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Pseudoloma neurophilia n. g., n. sp., a New Microsporidium from the Central Nervous System of the Zebrafish (*Danio rerio*)

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ABSTRACT. An unusual xenoma-forming microsporidium was discovered in the central nervous system of moribund zebrafish from a laboratory colony in Eugene, Oregon. Infected fish were often emaciated and lethargic, and histological examination commonly revealed severe myelitis and myositis associated with the infection. Based on its structure, development, and small subunit ribosomal DNA sequence it is unique among fish microsporidia. Spores are uninucleate, ovoid to pyriform, with a prominent posterior vacuole. Spores average 5.4 × 2.7 μm with 13–16 coils of the polar filament. The microsporidium produces xenomas within the spinal cord and hindbrain of fish, and xenomas contained sporophorous vesicles with up to 16 spores. Sporoblasts and presporoblast stages (probably sporonts) are found occasionally in small aggregates dispersed randomly throughout xenomas. It clustered in the "*Ichthyosporidium* group" along with other fish microsporidian genera based on rDNA sequence analysis. The rDNA sequence of the zebrafish microsporidium was most similar to that of *Ichthyosporidium,* but showed only 12.1% similarity and therefore this microsporidium can be considered a distinct genus and species, which we have named *Pseudoloma neurophilia* n. g., n. sp.

Key Words. *Ichthyosporidium*, *Loma*, myelitis, myositis, phylogeny, small subunit ribosomal DNA sequence, spores, sporophorous vesicles, xenoma.

MICROSPORIDIA infect essentially all animal groups and are common in fishes, in which some 100 species in about 20 genera have been described (Canning & Lom 1986). De Kinkelin et al. (1980) were the first to describe a microsporidian parasite as a cause of morbidity and mortality in zebrafish (*Danio rerio*), a model organism in the study of developmental biology (Baringa 1990; Eisen 1996). We first observed microsporidia in the central nervous system of zebrafish when disease investigations were initiated at the University of Oregon Zebrafish Facility, Eugene, Oregon, in 1993. We have since detected the infection in multiple other laboratory colonies and in zebrafish from pet stores.

MATERIALS AND METHODS

Specimen collection. Zebrafish from the University of Oregon have been maintained in a facility that utilizes both flow-through and recirculating water systems. Carbon-dechlorinated city water is used in the flow-through system. The main facility is a large recirculating system with particle-bead and biological-sand filters and ultraviolet sterilization post-filtration. Replacement water is generated by reverse osmosis of city water. The water systems are maintained at 28 °C.

Emaciated adult zebrafish were collected and euthanized (Eugenol; 200 μg/ml, 2× anesthetic dose) immediately prior to necropsy or fixation. The tail was removed and the abdomen opened, and then the fish were preserved whole for histology. For electron microscopy, cytology and molecular biology studies, the spinal cords were removed from fresh fish and examined by wet mount for the presence of the parasite.

Histology and cytology. Fish for histopathology were fixed in Dietrich's fixative (28.5% ethanol, 10% formalin, 2% glacial acetic acid) (Paul et al. 1981) for a minimum of three days. The day before processing, the fish were transferred to 5% trichloroacetic acid (TCA) in Dietrich's fixative and placed on a rotor overnight. The fish were then processed for paraffin sectioning by standard techniques. Histological sections were stained with hematoxylin and eosin.

Cytological preparations of spinal cord squash preparations were examined as wet mounts or stained with a Giemsa-like stain, DifQuick Stain (Jorgensen Laboratories, Inc., Loveland, CO).

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Electron microscopy. Spinal cord tissue was fixed overnight in 2.0% glutaraldehyde, 2.0% paraformaldhyde, 0.5% DMSO, 8 mM calcium chloride, and 80 mM cacodylate, pH 7.2. Tissues were postfixed in 2% osmium tetraoxide in 80 mM cacodylate, then dehydrated in ethanol and embedded in Spurr's resin. Reembedding and sectioning of thick sections was required to locate xenomas. Sections were stained with uranyl acetate and lead citrate, and examined with either a Phillips CM12 or EM300 electron microscope.

Spore measurements and prevalence. Twenty spores were measured from a wet mount preparation of fresh spinal cord tissue observed at 1,000× magnification. Digitalized images were measured using Diagnostic Instruments, Inc., Spot RT Software 3.0 (Sterling Heights, MI).

Prevalence was determined by examination of dissected spinal cords in wet mount preparations from 30 emaciated and 30 normal, healthy-appearing adult zebrafish (age: 1 yr ± 2 mos). Sections of an additional of eight emaciated fish and 16 apparently healthy fish from two different age groups, 4–5 mo (juvenile) and 2–3 yr of age (geriatric), were examined for the infection.

DNA isolation and PCR. DNA was extracted from infected zebrafish spinal tissue using two different protocols. The initial protocol was by proteinase K digestion at 56 °C overnight (10 mM Tris, pH 8.0; 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 200 μg/ml proteinase K). The mixture was extracted twice with phenol, chloroform and isoamyl alcohol (50:50:1 ratio). Linear polyacrylamide (15 μg) was added as a carrier (Gaillard and Strauss 1990) and the DNA precipitated with ammonium acetate and ethanol. The DNA was suspended in TE buffer (10 mM Tris-Cl pH 7.5, 1.0 mM EDTA) and stored at −20 °C. The second method of DNA extraction utilized the Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The Qiagen DNeasy protocol for animal tissues was followed. After elution the DNA was precipitated and suspended as previously noted.

A portion of the microsporidium rRNA gene was amplified from the isolated DNA using the universal panmicrosporidian primer set 530f:580r (Vossbrinck et al. 1993). The forward primer 530f (5′-GTGCCAGC(C/A)GCCGCGG-3′) and reverse primer 580r (5′-GGTCCGTGTTTCAAGACGG-3′) are located in the small subunit (SSU) and large subunit (LSU) rRNA genes, respectively. The amplified product was a 1553 bp rDNA fragment that includes the internal transcribed spacer region (ITS). The remaining 5′ end of the SSU was amplified using

the forward primer 18f (5′-CACCAGGTTGATTCTGCC-3′) (Zhu et al.1994) and two different reverse primers, 69r (5′-AGCTGTCTGTCACGCCATTC-3′) and 580r. The 69r reverse primer was designed from the sequence of the 530f:580r product. The primer pair 18f:69r yields a 529 bp fragment and the 18f:580r primer set yields a 1980 bp fragment.

PCR amplifications were performed with Platinum PCR SuperMIX (Life Technologies Inc., Rockville, MD). A standard 50-μl reaction contained 2.5 μl isolated DNA, 25 pmol of each primer, 20 U/ml complexed recombinant *Taq* DNA polymerase with PLATINUM *Taq* Antibody, 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dTTP, and 200 μM dCTP. The reactions were run in a PTC-100 thermal cycler (MJ Research, Watertown, MA). After initial denaturation and polymerase activation at 94 °C for 2 min, 35 cycles were performed with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s. The PCR was terminated with a final 10-min extension at 72 °C. DNA isolated from *Loma salmonae* was used as a positive PCR control (Docker et al. 1997a).

Cloning and sequencing of PCR products. PCR products were purified by agarose gel electrophoresis and QIAquick Gel Extraction (Qiagen Inc.) prior to sequencing and cloning. The purified PCR products from the primer pairs 530f:580r (1553 bp) and 18f:580r (1980 bp) were cloned into pGEM-T Easy Vector (Promega Corp., Madison, WI) using the manufacturer's protocol. The DNA was purified from overnight cultures using QIAprep Miniprep (Qiagen, Inc.). The PCR products and clones were sequenced by ABI Big Dye Terminator Cycle Sequencing in a ABI 377 DNA Sequencer (Perkin Elmer, Norwak, CT).

Phylogenetic analysis. The SSU rDNA sequence was aligned and analyzed using representative members of the *Ichthyosporidium* clade (Kent et al. 1999; Nilsen et al. 1998) with representative microsporidian outgroups. Alignment was based on the alignment given at the website http//www.pac.dfo-mpo.gc.ca/sci/sealane/micro/Index.htm, used in the species description of *Microsporidium prosopium* (Kent et al. 1999), with minor changes to incorporate new species and remove redundant gaps. In total, 23 microsporidia and 2 outgroup taxa were aligned. In addition to sequences available on this website alignment, we added *Loma acerinae* (GenBank AJ252951), the zebrafish microsporidium (GenBank AF322654), and a newly sequenced part of the SSU for *Loma embiotocia* (GenBank AF320310).

A preliminary phylogenetic analysis used only portions of sequences that were clearly conserved and easily aligned (1355 bp based on the zebrafish microsporidium). The taxa that grouped into close clusters from this tree were placed together in subgroups for alignment of the more variable regions that were difficult to align in the large block. This finer alignment of closely related subgroups used both CLUSTAL alignment and by eye. Phylogenetic trees were constructed using PAUP 4.0 beta3a version software (Swofford 2000). Phylogenetic trees were generated using all three generic types of optimality criterion: maximum parsimony, distance, and maximum likelihood, using parameters recommended in the documentation accompanying PAUP 4.0 (Swofford 2000). Bootstrap resampling was also performed with 100 replicates. Phylogenetic analysis was performed with and without an added data matrix containing "gap" or indel information. The gap matrix was generated with the recommendation of documentation in the PAUP 4.0 package, with a few modifications to allow analysis in maximum likelihood and distance analysis.

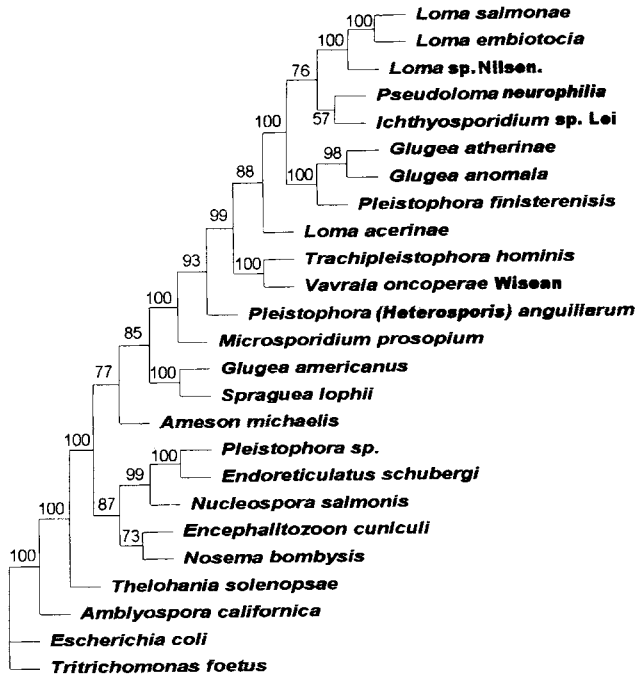

Fig. 1. Phylogeny of microsporidia based on small subunit rDNA analysis, emphasizing the "*Ichthyosporidium*" clade (*Loma salmonae-Ameson michaelis*). Maximum parsimony, with bootstrap values at nodes (100 replicates).

RESULTS

Pseudoloma n. g. (Fig. 2–9).

Description. Uninucleate spores occur in segregated aggregates of 8 to 16 in a sporophorous vesicle within xenomas that lack meronts. Signature sequences in small subunit rDNA at position 168–174, TTTTGTT and at position 1271–1276, TTATTT.

Type species. *Pseudoloma neurophilia* n. g., n. sp. described below.

Etymology. The genus name refers to the similarities with *Loma.*

Remarks. *Pseudoloma* n. g. is similar to the genus *Loma* in that in forms xenomas containing spores within sporophorous vesicles (SPOVs) in fish. However, the former differs from *Loma* species in that meronts were not seen throughout the xenoma, and rDNA analysis showed that the *Pseudoloma* n. g. clusters with *Ichthyosporidium* rather than the *Loma* clade (Fig. 1). The signature sequences in the rDNA, as indicate above, do not occur in other fish microsporidia. The presence of SPOVs and uninucleate spores distinguishes *Pseudoloma* from *Ichthyosporidium.* Ribosomal DNA analysis distinguishes *Pseudoloma* from other xenoma-forming microsporidian genera such as *Spraguea* and *Glugea.* In addition, *Spraguea* does not form SPOVs, while *Glugea* forms xenomas with developmental stages located at the periphery of the xenoma. No rDNA data are available for *Tetramicra,* but *Pseudoloma* differs from this genus in that the former has spores with a conspicuous inclusion in the posterior vacuole.

Pseudoloma neurophilia n. sp. (Fig. 2–9).

Description. Spores ovoid to pyriform; large posterior vacuole without an inclusion body; length 5.4 (4.8–5.9) μm, width 2.7 (2.3–3.1) μm; 13 to 16 coils of the polar filament; uninucleate. Xenomas occur in spinal cord (usually ventral) and

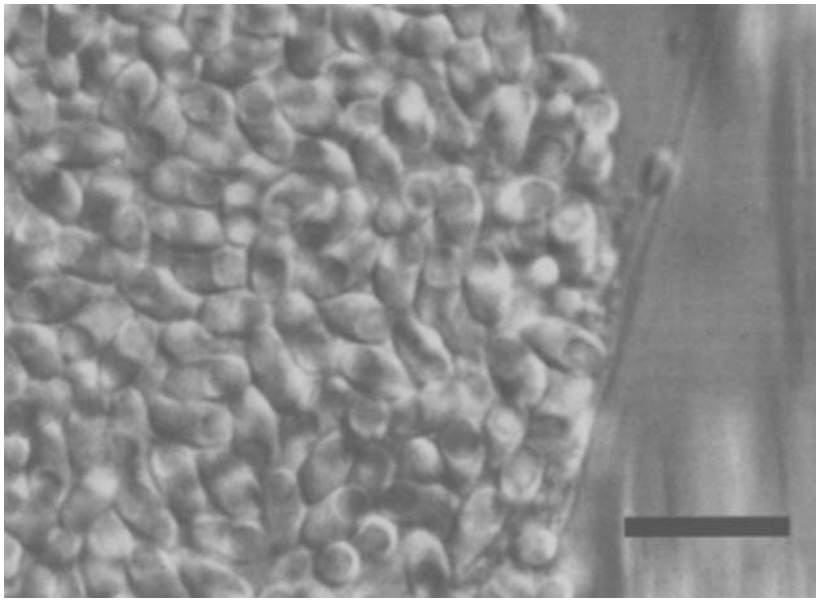

Fig. 2. The microsporidium, *Pseudoloma neurophilia* n. g., n. sp., in a wet-mount preparation of zebrafish spinal cord. Bar = 10 μm.

hind brain; occasionally observed in ventral nerve roots and musculature; comprised of sporophorous vesicles containing spores; variable in size, up to 200 μm in diameter; developmental forms rarely observed, sporoblasts occur randomly in sporophorous vesicles throughout xenoma.

Type host. *Danio rerio* (Hamilton and Buchanan 1822)

Location in host. Hindbrain, spinal cord, and ventral nerve roots of the central nervous system.

Type locality. Laboratory colony, University of Oregon, Eugene, Oregon, USA

Prevalence. Approximately 90% of emaciated fish and 10–30% of healthy fish.

Type material. Hapantotypes in histological sections in U.S. National Parasite Collection, Beltsville, Maryland (USNPC no. 090937.00) and the International Protozoan Type Slide Collection at the Smithsonian Institute, Washington, DC (USNM no. 51545).

Etymology. Denotes the primary site of infection, the central nervous system.

Remarks. Histological examination of moribund zebrafish revealed that this newly identified microsporidium occurs within multiple xenomas in the central nervous system (Fig. 3–5). Xenomas were commonly observed in axon rich regions of the ventral spinal cord and the peripheral ventral roots projecting into the surrounding somatic muscle. In addition, xenomas were occasionally observed in the medial longitudinal fasciculus. Developmental stages were observed dispersed throughout some of the spore-filled xenomas. Developmental stages and spores were clearly contained in aggregates within SPOVs with prominent walls (Fig. 4,5). Inflammation in the central nervous system was usually minimal and only occasionally seen surrounding xenomas or when dispersed spores were present in the CNS. In histological sections of heavily infected fish, an associated tissue reaction extended through the meninges and into the skeletal muscle associated with the adjacent vertebrae. Several of the emaciated fish had a characteristic massive chronic inflammation of the skeletal muscle, in which free spores occurred within phagocytes (Fig. 6). The myositis and myocytolysis seen in these infected fish, however, was often not directly associated with detectable parasites (i.e. only occasionally could parasites be detected in these inflammatory lesions either within xenomas or macrophages).

The earliest stage detected by electron microscopy was a single, uninucleate cell in contact with the host cytoplasm (Fig. 7). This presporoblast (probably a sporont) had a relatively thickened plasmalemma but was not surrounded by either host cell endoplasmic reticulum (ER) or an interfacial envelope. Free sporoblasts within an adjacent SPOV were uninucleate, spherical, and had abundant ER and a dense plasmalemma (Fig. 7). As seen in the previous stage, they had a moderately thickened plasmalemma. The mature spores exhibited coiled polar tubes with 13–16 coils (Fig. 8). The fully-formed spores and sporoblasts were clearly segregated within the SPOVs (Fig.7, 9).

The most common clinical sign associated with the microsporidian infection in zebrafish was emaciation. Lordosis and scoliosis were also commonly seen in infected fish. A survey of 30 emaciated and 30 normal appearing adult (age: 1 yr ± 2 mo) zebrafish by wet mount preparations revealed a prevalence of 29/30 (97%) and 10/30 (33%), respectively. The emaciated fish also exhibited heavier infections than the infected, normal appearing fish. Histological sections of eight additional emaciated fish and 16 additional apparently healthy fish from two different age groups (4–5 mo and 2–3 yr of age) showed emaciated 7/8 (87.5%) positive for microsporidiosis, while the parasite was not detected (0/8) in the juvenile group of normal appearing fish and in only one of the geriatric fish (1/8, 12.5%).

The small subunit rDNA (SSU rDNA) sequence of the unknown zebrafish microsporidian was aligned and analyzed using representative members of the *Ichthyosporidium* clade (Kent et al. 1999; Nilsen 2000; Nilsen et al. 1998;) and with representative microsporidian outgroups (Fig.1). Parsimony, distance, and maximum likelihood analyses delivered phylogenetic trees with the same topology, and trees were the same whether or not the gap matrix was added. Bootstrap support was higher with the gap matrix added, and the gap matrix generated largely the same topology when used as the only input data. Although all analyses showed that

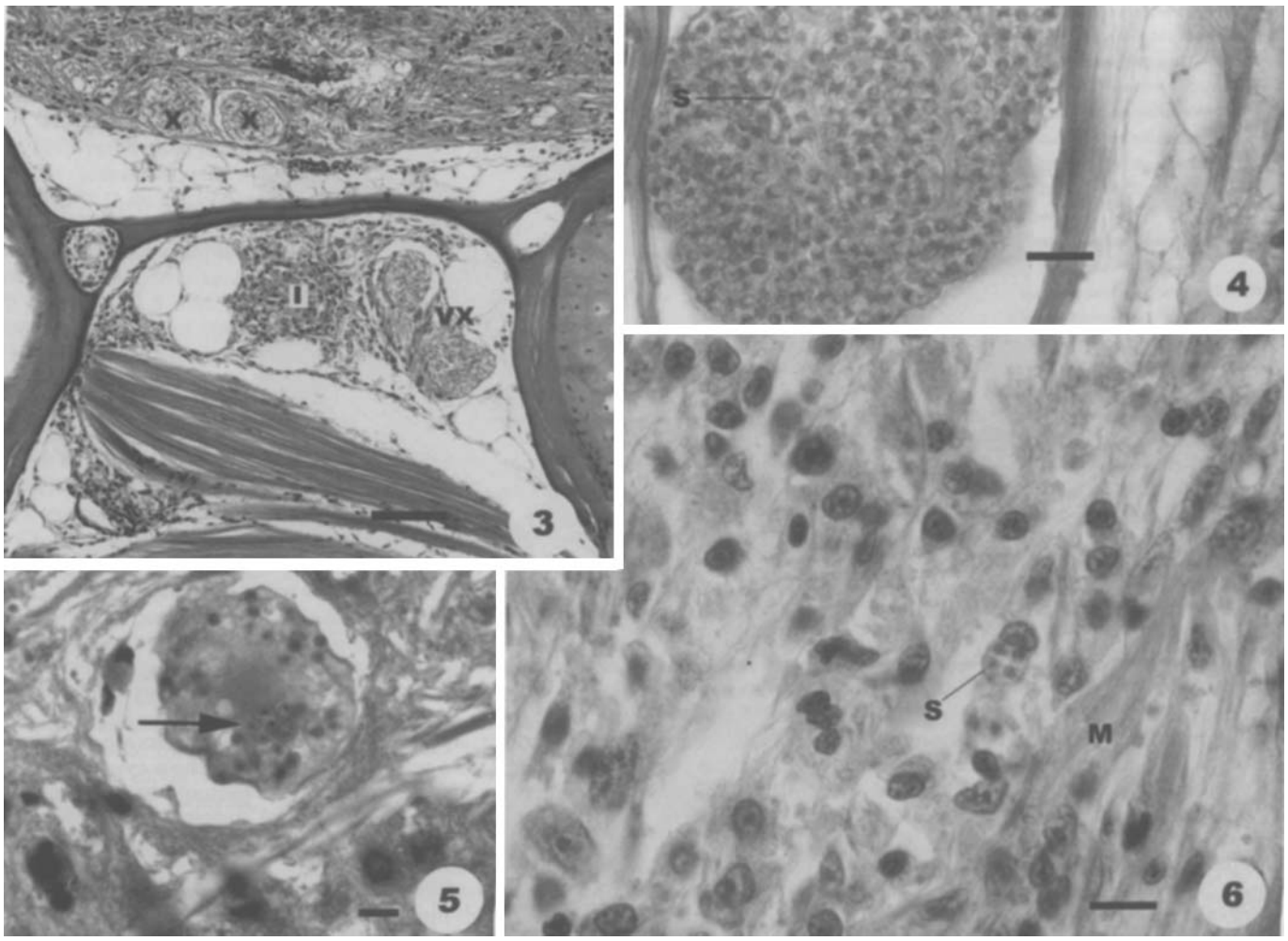

Fig. 3–6. Histological sections of zebrafish central nervous system infected with *Pseudoloma neurophilia* n. g., n. sp.. Hematoxylin and eosin. **3.** Xenomas (X) in the spinal cord and a ventral nerve root (VX), I = inflammation associated with released spores in phagocytes. Bar = 75 μm. **4.** Xenoma in spinal cord. S = septa formed by sporophorous vesicle. Bar = 10 μm. **5.** Xenoma with sporoblasts (arrow). Bar = 10 μm. **6.** Severe myositis in somatic muscle. M = muscle fiber. Spores (S) within phagocyte in muscle. Bar = 10 μm.

the microsporidium in this investigation clusters with *Ichthyosporidium* (Fig. 1), boostrap support at this node was reduced from 92% to 57% when *L. acerinae* was included in the data set. However, the zebrafish microsporidium was 12.1 % different from *Ichthyosporidium.*

DISCUSSION

Our study of this interesting microsporidium from zebrafish was initiated because of its high prevalence and association with morbidity in our laboratory colony. One of the most important conditions observed in zebrafish colonies is emaciation, often referred to as "skinny disease". This condition is not due solely to anorexia as affected fish often exhibit food in their digestive tracts. It should be noted that those infections confined to the central nervous system were usually not associated with prominent inflammatory changes, whereas spores within phagocytes were associated with inflammation around the ventral nerve root and severe myositis in the somatic musculature. Some infected fish also exhibited scoliotic changes, as was reported by de Kinkelin (1980). The considerably higher prevalence (87–97%) and intensity of *P. neurophilia* n. g., n. sp. in emaciated zebrafish compared to apparently healthy fish (0–33%) also suggests that the parasite may be linked to the disease.

Small subunit rDNA sequence comparison is a well-recognized technique for providing valuable information about phylogenetic relationships (Hillis and Dixon 1991). This technique is particularly important for the microsporidia because their spores have relatively few useful characteristics for taxonomic studies. Furthermore, developmental stages (which may provide

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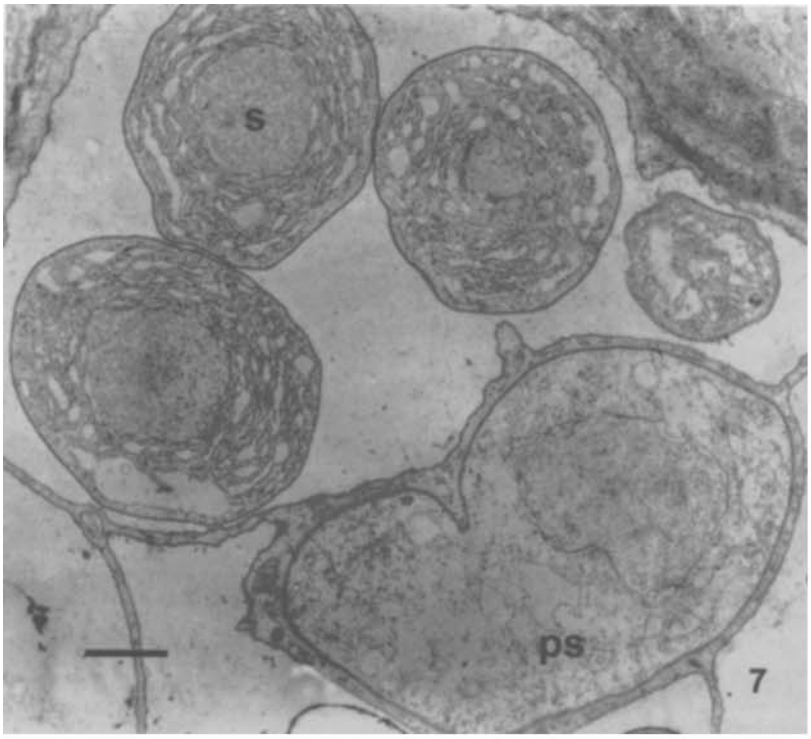
Fig. 7, 8. *Pseudoloma neurophilia* n. g., n. sp., TEM. **7.** Prespore development. Presporoblast (PS) (= sporont?) directly in contact with host cytoplasm. Note moderately thickened plasmalemma. S = sporoblast, free within SPOV. Bar = 1 μm. **8.** Fully developed spore. Bar = 1 μm.
Fig. 9. Xenoma of *Pseudoloma neurophilia* n. g., n. sp. with spores segregated into sporophorous vesicles, TEM. Bar = 10 μm.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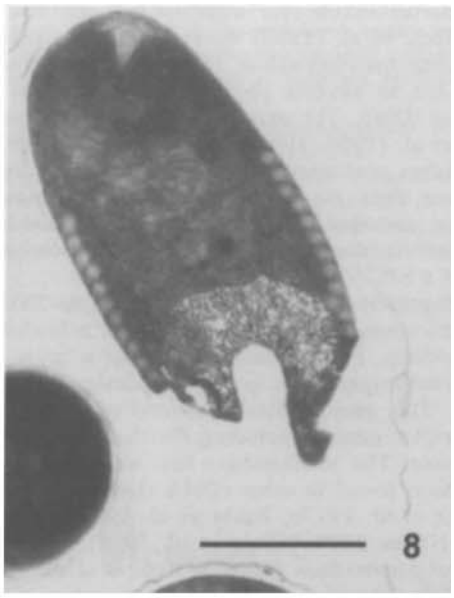
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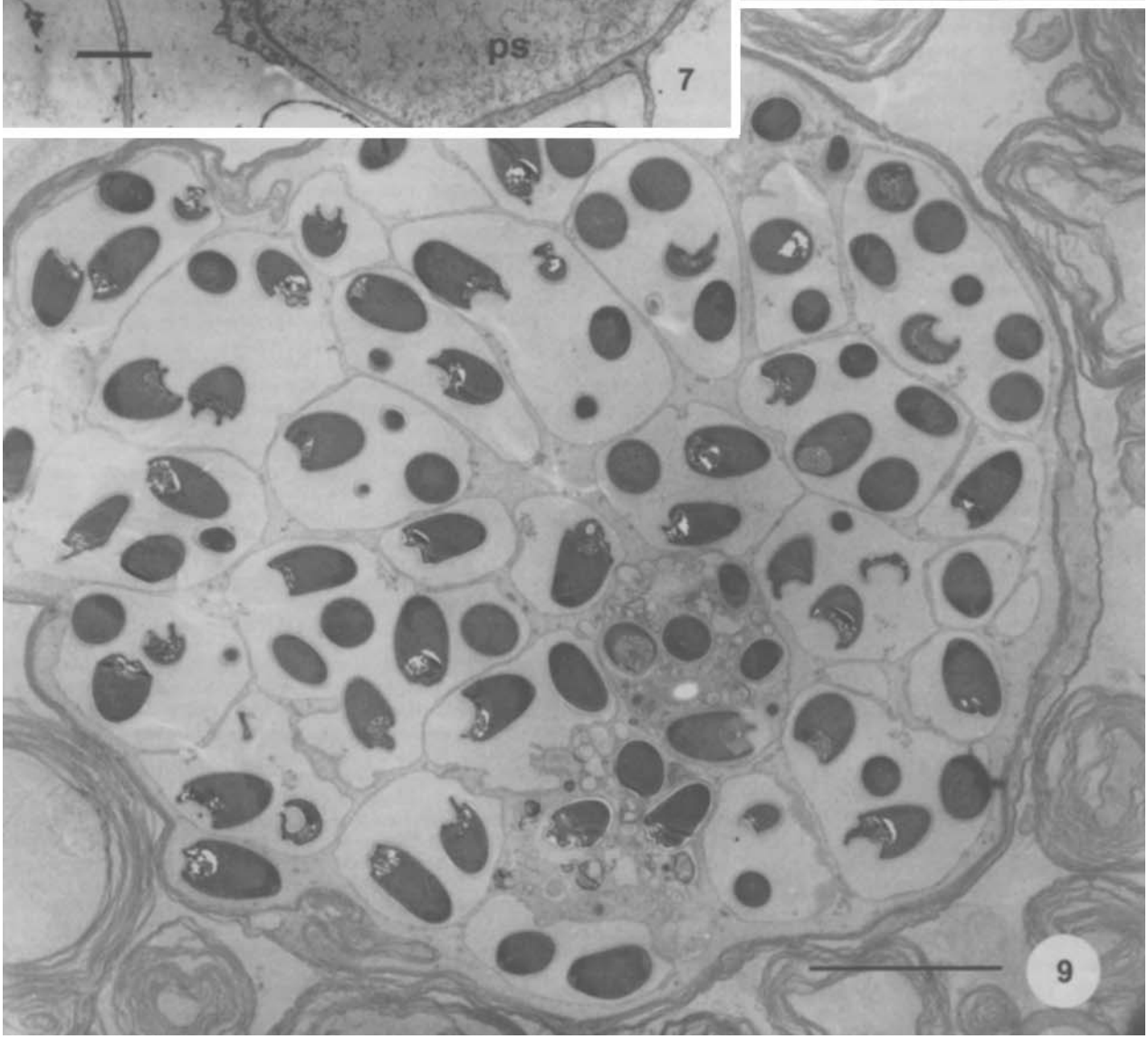
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more useful taxonomic information) are frequently absent. Vossbrinck et al. (1993) developed PCR primers capable of amplifying microsporidian rDNA directly from host tissues, which led to several phylogenetic studies of microsporidia based on rDNA. The most inclusive studies have been those of Baker et al. (1995, 1997), in which microsporidia from mammals, fishes, and insects were analyzed. They found that rDNA sequence data do not support the traditional taxonomic schemes, and that important morphological characters for use in higher classification (e.g. number of nuclei in spores, presence of a SPOV) appear to be convergent.

Phylogenetic analysis using comparable SSU sequences of other microsporidia suggested that the zebrafish-infecting microsporidium, *Pseudoloma neurophilia* n. g., n. sp., belongs to the "*Ichthyosporidium* group" as designated by Baker et al. (1995). This group contains several of the important fish microsporidian genera, including *Pleistophora, Glugea, Spraguea,* and *Loma.* The relationships that we observed are consistent with those found in other rDNA studies of fish microsporidia (Docker et al. 1997b; Baker et al. 1995, 1997; Cheney et al. 2000; Nilsen 2000; Nilsen et al. 1998). Previous analyses of the *Ichthyosporidium* group by Kent et al. (1999) and Nilsen et al. (1998) showed some similar relationships. For example, the genus *Pleistophora* was polyphyletic, and xenoma-forming microsporidia occurred in various clades.

Our study now brings into question the importance of the formation of SPOVs as well, with *Ichthyosporidium* (no SPOVs) clustering with *Pseudoloma* n. g. and *Loma* spp. (both with members that form SPOVs or related structures). Lom and Pekkarinen (1999) conducted a detailed ultrastructural study of *Loma acerinae,* and determined that its SPOVs were actually formed by the host, and thus were technically a "parasitophorous vesicle" rather than an SPOV. Within the genus *Loma,* the development of the sporogony vacuole has not been sufficiently studied for most species, and this vacuole in other species may also be of host origin (Lom and Pekkarinen 1999). These authors concluded that more *Loma* species should be reexamined, and that the presence and number of spores within SPOVs may be a less important taxonomic criterion than previously held. Our rDNA analyses and those of Cheney et al. (2000) support the views of Lom and Pekkarinen (1999) and suggest that *L. acerinae* should be transferred to another genus.

Spores of *Pseudoloma* n. g. were clearly segregated into clusters of up to 16, and appeared to develop within a true SPOV. In microsporidia that induce host-derived envelopes (parasitophorus vacuoles) the interfacial envelope is formed by a double membrane of host ER that surrounds the proliferating cells in very close proximity (Cali and Takvorian 1999). No evidence of this was seen in our study (Fig. 7). The earliest form observed by electron microscopy, probably a sporont, had a relatively thickened plasmalemma in direct contact with the host cell cytoplasm and there was no evidence of host ER surrounding this stage (Fig. 7). The next developmental stage that we detected was the sporoblast, which was clearly within an interfacial envelope. A more detailed ultrastructural study on the development of this microsporidium is warranted to more precisely identify these stages. As it was extremely difficult to find stages other than fully developed spores, this would best be accomplished using experimental hyper-infections. Merogony must be very brief with this species, or occur in another tissue location. In contrast, other xenoma-forming microsporidia, such as *Loma* and *Glugea,* exhibit numerous merogonic and sporogonic stages throughout xenoma development (Cali and Takvorian 1999). Transmission studies are underway to elucidate the association of the parasite with disease and to provide material for investigations of early parasite development.

Pseudoloma neurophilia n. g., n. sp. falls within a clade of two other xenoma-forming genera, *Loma* and *Ichthyosporidium,* being more closely related to the latter based on rDNA analysis. Although bootstrap support was relatively weak at the node separating *Pseudoloma* n. g. from *Ichthysporidium,* all forms of analyses supported it. We did not assign the microsporidium from zebrafish to *Ichthyosporidium* as the former forms SPOVs, and the latter forms diplokaryons. Moreover, the difference of 12.1% between these two genera is higher than that typically found in related microsporidian species within a genus (Docker et al. 1997b; Weiss & Vossbrinck 1998). *Pseudoloma* n. g. differs from *Loma* in that it forms SPOVs with up to 16 spores. Thus, based on molecular and morphological data available at present, it is most appropriate to assign the zebrafish microsporidium to a new genus and species.

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ANNOUNCEMENT

FASEB Ciliate Molecular Biology Meeting
July 28–August 2, 2001

Saxtons River, Vermont
Vermont Academy

Meeting Organizers:

Geoffrey Kapler, Texas A&M University, gkapler@medicine.tamu.edu
Jacek Gaertig, University of Georgia, jgaertig@cb.uga.edu
Laura Landweber, Princeton University, lfl@princeton.edu

Applications and abstracts accepted on line at
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Closing date for abstract submission:
June 14, 2001

ADDENDUM

In the review article by John R. Preer, Jr., *J. Eukaryot. Microbiol.*, **47**(6), 2000. pp. 515–524, **"Epigenetic Mechanisms Affecting Macronuclear Development in *Paramecium* and *Tetrahymena*"** the author notes:

"To begin this paper I stated that epigenesis includes all non-Mendelian inheritance, i.e. any inheritance that does not arise from DNA base changes. I should have noted that the inheritance of infectious elements and mitochondrial inheritance are non-Mendelian, but are based on DNA and should not be included in epigenesis."