REFERENCES

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A NEW MICROSPORIDIAN INFECTING THE MUSCULATURE OF THE ENDANGERED TIDEWATER GOBY (GOBIIDAE)

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ABSTRACT: A previously unrecognized microsporidian (*Kabatana newberryi* n. sp.) is described from the musculature of *Eucyclogobius newberryi* (Gobiidae) in Big Lagoon, Humboldt County, California. Spores are ovoid, ranging in size from 2.8 ± 0.3 μm in total length and 1.9 ± 0.4 μm in width (measurements of 30 spores made by calculation from micrograph). The polar filament has 9–10 coils in 1–2 rows. Development occurs in direct contact with host muscle cell cytoplasm, without xenoma or sporophorous vesicle. Phylogenetic analysis of the new species and of 35 other microsporidians known to infect fish using 1,115 base pairs of aligned 16S rRNA gene indicate the new species is most closely related to *Kabatana takedai*. However, the new species differs by 11% sequence divergence from *K. takedai*. Divergence in morphology and genetic data allow for diagnosis from all other fish-infecting microsporidia and supports recognition of a new species of microsporidian, *Kabatana newberryi* n. sp., presently known only from a suspected specific host, the endangered tidewater goby *Eucyclogobius newberryi*.

The tidewater goby *Eucyclogobius newberryi* (Teleostei: Gobiidae), is a species endemic to California coastal lagoons and estuaries. It ranges from Tillas Slough (mouth of Smith River, Del Norte County) south to Agua Hedionda Lagoon (San Diego County) (Moyle, 2000). Historic records suggest that the tidewater goby once occurred in as many as 134 locations (U.S. Fish and Wildlife Service, 2006). There are currently 96 known populations, of which 23 are thought to be unstable (U.S. Fish and Wildlife Service, 2006). The tidewater goby was first considered threatened by the State of California in 1987. By 1994, the tidewater goby was classified as an endangered species under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 1994). Little parasitology has been conducted on this species. The only documented parasite to infect the tidewater goby is the metacercaria stage of the trematode *Cryptocotyle* sp. (Swenson, 1999).

Some 15 genera of microsporidia have been reported to infect fish (Lom, 2002). Three of these have been documented to infect members of the diverse Gobiidae: *Glugea*, *Loma*, and *Pleistophora* (Canning and Lom, 1986; Lom, 2002). Three *Glugea* species are known to infect 16 different goby species (Canning and Lom, 1986; Lom, 2002). An undescribed *Glugea* species has been reported from the intestine of 10 species of marine gobies (Naidenova, 1974; Canning and Lom, 1986). Because this *Glugea* infects such a large number of hosts, it likely represents more than 1 species (Naidenova, 1974; Lom, 2002). *Glugea shulmani* Gaisimagomedov and Issi, 1970, infects the intestine of 3 marine gobies: *Neogobius caspius*, *Neogobius melanostomus*, and *Neogobius melanostomus affinis* (Canning and Lom, 1986). *Glugea anomala* Moniez, 1887, infects the subcutaneous tissue of *Neogobius syrman* and *Pomatoschistus minutus* (Canning and Lom, 1986). Only 1 species of *Loma*, i.e., *Loma dimorpha* Loubes, Maurand, Gasc, De Buron and Barral, 1984, is known to infect a member of Gobiidae. *Loma dimorpha* has been reported from the digestive tract of *Gobius niger* Leiro, Bos, Peris, Estevez, Santamarina and Sanmartin, 1996. Only 1 species of *Pleistophora* is reported from Gobiidae. *Pleistophora tuberifera* Gasimagodedov and Issi, 1970, infects the subcutaneous musculature of 2 marine goby species: *Neogobius kessleri gorlap* and *N. melanostomus affinisi* (Canning and Lom, 1986; Lom, 2002).

Herein, we describe a new species of microsporidian from the musculature of *Eucyclogobius newberryi*. The life cycle, morphological features, and phylogenetic relationships and genetic divergence based on 1,115 base pairs (bp) of aligned 16S rRNA sequence data of the parasite are discussed.

MATERIALS AND METHODS

Collection of specimens

Eighty *Eucyclogobius newberryi* (Gobiidae) were collected using a small mesh seine in the southeast corner of Big Lagoon at the mouth of a small unnamed freshwater tributary, 0.5 km north of the town of Big Lagoon, California (41°9′86″N, 124°7′85″W). Approximately 5 to 10 specimens were collected monthly as part of *Eucyclogobius newberryi* life history monitoring that occurred throughout 2005. The presence of microsporidians was visually determined by the presence of white opaque muscular tissue (healthy individuals have relatively clear muscular tissue). Fish were killed in MS-222 so that visibly infected muscle tissue could be removed for examination or fixation. Mature microsporidian spores were observed by excising muscle tissue and making a wet mount. Smears were examined using light microscopy at ×1,000; 30 spore measurements were calculated from electromicrographs.

Histology and electron microscopy

Infected muscle tissue was fixed in Bouin's fixative, rinsed, dehydrated in an ethanol–tertiary butyl alcohol–paraffin series, and embedded in paraffin. Histological sections were cut at 10 μm. Slides were stained with hematoxylin and eosin. Muscle tissue for electron microscopy was fixed in 4% glutaraldehyde buffered in 0.2 M sodium cacodylate buffer (pH 7.2) for 10 hr, rinsed overnight in sodium cacodylate buffer, and then postfixed in 2% osmium tetroxide in buffer for 4 hr. Tissue then went through a dehydration process in an ascending ethanol series and propylene oxide. Tissue was then embedded in Epon (10–12 hr in each change). Semithin sections were stained with methylene blue–Azure II and observed by DIC (Differential Interference Contrast) optics. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed under a JEOL 100CXII transmission electron microscope operated at 60 kV.

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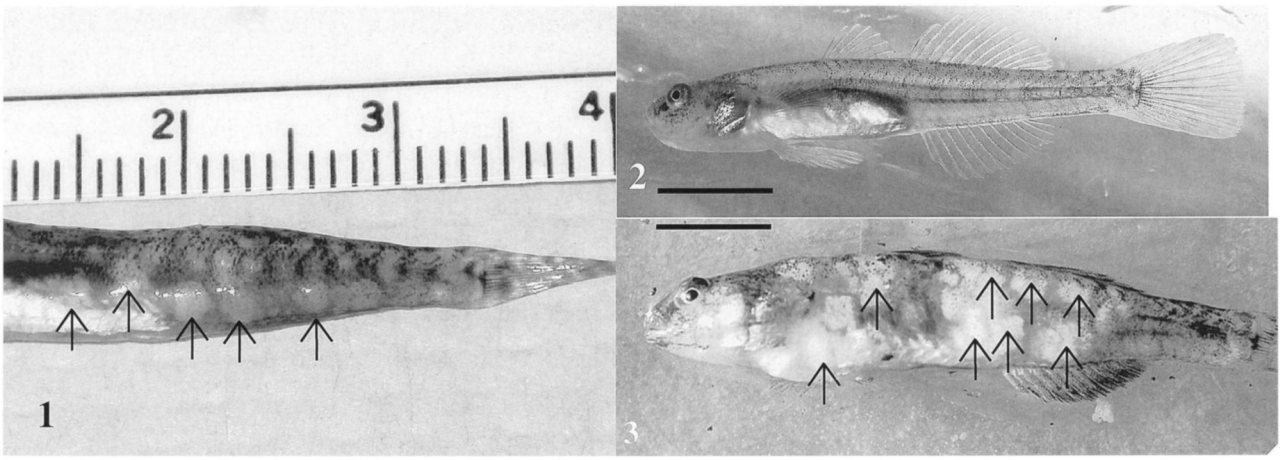


FIGURES 1–3. Macroscopic view of *Eucyclogobius newberryi* muscle tissue. (**1**) Individual infected with *Kabatana newberryi* from Big Lagoon, Humboldt County, California. Note white opaque muscular tissue (arrows). Ruler scale is in centimeters. (**2**) Healthy *E. newberryi* with "clear" muscle tissue. Scale bar = 5 mm. (**3**) Visibly infected individual from Rodeo Lagoon, Marin County, California. Scale bar = 5 mm.

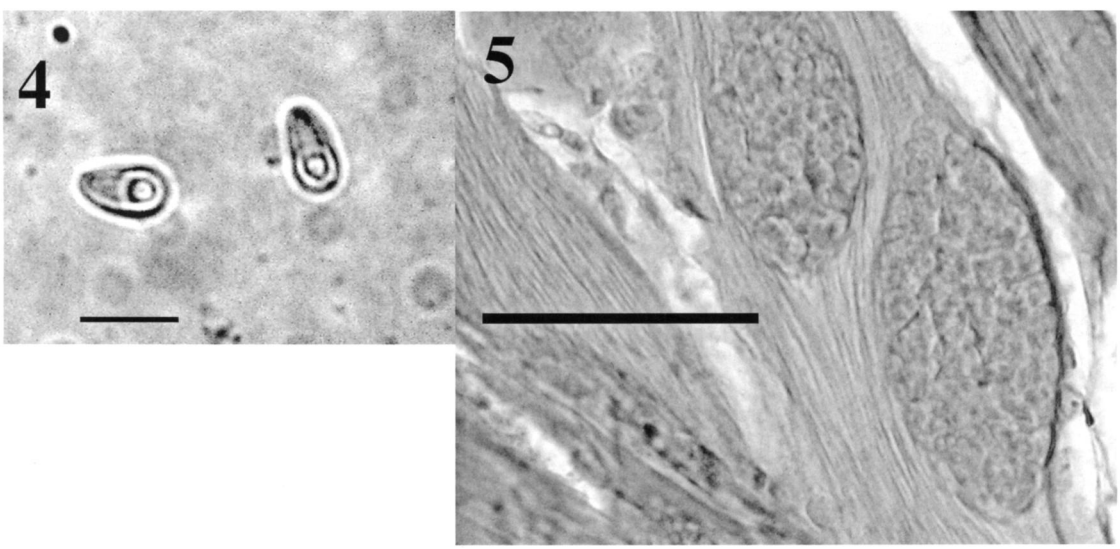
DNA isolation, polymerase chain reaction (PCR), and DNA sequencing

A wet mount was made of fresh, infected muscle tissue squashed between 2 glass slides to visually separate host tissue from microsporidian tissue using light microscopy at ×1,000. DNA extraction methods followed Miller and Kapuscinski (1996). Approximately 0.1 g of mature spores were isolated and added to 200 μl of 5% (weight/volume) Chelex (Sigma-Aldrich, St. Louis, Missouri) and 1 μl of 10 mg/m. proteinase-K in a 200-μl microcentrifuge tube. Samples were incubated at room temperature for 12–24 hr and boiled for 8 min.

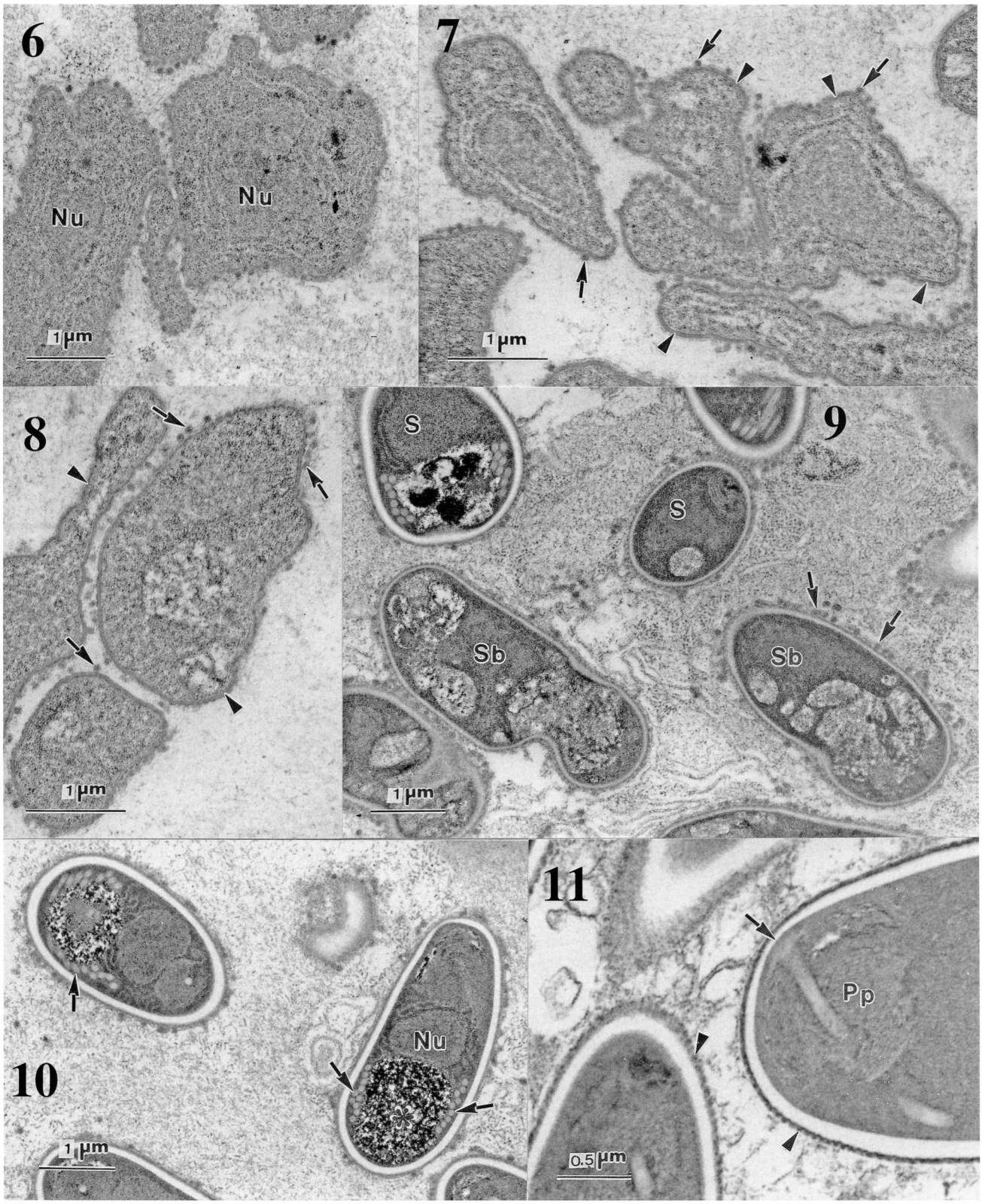
Microsporidian-specific primers 18 F and 1492 R were used to amplify a portion of the 16S rRNA gene (Vossbrinck et al., 1993). Each 50-μl PCR reaction tube contained 40 pmol of each primer, 400 μM of each dNTPs, 3 mM $MgCl_2$, 50 units of *Taq* DNA polymerase and proprietary reaction buffer (Promega, Madison, Wisconsin), and <250 ng of template DNA. Reactions were run on an ABI 2400 Thermocycler (Applied Biosystems, Foster City, California) for 35 cycles at 94 C for 45 sec, 50 C for 30 sec, and at 72 C for 90 sec. This cycling routine was preceded by a denaturation at 94 C for 3 min, and followed by a final extension at 72 C for 10 min. The amplified PCR product, approximately 1,400 bp in length, was sized using an ethidium bromide–stained 2.5% agarose gel and a 100-bp DNA ladder (Promega). This band was excised and then purified using a QIAquick PCR Purification Kit (Qiagen Company, Valencia, California) following manufacturer's instructions. Sequence data were generated using dye-terminated cycle sequencing chemistry (Beckman Coulter DTCS Quick Start kit, Fullerton, California) with 30 cycles of 96 C for 20 sec, 50 C for 20 sec, and 60 C for 4 min. Sequence data were generated in 2 segments using 1492 R (Vossbrinck et al., 1998) and 1047 R (Vossbrinck et al., 2004). Sequences were read on a Beckman Coulter CEQ 8000 XL sequencer.

Phylogenetic analyses

The rDNA sequence generated from the microsporidian infecting *E. newberryi* was combined with 35 fish-infecting microsporidians from GenBank (http://www.ncbi.nlm.nih.gov/) and aligned using Clustal X (Thompson et al., 1997). *Endorecticulatus schubergi* and *Vittaforme corneae* were selected as outgroup taxa as in previous phylogenetic analyses of fish-infecting microsporidia (Lom and Nilsen, 2003). GenBank sequence data were trimmed to be the same length as the *E. newberryi* sequence, with the aligned sequence data set consisting of 1,115 aligned positions including insertions and deletions. A gene tree was reconstructed under parsimony optimality criteria using a heuristic search algorithm in PAUP (version 4.0b10, Sinauer Associates, Sunderland, Massachusetts) with 1,000 random additional sequences. In-



FIGURES 4–5. Light microscopy of *Kabatana newberryi* at ×1,000 magnification in host muscle cell cytoplasm. (**4**) Mature spores in wet mount. Scale bar = 3 μm. (**5**) Multiple foci of infections in single host cell. Scale bar = 0.05 mm.



FIGURES 6–11. Transmission electron microscopy of life cycle stages of *Kabatana newberryi*. (**6**) Round-to-ovoid meronts in close contact with cytoplasm of the host cell. Nuclei (Nu). (**7**) Early sporont cells differentiating the coat (arrowheads) and numerous blisters (arrows) adhering to the coat. (**8**) Sporogonic plasmodia after the division showing the thickness coat (arrowheads) and some blisters (arrows). (**9**) Young sporoblast cells (Sb) differentiating the spore organelles and mature spores (S). Several blisters adhering to the exospore wall (arrows). (**10**) Mature spores showing 10 polar filament coils arranged in 1 or 2 rows (arrows), central nucleus (Nu), and posterior vacuole (*). (**11**) Ultrathin detail of the anterior pole of the spore showing the anchoring disk (arrow) and polaroplast (Pp). Tangential section of a spore wall showing the exospore with an irregular surface (arrowheads).

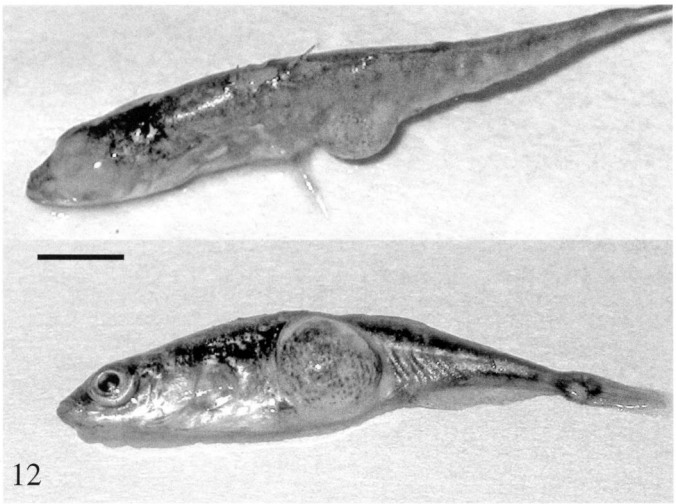


FIGURE 12. *Glugea anomala* infection in *Gasterosteus aculeatus*; Big Lagoon, California. Scale bar = 5 mm.

sertions and deletions were treated as missing data. Branch support was assessed by means of bootstrapping (with 10,000 pseudoreplicates).

DESCRIPTION

Kabatana newberryi n. sp.
(Figures 1–11, 13)

General diagnosis: Macroscopic lesions varying in diameter from 1 mm to 3 mm spread throughout musculature of *Eucyclogobius newberryi* found in Big Lagoon, Humboldt County, California (Fig. 1). Lesion border indiscriminant from surrounding healthy muscle tissue. Healthy *E. newberryi* with clear muscular tissue (Fig. 2). Similar infection observed in individuals from Rodeo Lagoon, Marin County, California (Fig. 3). Wet mounts viewed under ×1,000 magnification revealed mature ovoid microsporidian spores within lesions (Fig. 4). In advanced infections, multiple foci of infection present in a single host muscle cell (Fig. 5).

Ultrastructural details obtained by transmission electron microscopy showed that parasite development occurred in cytoplasm of muscle cells without presence of sporophorous vesicles during all life cycle stages. Meronts round to ovoid in shape, with 1 to multiple unpaired nuclei (Fig. 6). Cells divide by plasmotomy or binary fission giving rise to sporonts (Fig. 7). Plasmalemma of sporont cells acquire gradual densification because of deposition of amorphous material (Fig. 8). Numerous blisters adhering to coat observed (Figs. 8–10). In late sporont stage, coat becomes continuous and transforms to sporoblast cell (Fig. 9). In early sporoblast stage, differentiation of spore organelles and endospore wall becomes evident (Fig. 9). Mature spores from 2.8 ± 0.3 μm in total length and 1.9 ± 0.4 μm in width (Figs. 4, 10). Nucleus occupies central position in spore, surrounded by polyribosomes. Vacuole in a posterior position, with a posterosome among an electron-dense mass. Polar filament with 9–10 coils arranged in 1–2 rows (Fig. 10). Anchoring disk and anterior portion of polar filament located in apical region of mature spore. Exospore wall with irregular surface (Fig. 11).

Data matrix with 893 variable characters; 587 parsimony informative. Tree topology generated using Clustal X alignment and heuristic parsimony search (Fig. 13), similar to previous phylogenetic analysis of microsporidia infecting fish (Lom and Nilsen, 2003). Fish-infecting microsporidia broken into 4 clades (Lom and Nilsen, 2003). Clades resolved in current analysis were similar. The undescribed microsporidian resolved within clade "four" (see Lom and Nilsen, 2003) containing *Microgemma caulleryi*, *Tetramicra brevifilum*, *Sparguea lophii*, *Microsporidium seriolae*, *Microgemma* sp., and *Kabatana takedai*. Within this clade, the new microsporidian resolved as sister to *Kabatana takedai*; however, new species differs by 11% sequence divergence (uncorrected p-distance) from *K. takedai*. The phylogeny of fish-infecting microsporidians has changed significantly since the Lom and Nilsen (2003) phylogeny: *Kabatana newberryi* is now sister taxa to *Kabatana takedai*; *Kabatana* (*Microsporidium*) *seriolae* is now sister taxa to *Microsporidium* sp.

Taxonomic summary

Type host: Tidewater goby, *Eucyclogobius newberryi* (Girard, 1856).

Hapantotypes material: U.S. National Parasite Collection 99339–99341.

Site of infection: Musculature.

Type locality: Big Lagoon, Humboldt County, California (41°9′86″N, 124°7′85″W).

Prevalence: 105 of 1,513 (6.9%) tidewater gobies were visibly infected.

Etymology: The species is named after the specific epithet of the type host.

Remarks

Species of *Glugea*, *Heterosporis*, *Kabatana*, *Pleistophora*, *Tetramicra*, and the collective group, *Microsporidium*, are all known to infect muscle tissue (Dykova and Lom, 2000; Lom, 2002; Yokoyama et al., 2002). Among these genera, only species of *Pleistophora* and *Kabatana* develop within the host cell cytoplasm, as does *K. newberryi* (Fig. 2) (Lom, 2002). Several features differentiate species of *Kabatana* from *Pleistophora*. *Pleistophora* spp. meronts have a thick amorphous wall, with sporogony occurring within a well-defined sporophorous vesicle (Lom and Dykova, 1992; Lom, 2002). Conversely, *Kabatana* spp. occur in direct contact with degraded host cell cytoplasm, with sporogony occurring without a sporophorous vesicle (Lom, 2002). Our morphological analyses indicate the new species of the collective group, *Microsporidium*, develops in direct contact with host cell cytoplasm and lacks a sporophorous vesicle, consistent with species of *Kabatana*. Further, *Pleistophora* and *Kabatana* spp. have been shown to be genetically distinct from each other (Bell et al., 2001; Lom and Nilsen, 2003). Our genetic analyses indicated a close relationship between *K. newberryi* and *Kabatana* spp. Thus, we assign the new species to *Kabatana*.

Kabatana newberryi can be morphologically distinguished from the 3 other described members of the genus using a combination of several characters. Spores of *Kabatana takedai* are ovoid, averaging 3.4 × 2 μm in size and have 3–4 coils in 1 row (Lom et al., 2001). Spores of *Kabatana arthuri* are rounded, pyriform, often curved, and 3.1 × 2.1 μm in size: they have 5 coils (Lom and Dykova, 1992; Lom et al., 1999). Spores of *Kabatana seriolae* are ovoid to bent pyriform, averaging 3.3 × 2.2 μm in size (Lom et al., 1999). Mature spores of *K. newberryi* are distinct. They have a high number of polar filament coils (9–10 in 1–2 rows) and a smaller, ovoid shape (2.8 × 1.9 μm in size) than other mature spores in *Kabatana* spp.

All currently described species of *Kabatana* occur in trunk muscle cells (Lom, 2002). Some species cause serious problems in fish farms in Japan and Thailand. *Kabatana seriolae*, first described as *Microsporidium seriolae*, is the cause of "Beko" disease of farmed yellowtail (*Seriola quinqueradiata*) and red seabream (*Pagrus major*) in Japan (Egusa et al., 1988; Lom and Dykova, 1992). Infection is characterized by macroscopic concave skin depressions, caused by liquefaction of underlying muscle tissue (Lom and Dykova, 1992). *Kabatana takedai*, first described as *Microsporidium takedai*, infects several salmonids, including *Oncorhynchus masou*, *Oncorhynchus mykiss*, *Oncorhynchus keta*, and *Salvelinus leucomaenis* (Lom and Dykova, 1992). Lesions are in the form of cystlike bodies. The disease causes high mortality in cultured and wild trout and salmon in Japan (Lom and Dykova, 1992). *Kabatana arthuri*, formerly described as *Microsporidium arthuri*, infects musculature of sutchi catfish (*Pangasius sutchi*) in Thailand (Lom and Dykova, 1992). Spores of *Kabatana arthuri* are contained within host cell macrophages. This apparently helps transport spores through the muscle to the surface of the skin, where they may be released into the water. This is an efficient way to spread the infection while the host is still alive (Dykova and Lom, 2000). *Kabatana newberryi* infects muscle cytoplasm of *E. newberryi*, but has not been observed to cause muscle liquefaction, or to have spores contained in host cell macrophages as in other species of *Kabatana*. Minimum prevalence of *E. newberryi* in Big Lagoon, based on visual observations of live specimens, is about 7%. The highest prevalence of visibly infected individ-

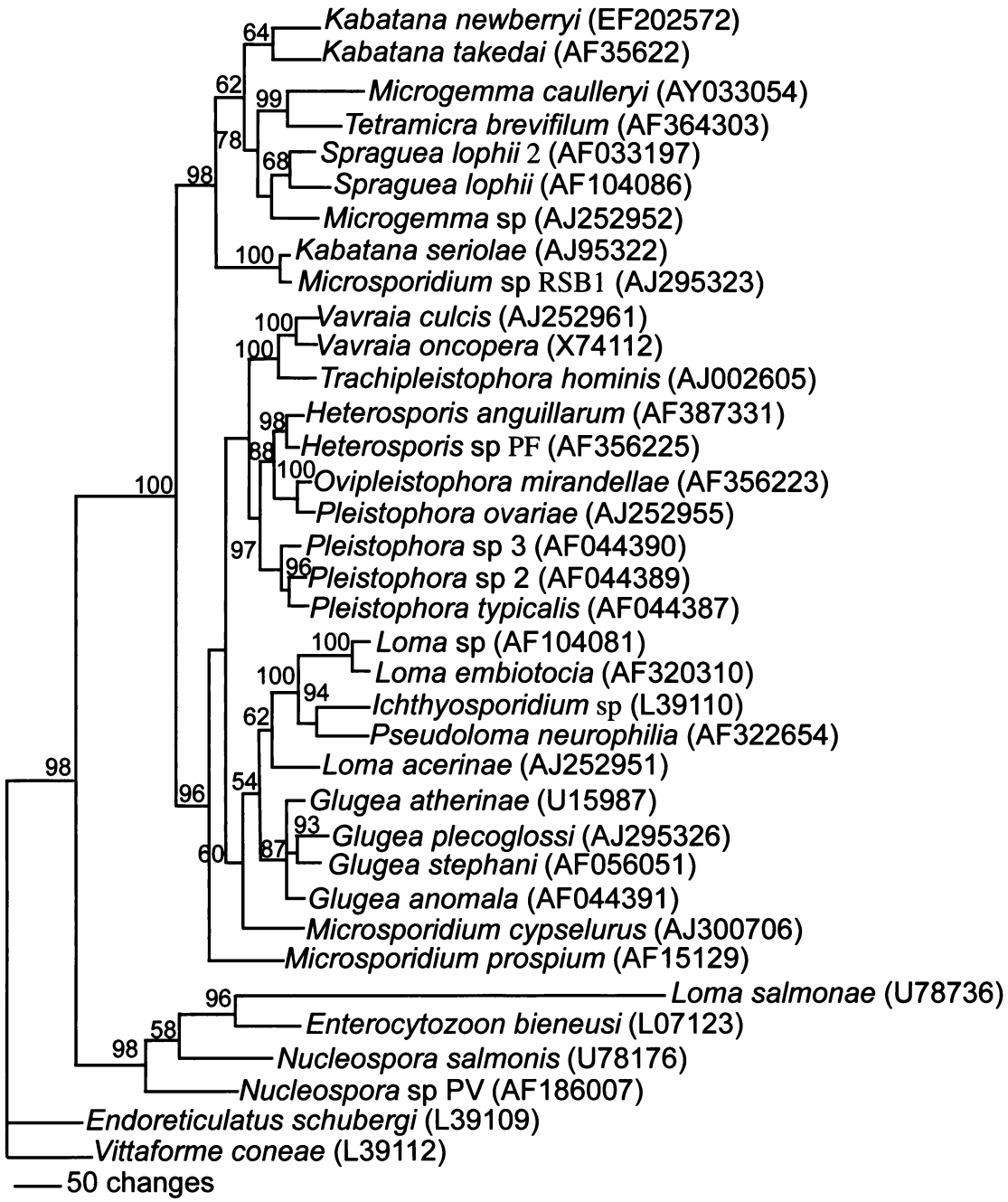


FIGURE 13. Maximum parsimony phylogram resulting from analysis of 1,115 aligned base pairs of rRNA sequence data (3,103 steps). GenBank accession numbers provided in parentheses. Bootstrap values above branches.

uals was observed December 2004 (23%), September 2005 (20%), and October 2005 (18%). The lowest prevalence of infected individuals was observed in February 2005 (0%), November 2005 (1%), and December 2005 (3%). The pathology induced by *K. newberryi* on the endangered *E. newberryi* is uncertain and additional studies are greatly needed to determine whether this parasite may be contributing to declines in *E. newberryi* throughout its range.

It is suspected that *Kabatana newberryi* is a host-specific parasite occurring on a federally endangered host, *Eucyclogobius newberryi*. Preliminary data suggest *K. newberryi* may be geographically widespread, occurring sympatrically with *E. newberryi* throughout northern California. *Eucyclogobius newberryi* presence–absence surveys conducted during the summers of 2003 and 2004 reported individuals with white, opaque muscular tissue infected with the microsporidian provisionally identified as *Kabatana newberryi*, throughout northern California. A similar microsporidian infection in *E. newberryi* was observed in Rodeo Lagoon, Marin County, California in 2005 (D. Fong, pers. comm.). No specific identification was made because the voucher specimens were preserved in formalin. However, the parasite appears very similar to *K. newberryi* in that it infects the cytoplasm of muscle cells (Fig. 1C) (D. Elliott, pers. comm.). *Kabatana newberryi* has not been observed in southern California, where populations of *E. newberryi* are especially declining (Lafferty et al., 1999). The dispersion mechanism of *K. newberryi* is not well understood at this time. Surveys evaluating

the presence and potential role of *K. newberryi* in tidewater goby declines are needed to assess whether this parasite represents a significant threat to its endangered host.

In addition to *Kabatana newberryi*, another unidentified microsporidian species was observed in Big Lagoon; this species was similar to *Glugea anomala*, which infected *Gasterosteus aculeatus*. This microsporidian forms a xenoma (Fig. 12) and mature spores range in size from 3–6 × 1.9–2.7 μm (larger than the mature spores mentioned in *G. anomala* literature). *Kabatana newberryi* can be distinguished from this unidentified microsporidian because it does not form a xenoma and has much smaller mature spores (2.8 × 1.9 μm). Thus, 2 microsporidian species coexist in Big Lagoon, an unidentified microsporidian similar to *Glugea anomala* and *Kabatana newberryi*.

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