J. Parasitol., 85(6), 1999 p. 1114–1119

A NEW *MICROSPORIDIUM* SP. (MICROSPORIDIA) FROM THE MUSCULATURE OF THE MOUNTAIN WHITEFISH *PROSOPIUM WILLIAMSONI* FROM BRITISH COLUMBIA: MORPHOLOGY AND PHYLOGENY

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ABSTRACT: Few microsporidia have been reported from whitefish species (subfamily Coregoninae). For the most part, these microsporidia have been incompletely described. In a survey of parasites of mountain whitefish *Prosopium williamsoni* collected from Kootenay Lake, British Columbia, we encountered an unusual microsporidium infecting the endomysium of the skeletal musculature. Spores were uninucleate, ovoid to pyriform, and were 5.6 (5–7) μm × 3.2 (3–4) μm with 13–16 coils in the polar filament. We describe here this organism as a new species based on its site of development and its relationship among fish microsporidia based on small subunit ribosomal DNA sequence data, i.e., our analysis showed that it is not closely related to other microsporidia for which ribosomal DNA sequence is available thus far.

Microsporidia infect essentially all animal groups and are common in fishes, representing about 100 described species in some 20 genera (Canning and Lom, 1986). Several fish microsporidia have been provisionally placed in *Microsporidium* because inadequate information is available on their developmental stages to assign them to a specific genus. Ribosomal DNA sequence analysis has recently been used to resolve the taxonomy of certain microsporidia (Baker et al., 1995, 1997) and may be useful for more definitive identifications in cases where developmental stages are not observed.

We observed a heavy infection by an unusual microsporidium in the somatic muscle of a mountain whitefish *Prosopium williamsoni* from British Columbia, Canada. As only spores were observed it was difficult to determine its precise generic status, and thus it was placed in the *Microsporidium.* Ribosomal DNA analysis of this microsporidium suggests that it belongs to *Ichthyosporidium* as designated by Baker et al. (1997) but shows no close relationship to any sequenced microsporidia including members of the *Pleistophora, Glugea,* and *Spraguea.*

MATERIALS AND METHODS

Twenty-three adult mountain whitefish (~1–2 kg) were collected by seine from Kootenay Lake, British Columbia, Canada (N49°30″W 117°30″) on 17 March 1995. The fish were transported on ice to the Pacific Biological Station, Nanaimo, British Columbia, Canada for examination the following day.

Histology and electron microscopy

One fish was found to be infected with the microsporidium described below. Microsporidian spores (n = 30) from the skeletal muscle were measured from wet-mount preparations using an ocular micrometer. Infected muscle and other organs were preserved in Davidson's fixative, processed for histology, and slides were stained with Harris' hematoxylin and eosin or periodic acid Schiff's. Muscle tissue was also preserved in 4% glutaraldehyde in Millonig's buffer and processed for transmission electron microscopy using standard techniques.

Received 4 November 1998; revised 28 April 1999; accepted 28 April 1999.

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Spore isolation

Muscle tissue was chopped finely, passed through a wire mesh in Earl's buffered saline solution (EBSS), and centrifuged at 3,000 rpm for 10 min. The pellet was resuspended in EBSS, homogenized at low speed, and centrifugation and resuspension were repeated. Large pieces of tissue debris were removed by successive filtration through 300-μm and then 50-μm nylon screens. The filtrate was layered onto a 34/51% Percoll gradient and centrifuged at 1,900 rpm for 45–60 min. The layers and pellet were removed separately, concentrated by a further 10 min centrifugation at 3,000 rpm, and the spores were stored at −70 C. Throughout the process, visual inspection verified the presence of the intact spores at each step.

DNA isolation and polymerase chain reaction (PCR)

DNA was extracted from 30 μl (packed volume) of purified spores washed and resuspended in TE buffer (10 mM Tris, 1 mM EDTA), pH 8.0, with the addition of 1 mg/ml yeast tRNA prior to bead-beating to enhance DNA recovery. Shaking was performed with 0.5-mm silica beads for 3 min in a Mini-Beadbeater (Biospec Products, Bartlesville, Oklahoma). The suspension was extracted twice with phenol–chloroform–isoamyl alcohol and the DNA precipitated on ice with sodium acetate and ethanol. The DNA was resuspended in TE, quantified, and stored at 4 C.

The microsporidian DNA was originally amplified by PCR with primers described by 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 rDNA genes, respectively, and have been used successfully in several other microsporidia to amplify rDNA fragments of 1,350–1,550 bp in length (Vossbrinck et al., 1993; Zhu et al., 1994).

The remaining 5′ end of the SSU was amplified using the forward primer 18eMIC (5′-CACCAGGTTGATTCTGCC-3′) described by Docker et al. (1997) along with the reverse primer Mpro1r (5′-CTTGATTTCTCTTCCGCACTA-3′) located 3′ of 530f, generating a fragment of about 700 bp.

Each 50-μl PCR reaction used standard PCR buffer (Gibco BRL, Gaithersburg, Maryland), 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, 25 pmol each primer, 1.25 units Taq DNA polymerase (Gibco BRL), and 600 ng template DNA. The reactions were run in a GeneAmp 9600 Thermocycler (Perkin Elmer Cetus, Norwalk, Connecticut) for 30 cycles consisting of denaturation at 94 C for 1 min, primer annealing at 50 C for 1 min, and extension at 72 C for 3 min. These steps were preceded by an initial 3 min denaturation at 95 C and followed by a final 10-min extension at 72 C. The microsporidian-specific PCR product was excised from low melting point agarose and purified according to the freeze–thaw method of Qian and Wilkinson (1991) for subsequent cloning, sequencing, or both.

Cloning and sequencing

Primers 530f and 580r were synthesized to include *EcoR*I and *BamH*I restriction sites, respectively, at their 5′ ends. The purified PCR product from isolated spores was cut with these restriction enzymes and cloned

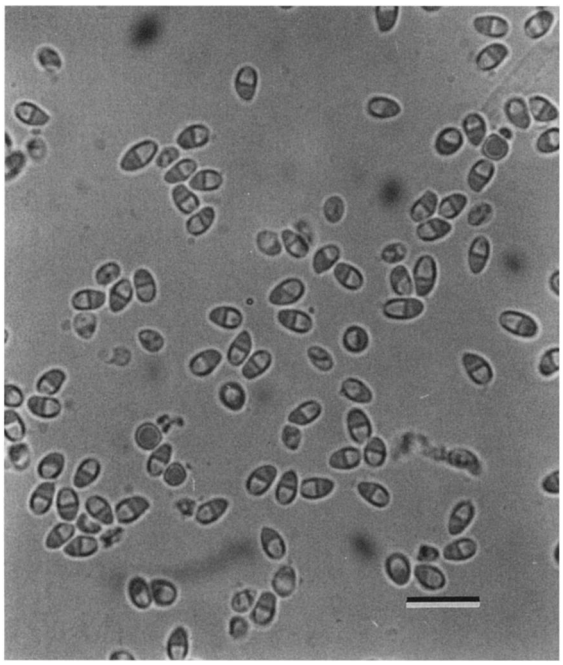


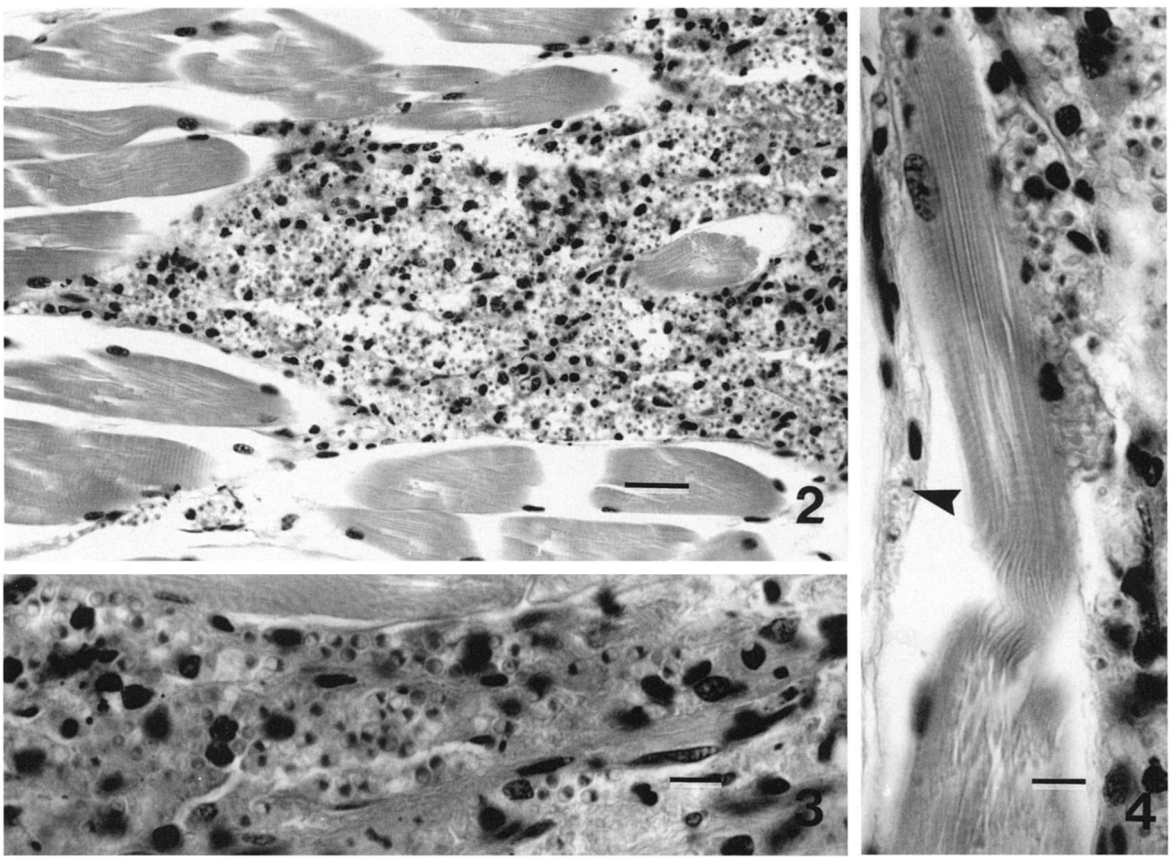
FIGURE 1. Wet-mount preparation of *Microsporidium prosopium* spores. Bar = 10 μm.

into the phagemid vector pBluescript II (SK− and SK+) (Stratagene Corp., La Jolla, California) by standard procedure. DNA was purified from overnight cultures by standard alkaline lysis miniprep protocol.

One clone with each of the SK− and SK+ vectors was selected and the presence of the desired inserts was verified by restriction enzyme digests. Single-stranded DNA was generated from both clones using VCS-M13 helper phage (Stratagene). Both strands of the 2 clones were sequenced from single- and double-stranded DNA using the Sequenase Version 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, Ohio). The 18eMIC-Mpro1r PCR fragment was sequenced directly using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, Ohio).

Phylogenetic analysis

The SSU rDNA was aligned and analyzed in 2 separate groups. First, representative microsporidia from all groups for which a complete SSU rDNA (18f to 1492r) (Weiss and Vossbrinck, 1998) has been published were aligned by eye along with that for the microsporidium from whitefish being analyzed in this study. Regions of uncertain alignment were eliminated from the analysis. These alignments are available at http://www.pac.dfo-mpo.gc.ca/sci/sealane/micro/Index.htm. Second, a number of partial SSU rDNA sequences of fish and related microsporidia were available from GenBank. These sequences were aligned with the homologous region of the whitefish microsporidium and the sequences were analyzed in this region for which all species presented had been sequenced. In both cases the aligned sequence data were analyzed using the computer package PAUP (beta version 4.0b1, Sinauer Associates, Sunderland, Massachusetts). The analyses used were the branch-and-bound algorithm for maximum parsimony analysis, the neighbor-joining algorithm for distance analysis, and the heuristic search with the maximum-likelihood algorithm.



FIGURES 2–4. Histological sections of whitefish muscle infected with *Microsporidium prosopium* n. sp. Arrowhead = microsporidian spores. Hematoxylin and eosin. **2.** Heavy infection of the endomysium showing a massive number of spores between myofibers. Bar = 50 μm. **3.** High magnification showing spores in the cytoplasm of cells within endomysium. Bar = 10 μm. **4.** Light infection of spindle-shaped cells suggestive of fibrocytes within the endomysium. Bar = 10 μm.

FIGURE 5. Electron microscopy of *Microsporidium prosopium* in the endomysium. Spores are free in the cytoplasm of putative fibrocytes (f); m = muscle fiber. Bar = 5 μm.

DESCRIPTION

Microsporidium prosopium n. sp.
(Figs. 1–6)

One of 23 adult mountain whitefish exhibited a massive infection of the skeletal muscle by the microsporidium, resulting in numerous, white macroscopic lesions (about 5 mm × 2 mm) throughout the musculature. Wet-mount preparations revealed that the lesions were comprised primarily of microsporidian spores (Fig. 1).

Histological examination did not reveal the microsporidium within mature muscle fibers, and it was difficult to ascertain the exact cell type that was infected. In areas of light infections, i.e., cells with the fewest number of spores, the cells were spindle shaped and thus were suggestive of fibroblasts or fibrocytes (Figs. 2–4). However, many of these infected cells appeared to occur just inside the basal lamina of myocytes.

Multiple foci of massive infection occurred between intact muscle fibers, apparently within the connective tissue of the endomysium. Heavily infected cells were rounded with an eccentric nucleus, and were replete with 20–30 spores. Consistent with fibroblasts, many of the heavily infected cells were stained bluish with periodic acid Schiff (PAS) stain. Intercellular collagen fibers that stained rose to purple with

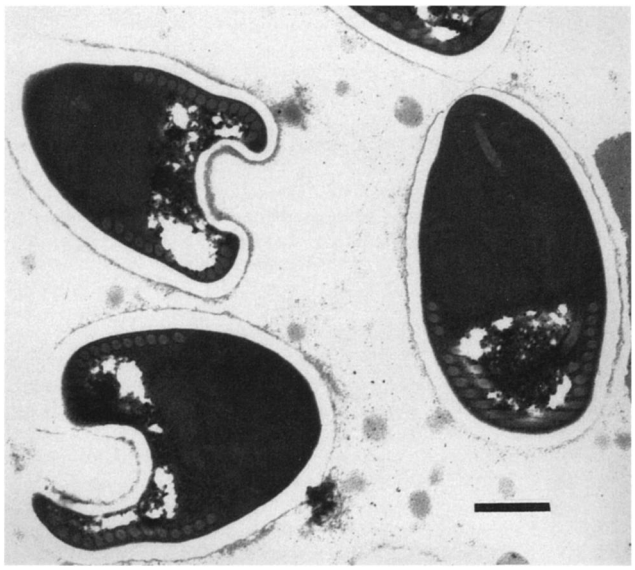


FIGURE 6. Electron micrograph of spore of *Microsporidium prosopium*. Bar = 1 μm.

PAS were observed throughout the lesions. The infection was not observed in any other organ.

Electron microscopy of the infected tissue (Figs. 5, 6) revealed round and smooth host cell nuclei with a pachychromatic pattern and no visible nucleolus. Inclusions suggestive of lysosomes were observed in some cells, whereas others exhibited abundant rough endoplasmic reticulum. Uninfected cells consistent with mature fibrocytes were observed mixed with the infected cells. Extracellular collagen was not abundant. The microsporidian spores were free in the cytoplasm; they were not encased in a sporophorous vesicle. Other ultrastructural characteristics of the microsporidium are described below.

Similar phylogenetic trees were generated using all 3 algorithms, and all suggested that the microsporidium in study is not closely related to other fish microsporidia examined thus far (Fig. 7). These analyses place *M. prosopium* n. sp. in the *Ichthyosporidium* group, being least distant from *Pleistophora anguillarum* Hoshina, 1951 from eels.

Taxonomic summary

Type host: Mountain whitefish *P. williamsoni (Girard).*
Location in host: Skeletal muscle endomysium.
Locality: Kootenay Lake, British Columbia.
Prevalence: One of 23.
Type material: Hapantotypes in histological sections and heavily infected tissue preserved in formalin, Canadian Museum of Nature, Invertebrate Collection (Parasites), Ottawa, Canada; catalogue numbers CMNP 1999–0012.
Etymology: Denotes the generic name of the host.

Remarks

The microsporidium infected the connective tissue, rather than muscle fibers, in the somatic muscle. Fibrocytes were infected primarily. However, the infection also appeared to occur just within the basal lamina of myocytes, which is the area occupied by myocyte nuclei and satellite cells. The latter cells are also spindle shaped, and thus these cells also have been infected.

The microsporidium described herein is apparently an undescribed species and may belong to a new genus. There are a few incomplete descriptions of microsporidia in whitefishes, and our isolate appears to be different from these organisms. *Glugea hertwigi* Weissenberg, 1911 has been reported to infect the muscle, urinary bladder, and subcutaneous connective tissue of *Coregonus lavaretus* in Russia (Shulman and Shulman-Albova, 1953; Lom and Dyková, 1992); *Thelohania ovicola* Auerbach, 1910 was described from the ovaries of *Coregonus exiguus*

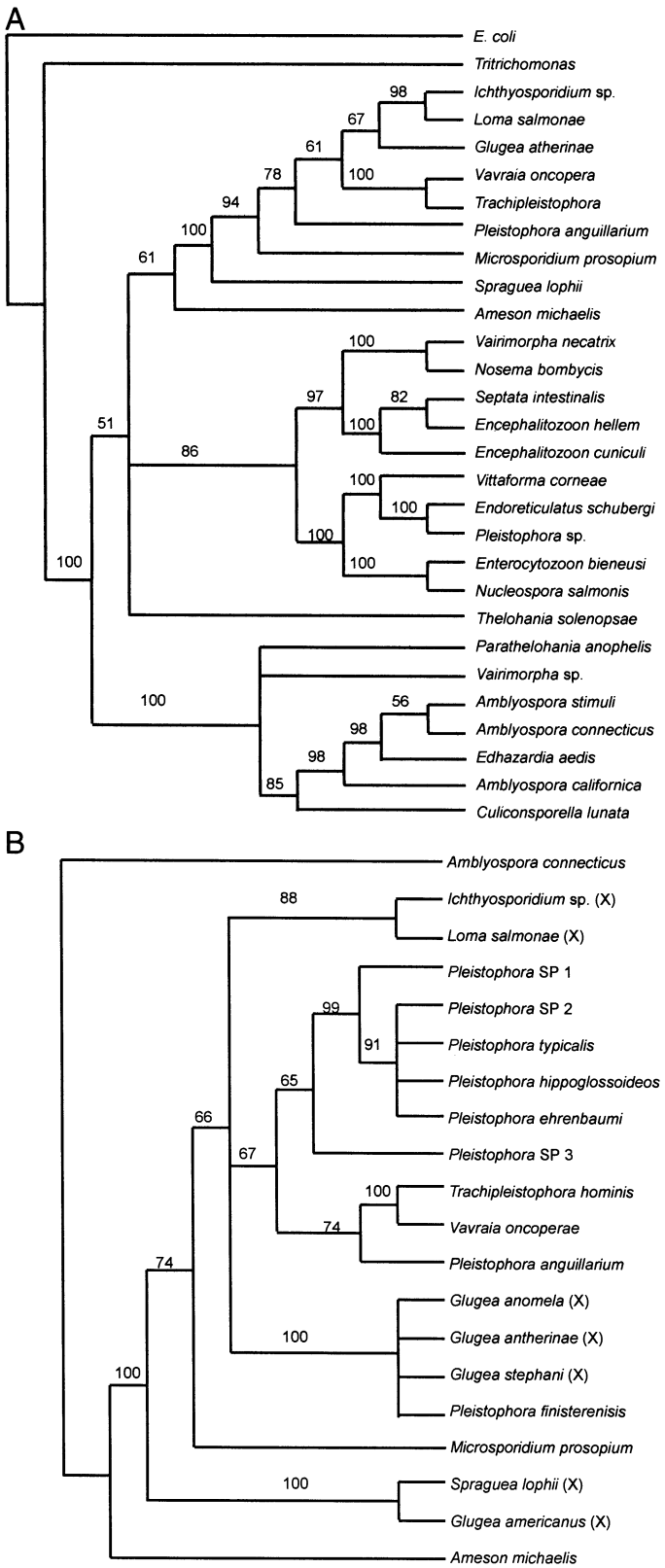


FIGURE 7. Phylogeny of microsporidia based on small subunit rDNA analysis. Maximum likelihood analysis. A. Representatives of all available microsporidian genera. B. The *Ichthyosporidium* group as designated by Baker et al. (1997). X = microsporidia that form xenomas.

from Switzerland (Auerbach, 1910); Dechtiar et al. (1989) reported a *Pleistophora* species in the muscle of *Coregonus aleuticus* from Ontario, Canada; and Arai and Mundry (1983) found a microsporidium that they identified as a *Nosema* sp., in the intestines of the mountain whitefish *P. williamsoni* in British Columbia, Canada. *Microsporidium prosopium* n. sp. differs from all these species except *G. hertwigi* in that the former infects connective tissue cells, i.e., fibroblasts, and it differs from *G. hertwigi* in that the latter forms xenomas and its spores are enclosed in a sporophorous vesicle.

Other microsporidian genera of fishes that infect connective tissues include *Heterosporis, Ichthyosporidium,* and *Tetramica* (cf. Dkyová, 1995). These microsporidia differ from the one described here in that the former form xenomas, in addition to differences in spore morphology (Schubert, 1969; Matthews and Matthews, 1980; Sprague and Hussey, 1980). *Microsporidium peponoides* also infects connective tissue, but differs from *M. prosopium* in that it forms sporophorous vesicles (Canning and Lom, 1986). In addition, *M. prosopium* is not a member of *Pleistophora,* a common genus that infects the muscle of fishes, in that the latter forms a distinctive sporophorous vesicle.

Prespore developmental stages were not observed, and thus we were unable determine more specific information concerning the taxonomic status of the microsporidium in studies based on morphological criteria. Therefore, we provisionally place it in *Microsporidium.* The generic name *Microsporidium* is a collective group name used for "identifiable species of which generic positions for the time being uncertain" (Sprague, 1977). No sporophorous vesicles were seen, the spores were dispersed free in the cytoplasm, and thus we provisionally place *M. prosopium* in the Apansporoblastina.

Small subunit rDNA sequence comparisons 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 more useful taxonomic information) are frequently absent. Vossbrinck et al. (1993) developed PCR primers capable of amplifying microsporidia rDNA directly from host tissues. This 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 and presence of a sporophorous vesicle, appear to have multiple origins.

Phylogenetic analysis using comparable SSU sequences of other microsporidia suggested that *M. prosopium* 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. Microsporidium prosopium* did not cluster closely with any of the genera in this group, e.g., it was only 84% similar to its most closely related microsporidium (*Pleistophora anguillarum* from eels), a value generally below the intrageneric similarity seen with other microsporidia (Baker et al. 1997; Docker et al., 1997).

Our analysis of the *Ichthyosporidium* group shows some interesting relationships. We see, for example, that *Pleistophora finisterensis* Leiro, Ortega, Iglesias, Estévez and Sanmartín, 1996 clusters with the *Glugea* clade. This has been shown quite recently by Nilsen et al. (1998) using essentially the same analytical methods but by developing separate alignments from us. We therefore, show that the production of xenomas must have been lost in *Pleistophora finisterensis.* Nilsen et al. (1998) discussed the differences as the production of an amorphous coat rather than the induction or loss of induction of a xenoma in the host and concluded that the pleistophorans do not form monophyletic groups.

Glugea americanus from the goosefish *Lophia americanus,* was originally classified as *Spraguea lophii* (Doflein, 1898) because of its similar appearance to this microsporidium that parasitizes European anglerfishes *Lophius budegassa* and *Lophius piscatorius.* Based on ultrastructural evidence, Takvorian and Cali (1986) reclassified the microsporidium from the goosefish to its original genus, *Glugea* and gave it the species designation *G. americanus* Takvorian and Cali, 1986. *Spraguea lophii* differs from *G. americanus* in several ways. *Spraguea lophii* has 2 spore types; a large spore containing a diplokaryon and 3–4 polar tube coils plus a smaller uninucleate spore with 5 and 6 polar tube coils. In contrast, the spores of *G. americanus* are monomorphic and are uninucleate. *Spraguea* and *Glugea* differ in that members of the latter genus form sporophorous vesicles, but it has not been determined if these occur in the development of *G. americanus.* Our rDNA analysis indicates that *G. americanus* is closely related to, but distinct from, *S. lophii* and is not closely related to other *Glugea* species. Determination of whether *G. americanus* forms sporophorous vesicles is needed to ascertain if a new genus should be erected for this microsporidium or if it should be reclassified as a *Spraguea* species.

Microsporidia lack enough characters at the light microscopic level to be useful for classification, and as more electron microscopy studies have been done, the meaning of these ultrastructural and ecological details for taxonomic purposes have come into question. As newly characterized microsporidia are sequenced, it is becoming more apparent that characters such as the diplokaryon, the spore covering, and the number of spores/ sporont are not characters that can be used to erect or maintain monophyletic taxa. It appears to us that the ability to control the number of nuclei, or the ability to secrete a thick membrane around the sporoblast, e.g., *Pleistophora,* or the ability to induce a xenoma, etc., may be of fundamental ecological and evolutionary importance to the survival of microsporidia. They may maintain the ability to switch from 1 state to another depending on which tissue or cell type they are invading. This suggests that what we characterized as very basic morphological features of the microsporidia are more analogous to feeding structures in birds or insects and evolved rather rapidly. Whereas rDNA sequence comparisons are elucidating the relationships of microsporidia, only a few of the 110 genera, representing some 1,000 species, have been sequenced thus far. More comparative studies utilizing both ultrastructural and sequence character comparisons will give further insight to this question.

In conclusion, the parasite from mountain whitefish muscle appears to represent a new genus of fish microsporidia within the *Ichthyosporidium* group, but we must await description of its developmental stages before this probable new genus is designated. Furthermore, the SSU rDNA sequence analysis also shows that it is not closely related to other members of the *Ichthyosporidium* group. Therefore, we are compelled to place this organism in the collective group genus *Microsporidium.*

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