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

Author(s): J. S. Khattra, S. J. Gresoviac, M. L. Kent, M. S. Myers, R. P. Hedrick and R. H. Devlin

Source: *The Journal of Parasitology*, Vol. 86, No. 4 (Aug., 2000), pp. 867-871
Published by: Allen Press on behalf of The American Society of Parasitologists
Stable URL: https://www.jstor.org/stable/3284984
Accessed: 13-04-2020 13:41 UTC

cruzi trypomastigote surface antigen-1 protein that mask protective epitopes. Journal of Immunology **153:** 3148–3154.

YOKOYAMA-YASUNAKA, J., E. M. F. PRAL, O. C. OLIVEIRA, S. C. ALFIERI, AND A. M. S. STOLF. 1994. *Trypanosoma cruzi*: Identification of proteinases in shed components of trypomastigote forms. Acta Tropica **57:** 307–315.

YOSHIDA, N. 1983. Surface antigens of metacyclic trypomastigotes of *Trypanosoma cruzi*. Infection and Immunity **40:** 836–839.

ZINGALES, B., M. E. S. PEREIRA, K. ALMEIDA, E. S. UMEZAWA, N. S. NEHME, R. P. OLIVEIRA, A. MACEDO, AND R. P. SOUTO. 1997. Biological parameters and molecular markers of clone CL Brener—The reference organism of *Trypanosoma cruzi* genome project. Memórias do Instituto Oswaldo Cruz **92:** 811–814.

———, ———, R. P. OLIVEIRA, K. ALMEIDA, E. S. UMEZAWA, R. P. SOUTO, N. VARGAS, M. I. CANO, J. F. DA SILVEIRA, N. S. NEHME, C. M. MOREL, Z. BRENER, AND A. MACEDO. 1997. *Trypanosoma cruzi* genome project: Biological characteristics and molecular typing of clone CL Brener. Acta Tropica **68:** 159–173.

J. Parasitol., 86(4), 2000, p. 867–871

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

J. S. Khattra, S. J. Gresoviac*, M. L. Kent†, M. S. Myers‡, R. P. Hedrick*, and R. H. Devlin, West Vancouver Laboratory, Department of Fisheries and Oceans, 4160 Marine Drive, West Vancouver, British Columbia, Canada V7V 1N6; *Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616; †Pacific Biological Station, Department of Fisheries and Oceans, Nanaimo, British Columbia, Canada V9R 5K6; and ‡Northwest Fisheries Science Center, Environmental Conservation Division, 2725 Montlake Boulevard East, Seattle, Washington 98112-2013

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 an 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 *Nucleospora salmonis* and branched within the Enterocytozoonidae. Re-examination of the original tissues used for DNA extractions revealed the presence of putative microsporidian spores in PCR-positive samples. These observations reiterate the highly sensitive diagnostic feature of PCR, allowing detection of organisms overlooked by conventional methods and demonstrate the occurrence of rare, coinfecting organisms.

Pseudotumors composed of unusual "X-cells" (Brooks et al., 1969) were observed in English sole (*Pleuronectes vetulus*) collected from Washington State. We attempted to characterize the X-cell organism of perhaps protozoan origin (Dawe, 1981; Myers, 1981; Watermann et al., 1982; Harshbarger, 1984; Kent et al., 1988) via ribosomal DNA (rDNA) sequence analysis (Hillis and Dixon, 1991). Initially, a general assortment of primers capable of amplifying rDNA sequences from a wide range of organisms were used. We identified the presence of rDNA sequences similar to those found in the microsporidian *Nucleospora*. Here, we present a phylogenetic placement of this microsporidian within the Enterocytozoonidae based primarily on rDNA data.

Five English sole, exhibiting X-cell lesions (Fig. 1), were collected from 3 sites in Washington State in the fall of 1994. Various tissue samples (tumor, skin, muscle, liver, kidney, spleen, ovary, testis, heart) were processed for microscopic and histological examination and portions frozen for DNA analysis. The original specimen from which we amplified the microsporidian in question was a male English sole, measuring 125 mm in length and weighing 17 g, with a spherical lesion (10 mm in diameter); it was histologically characterized as an angioepithelial nodule, a condition initially described by Wellings et al. (1964). This stage of the X-cell tumor precedes the typical papillomatous pseudotumor condition when the nodules contain abundant inflammatory cells in addition to X-cells.

Genomic DNA was extracted and quantified from tumor and nontumor samples by standard methods using proteinase K digestion and phenol–chloroform extraction, as described by Devlin et al. (1991). A first 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 $MgCl_2$, 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 the magnesium concentration, annealing temperature, and cycling profile were also performed to check for possible differences in the banding pattern generated between tumor from nontumor samples. The second set of primers, 530f and 580r, are microsporidian general primers (Vossbrinck et al., 1987) that are located in the SSU and large subunit (LSU), respectively, and give rise to a fragment of approximately 1,500 bp. PCR conditions were as above except for a 50 C annealing temperature and 35 cycles of amplification. A third PCR assay was based on the 4 *Nucleospora salmonis* SSU diagnostic primers ES-A, ES-B, ES-C, and ES-D (Barlough et al., 1995). These were used to amplify the entire SSU and perform a nested PCR (Gresoviac et al., 1999). A fourth set of primers, Microlsu-1f (5′-CTTCTAAAGCTAAATATCG) and Microlsu-2r (5′-GTATTTTCACTTTTCACAG), were designed from preliminary 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

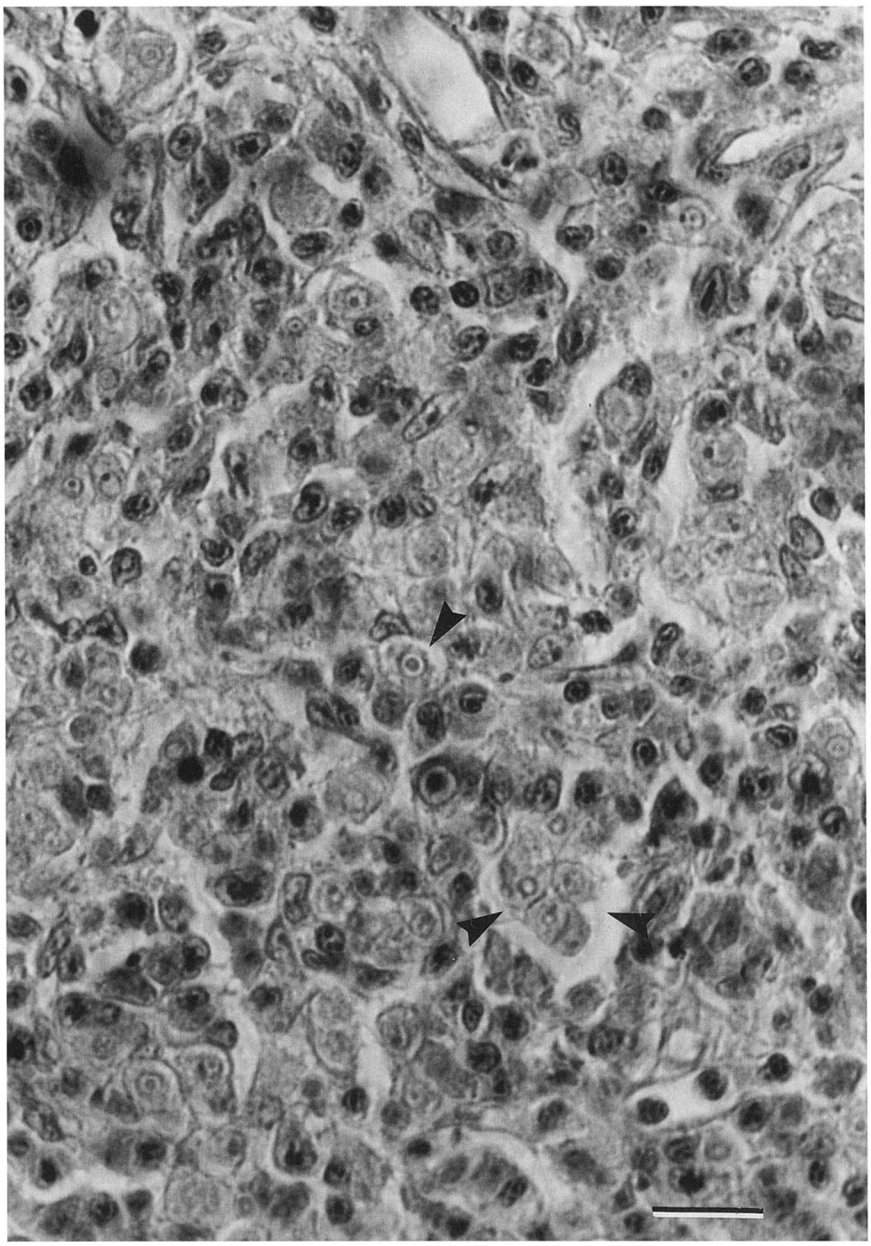

FIGURE 1. Histological section of hematoxylin and eosin stained X-cell pseudotumor (angioepithelial nodule type) from English sole. Note abundant leukocytes intermixed with X-cells (arrows). Bar = 20 μm.

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

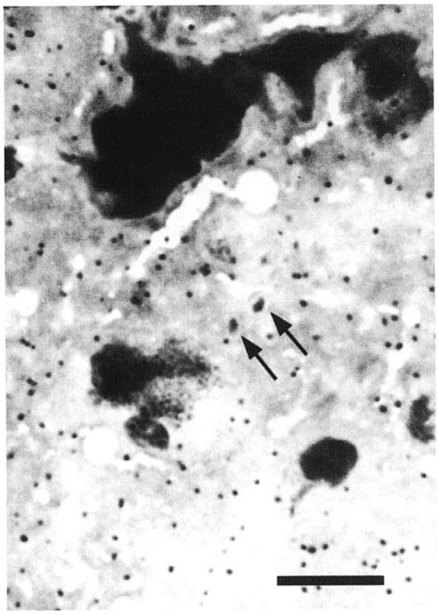

FIGURE 2. Gram stain of PCR-positive sample. Bar = 10 μm.

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-

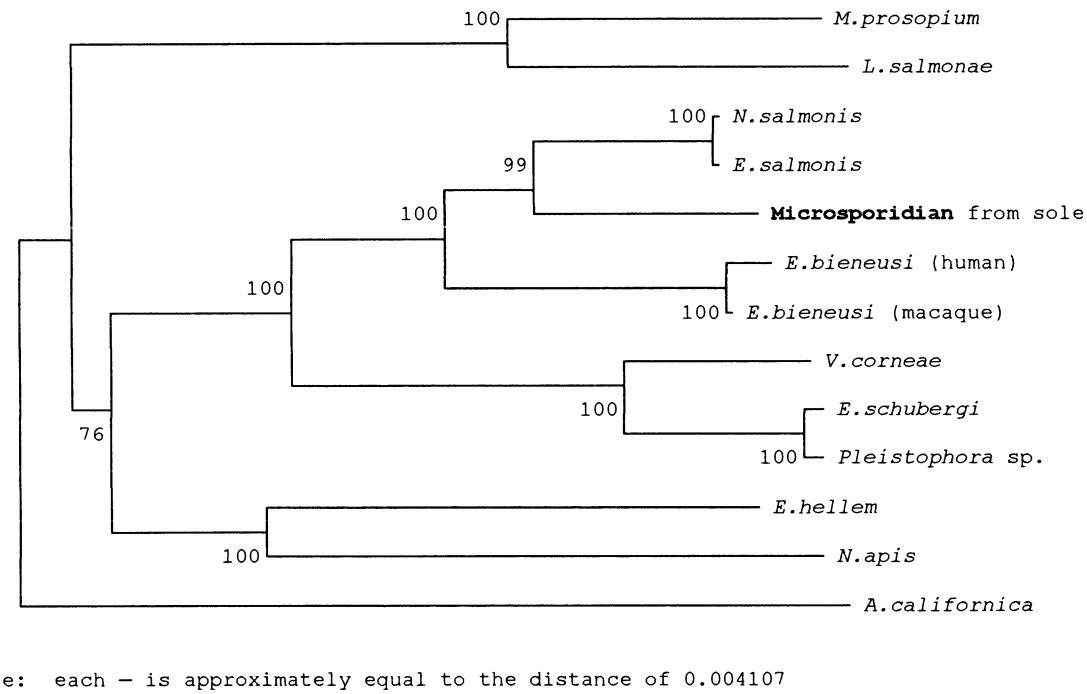

FIGURE 3. Phylogenetic tree of SSU rDNA sequence data using the neighbor-joining distance method. Numbers at nodes indicate bootstrap confidence levels. The GenBank accession numbers are: 1, Microsporidian from English sole AF201911; 2, *Nucleospora salmonis** U78176; 3, *Enterocytozoon salmonis** U10883; 4, *Enterocytozoon bieneusi* (human) L07123; 5, *E. bieneusi* (macaque) AF023245; 6, *Endoreticulatus schubergi* L39109; 7, *Pleistophora* sp. D85500; 8, *Vittaforma corneae* U11046; 9, *Encephalitozoon hellem* L19070; 10, *Nosema apis* U26534; 11, *Loma salmonae* U78736; 12, *Microsporidium prosopium* AF151529; 13, *Amblyospora californica* U68473, outgroup. *These two are the same species (see Docker et al., 1997).

TABLE I. Genetic distances (Jukes–Cantor) for 1,250 by of small subunit rDNA sequence data.*

	1	2	3	4	5	6	7	8	9	10	11	12	13
1		0.1290	0.1290	0.2134	0.2007	0.3202	0.3161	0.3016	0.4063	0.4486	0.4428	0.4517	0.4934
2	0.0109		0.0024	0.1853	0.1734	0.2935	0.2907	0.2805	0.4287	0.4527	0.4698	0.4448	0.4844
3	0.0109	0.0014		0.1843	0.1724	0.2935	0.2907	0.2781	0.4258	0.4496	0.4714	0.4463	0.4828
4	0.0148	0.0136	0.0135		0.0157	0.3334	0.3292	0.3335	0.4241	0.4638	0.4807	0.4494	0.4771
5	0.0141	0.0129	0.0129	0.0036		0.3155	0.3114	0.3170	0.4205	0.4517	0.4679	0.4359	0.4675
6	0.0194	0.0183	0.0183	0.0201	0.0193		0.0067	0.1180	0.4263	0.4340	0.4582	0.4687	0.5294
7	0.0193	0.0182	0.0182	0.0199	0.0191	0.0024		0.1212	0.4277	0.4324	0.4598	0.4719	0.5211
8	0.0186	0.0177	0.0176	0.0200	0.0193	0.0105	0.0107		0.4156	0.4121	0.4779	0.4935	0.5507
9	0.0228	0.0238	0.0236	0.0237	0.0234	0.0240	0.0241	0.0235		0.3331	0.4520	0.4665	0.5145
10	0.0251	0.0253	0.0251	0.0259	0.0252	0.0249	0.0248	0.0238	0.0202		0.4996	0.4740	0.5010
11	0.0243	0.0255	0.0256	0.0262	0.0254	0.0254	0.0255	0.0262	0.0245	0.0274		0.2038	0.5160
12	0.0248	0.0245	0.0245	0.0249	0.0241	0.0259	0.0260	0.0269	0.0252	0.0263	0.0139		0.5296
13	0.0266	0.0262	0.0261	0.0260	0.0255	0.0286	0.0282	0.0294	0.0276	0.0275	0.0274	0.0279	

* 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 corneae*; 9, *Encephalitozoon hellem*; 10, *Nosema apis*; 11, *Loma salmonae*; 12, *Microsporidium prosopium*; 13, *Amblyospora californica.*

ples were positive. The tumor samples appeared to amplify stronger than the nontumor samples that tested positive. Two tumor samples that tested negative by PCR were from a fish collected at a different site than the other fish examined. This fish was also PCR negative for all nontumor tissue samples. The observed difference in the intensity of amplification between tumor and nontumor samples may be due to the relative prevalence of leukocytes because, as is the case for *N. salmonis,* the flatfish microsporidian may show a propensity to infect nuclei of leukocytes. However, close microscopic examination of tissue sections from the X-cell tumors did not reveal microsporidia within host cell nuclei.

PCR analysis utilizing *Hematodinium*-type dinoflagellate primers did not amplify any positive bands from tumor or nontumor samples, suggesting the X-cell organism's rDNA sequence may be quite different from *Hematodinium*-type dinoflagellates. These primers were used to test the possibility of the X-cell organism being closely related to this group of dinoflagellates.

These results do not demonstrate that the X-cell has been identified as a microsporidian, only that the microsporidian detected here co-occurs with X-cells, possibly due to the high proportion of inflammatory cells in the lesions. The phylogenetic inferences from the SSU rDNA data place this flatfish microsporidian in the Enterocytozoonidae. The difference between the sequences of the flatfish microsporidian and other known microsporidian sequences appears large enough to withhold placement of this microsporidian into any of the existing genera in group II microsporidia.

We thank Sheila Dawe, from the Department of Fisheries and Oceans Canada, for performing the Gram-stain analysis.

LITERATURE CITED

ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHAFFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research **25:** 3389–3402.

BAKER, M. D., C. R. VOSSBRINCK, J. J. BECNEL, AND J. V. MADDOX. 1997. Phylogenetic position of *Amblyospora* Hazard & Oldacre (Microspora: Amblyosporidae) based on small subunit rDNA data and its implication for the evolution of the Microsporidia. Journal of Eukaryotic Microbiology **44:** 220–225.

BARLOUGH, J. E., T. S. MCDOWELL, A. MILANI, L. BIGORNIA, N. J. PIENIAZEK, AND R. P. HEDRICK. 1995. Nested polymerase chain reaction for detection of *Enterocytozoon salmonis* genomic DNA in chinook salmon *Oncorhynchus tshawytscha.* Diseases of Aquatic Organisms **23:** 17–23.

BROOKS, R. E., G. E. MCCARN, AND S. R. WELLINGS. 1969. Ultrastructural observations on an unidentified cell type found in epidermal tumors of flounders. Journal of the National Cancer Institute **43:** 97–110.

DAWE, C. J. 1981. Polyoma tumors in mice and X-cell tumors in fish, viewed through telescope and microscope. *In* Phyletic approaches to cancer, C. J. Dawe, J. C. Harshbarger, S. Kondo, T. Sugimura, and S. Takayama (eds.). Japan Scientific Society Press, Tokyo, Japan, p. 19–49.

DEVLIN, R. H., B. K. MCNEIL, T. D. D. GROVES, AND E. M. DONALDSON. 1991. Isolation of Y-chromosomal DNA probe capable of determining genetic sex in chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences **48:** 1606–1612.

DOCKER, M. F., M. L. KENT, D. M. L. HERVIO, J. S. KHATTRA, L. M. WEISS, A. CALI, AND R. H. DEVLIN. 1997. Ribosomal DNA sequence of *Nucleospora salmonis* Hedrick, Groff and Baxa, 1991 (Microsporea: Enterocytozoonidae): Implications for phylogeny and nomenclature. Journal of Eukaryotic Microbiology **44:** 55–60.

GRESOVIAC, S. J., J. S. KHATTRA, M. L. KENT, R. H. DEVLIN, C. P. VIVARES, AND R. P. HEDRICK. 2000. Comparison of small subunit ribosomal RNA gene and internal transcribed spacer region sequences among isolates of the intranuclear microsporidian *Nucleospora salmonis.* Journal of Eukaryotic Microbiology (in press).

HARSHBARGER, J. C. 1984. Pseudoneoplasms in ectothermic animals. National Cancer Institute Monogram **65:** 251–273.

HILLIS, D. M., AND M. T. DIXON. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. Quarterly Review of Biology **66:** 411–453.

HUDSON, D. A., AND R. D. ADLARD. 1996. Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium* cf. *perezi* and *Hematodinium*-like dinoflagellates. Diseases of Aquatic Organisms **24:** 55–60.

JUKES, T. H., AND C. R. CANTOR. 1969. Evolution of protein molecules. *In* Mammalian protein metabolism, H. N. Munro (ed.). Academic Press, New York, New York, p. 21–132.

KENT, M. L., M. S. MYERS, S. R. WELLINGS, AND R. A. ELSTON. 1988. An internal X-cell pseudotumor in a black croaker (*Cheilotrema saturnum*). Journal of Wildlife Diseases **24:** 142–145.

KUMAR, S., K. TAMURA, AND M. NEI. 1993. MEGA: Molecular evolu-

tionary genetics analysis, version 1.01. Pennsylvania State University Press, University Park, Pennsylvania, 130 p.
MYERS, M. S. 1981. Pathologic anatomy of papilloma-like tumors in the Pacific Ocean perch, *Sebastes alutus,* from the Gulf of Alaska. M.S. thesis. University of Washington, Seattle, Washington, 98 p.
NILSEN, F., C. ENDRESEN, AND I. HORDVIK. 1998. Molecular phylogeny of microsporidians with particular reference to species that infect the muscles of fish. Journal of Eukaryotic Microbiology **45:** 535–543.
SAIKI, R. S. 1990. Amplification of genomic DNA. *In* PCR protocols, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). Academic Press, San Diego, California, p. 13–20.
SAITOU, N., AND M. NEI. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:** 406–425.
THOMPSON, J. D., D. G. HIGGINS, AND T. J. GIBSON. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673–4680.
VOSSBRINCK, C. R., J. V. MADDOX, D. FRIEDMAN, B. A. DEBRUNNER-VOSSBRINCK, AND C. R. WOESE. 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature **326:** 411–414.
WATERMANN, B., AND V. DETHLEFSEN. 1982. Histology of pseudobranchial tumors in Atlantic cod (*Gadus morhua*) from the North Sea and the Baltic Sea. Helgolander Meeresuntersuchungen **35:** 231–242.
WELLINGS, S. R., R. G. CHUINARD, R. T. GOMLEY, AND R. A. COOPER. 1964. Epidermal papillomas in the flathead sole, *Hippoglossoides classodon,* with notes on the occurrence of similar neoplasms in other pleuronectids. Journal of the National Cancer Institute **33**: 991–1004.

J. Parasitol., 86(4), 2000, p. 871–872

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

M. Køie, Marine Biological Laboratory, University of Copenhagen, DK-3000 Helsingør, Denmark

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 10–12μm. 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 found in the musculature and parapodia but not in 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 actinosporeans 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

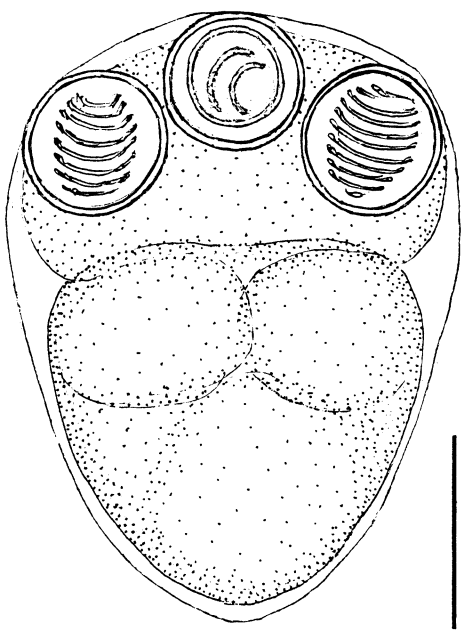

FIGURE 1. Drawing of an actinospore from *Nereis diversicolor.* Scale bar, 5 μm.