., 1998; Hallett and Lester, 1999), it is likely that most of the numerous myxosporeans that occur in marine fish (Lom and Dyková, 1992) use other invertebrate (alternate) hosts. The polychaete annelids are the most likely candidates as invertebrate hosts for marine species of Myxozoa. In support of this, 2 types of actinosporeans have been found in a freshwater polychaete (Bartholomew et al., 1997).

One, 2-cm-long Nereis (Hediste) diversicolor O. F. Müller (Annelida, Polychaeta, Nereididae) from 50 specimens (2–8 cm long) was infected with an undescribed actinosporean. The polychaetes were collected from a sandy bottom at 0.5 m depth in the Øresund (Nivå Bay) in July 1999, placed in aquaria with recirculating seawater (30‰ salinity, 10 °C), and examined in September 1999. The infected polychaete did not differ from uninfected specimens. The spores were revealed in flattened tissue examined under high magnification. Pansporocysts and free actinospores (Fig. 1) were found in the coelomic cavity, including the parapodia (Fig. 2), and between muscle fibers (Fig. 3). Eight actinospores develop in each pansporocyst. Actinospores were not found in the epidermal layer, in the intestinal epithelium, or in the lumen of the intestine. The body of the pyriform actinospores is 12–16 μm long and 10–12 μm in maximum width. The spores are triangular in apical view, with the 3 spherical polar capsules distally, each 3–4 μm in diameter. There are no caudal processes. Giemsa-stained smears revealed 2 sporoplasm nuclei. The free spores remained unchanged for a few days in seawater.

The presence of a binucleate sporoplasm is the definition of the genus Tetractinomyxon Ikeda, 1912. However, this common genus