FOLIA PARASITOLOGICA 49: 9-15, 2002

Occurrence of a new microsporidium in the skeletal muscle of the flying fish *Cypselurus pinnatibarbatus japonicus* (Exocoetidae) from Yakushima, Japan

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Key words: Microspora, microsporidium, flying fish, *Cypselurus pinnatibarbatus*

Abstract. A new microsporidium was observed in the flying fish *Cypselurus pinnatibarbatus japonicus* (Franz) (Exocoetidae) from Yakushima, Japan. Visual examination revealed the microsporidium to form white elongate nodules in the host's trunk muscle. Monomorphic spores were ovoid to pyriform in shape, with average dimensions of 4.1 × 2.2 μm and possessing a polar tube describing 13-15 coils. Histological observations showed that each parasite focus of infection was encapsulated by a host-produced fibrous membrane. The presence of sporophorous vesicles was not clearly determined. Ribosomal DNA sequence analyses showed the microsporidium to be discrete from other known fish muscle-infecting species and to be most closely related to a clade comprising the Pleistophoridae and *Glugea* spp. The parasite is provisionally placed as *Microsporidium cypselurus* sp. n.

Microsporidia infecting the musculature of commercially important fish species have a negative economic impact on fish products. Such microsporidia species, thus far reported (see Dyková 1995), belong to the genera *Heterosporis*, *Pleistophora*, *Kabatana* and the collective group *Microsporidium*. In Japan, *Microsporidium* infections in the trunk muscle of farmed yellowtail (Japanese amberjack) *Seriola quinqueradiata* and red seabream *Pagrus major* have caused serious damage to seed production (Egusa 1982, Egusa et al. 1988, Sano et al. 1998). *Microsporidium seriolae* forms elongate whitish nodules in the musculature of yellowtail, causes liquefaction of muscle fibres resulting in a characteristic concave body surface and, in extreme circumstances, may result in the death of the host fish.

The lack of reliable morphological characteristics has made it difficult to identify microsporidia at the species level and to discriminate between related species. Recent studies on the phylogeny of the Microspora using ribosomal DNA sequence analyses have revealed valuable information about the classification of fish-infecting microsporidians (Nilsen et al. 1998, Bell et al. 2001). Nilsen et al. (1998) showed the genus *Pleistophora* to be polyphyletic, whereas the work of Bell et al. (2001) identified a close relationship between *Microsporidium seriolae*, the *Microsporidium* sp. infecting red seabream and an unidentified microsporidium from Maltese gilthead seabream *Sparus aurata*. Further, Bell et al. (1999) developed a highly sensitive PCR assay for the detection of *M. seriolae*.

In the present study an unidentified microsporidium infection was found in the musculature of feral flying fish from Kagoshima Prefecture in Japan, a site of intensive yellowtail culture. Thus, it was suspected that the flying fish might act as a vector or as an alternative natural host for *M. seriolae.* Therefore, morphological and molecular analyses were conducted on this parasite in an attempt to establish its taxonomic position and more specifically its relationship with *M. seriolae*.

MATERIALS AND METHODS

A flying fish *Cypselurus pinnatibarbatus japonicus* (Franz) caught by an angler on the coast of Yakushima, Kagoshima Prefecture, Japan (30°20'N, 130º30'E) in April 2000, was found to be infected with an unidentified microsporidium, and was subsequently transported to the laboratory on ice. Microsporidian spores were freshly isolated from the skeletal muscle, examined in wet mount preparations and photographed using an oil immersion lens. Spore measurements (n = 30) were taken from enlarged photographs. Infected muscle tissues were fixed in 10% formalin, processed routinely for histology, and sections stained with Uvitex 2B and H&E (see Yokoyama et al. 1996). Tissues were also preserved in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. After dehydration, the tissues were embedded in Spurr's resin, and processed for transmission electron microscopy (TEM) using standard techniques.

Spore purification and DNA isolation

Parasite masses were excised from the musculature, squashed and passed through a steel mesh (Cell dissociation sieve-grinder kit, Sigma, USA) and then a nylon mesh series (100, 75, 50, 25, 10 and 5 μm) to remove large pieces of tissue debris. The filtrate was layered onto an equal volume of 50% Percoll and centrifuged at 750 × g for 15 min. The pellet was removed and re-suspended in distilled water. Purified spores

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were pelleted, washed in high concentration urea buffer (TNES-Urea: 10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 0.5% SDS; 4 M urea – modified from Asahida et al. 1996), resuspended in 0.5 ml of the same medium and vortexed (in a manner to maximise shearing directions) for 1 min with 0.4 g of 0.5-mm silica beads to facilitate the mechanical disruption of mature spore walls. After centrifugation at 1,000 × g for 3 min, the supernatant was removed, its volume adjusted to 500 µl, proteinase K added to a concentration of 100 µg/ml, and digestion allowed to occur overnight in a 37°C tilting waterbath. DNA was subsequently extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1) and diethyl ether, then precipitated at –80°C with 2.5 × volume of 95% cold ethanol and 0.1 × volume of 3M sodium acetate, and finally re-suspended in sterile water.

PCR and sequencing

Targeted DNA was amplified using the PCR primers described by Vossbrinck et al. (1993): forward primer 530f (5' GTGCCATCCAGCCGCGG 3'), reverse primer (5' GGTCCG TGTTTCAAGACGG 3'). Each 25 µl PCR reaction contained ~10 ng of genomic DNA, 25 pmol of each primer and utilised Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech UK Limited) which comprise ~1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP and stabilisers including bovine serum albumin. After an initial denaturation at 95°C for 5 min, samples were subjected to 30 cycles of amplification (denaturation at 95ºC for 30 s, primer annealing at 43ºC for 30 s, and extension at 72ºC for 1 min), followed by a 10 min terminal extension at 72ºC. All amplifications were performed on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The PCR product obtained was visualised in an ethidium bromide-stained 1% agarose gel, the DNA band excised and purified using the Prep-A-Gene DNA purification kit (Bio-Rad Laboratories, CA, USA).

All sequences were determined directly from the PCR products. Cycle sequencing reactions using the BigDye Terminator™ Sequencing Kit (Perkin-Elmer Corporation) and incorporating the same primers as those used in the initial PCRs were performed according to the manufacturer's instructions. Sequencing products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corporation). The consensus sequence was based upon both sense and anti-sense strands of isolates originating from four separate PCR products.

Phylogenetic analyses

CLUSTAL W (Thompson et al. 1994) was used for initial sequence alignments with default settings for gap and weighting values; alignments were subsequently edited manually. Alignment files were converted into distances by the Kimura 2 parameter and trees constructed using the neighbour-joining (N-J) algorithm (Saitou and Nei 1987) within the Phylogeny Inference Package (PHYLIP version 3.57; Felsenstein J. 1993). In addition, cladograms were produced from the alignment files using the Maximum-Likelihood (M-L) algorithm within Puzzle (Strimmer and Haeseler 1996). For Puzzle analyses rate heterogeneity was set at the uniform (default) rate. Numerical values at branch points indicate the percentage of tree topologies that support the observed tree. The insect-infecting microsporidian *Nosema apis* was used as an outgroup in the phylogenetic analyses. The *Microsporidium cypselurus* rDNA sequence determined in the current study was ascribed the accession number AJ300706 in GenBank. GenBank accession numbers of additional sequences utilised in the analyses were: *Ameson michaelis* (L15741), *Glugea americanus* (AF056014), *Glugea anomala* (AF044391), *Glugea atherinae* (U15987), *Glugea plecoglossi* (AJ295326), *Glugea* sp. from Scottish sticklebacks (AJ295325), *Glugea stephani* (AF056015), *Heterosporis anguillarum* (Kamaishi T. 1996), *Ichthyosporidium* sp. (L39110), *Loma salmonae* (U78736), *Microsporidium prosopium* (AF151529), *Microsporidium seriolae* (AJ295322), *Microsporidium* sp. from red seabream (AJ295323), *Nosema apis* (U97150), *Pleistophora ehrenbaumi* (AF044392), *Pleistophora finisterrensis* (AF044393), *Pleistophora hippoglossoides* (AF044388), *Pleistophora mirandellae* (AJ295327), *Pleistophora* sp. I (AFO44394), *Pleistophora* sp. II (AF044389), *Pleistophora* sp. III (AF044390), *Pleistophora typicalis* (AF044387), *Spraguea lophii* (AF033197), *Trachipleistophora hominis* (THAAJ2605), an unidentified microsporidium from gilthead seabream (AJ295324), an unidentified microsporidium from the metapenaeid shrimp *Metapenaeus joyneri* (AJ295328), and *Vavraia oncoperae* (X74112).

RESULTS

Microsporidium cypselurus sp. n.

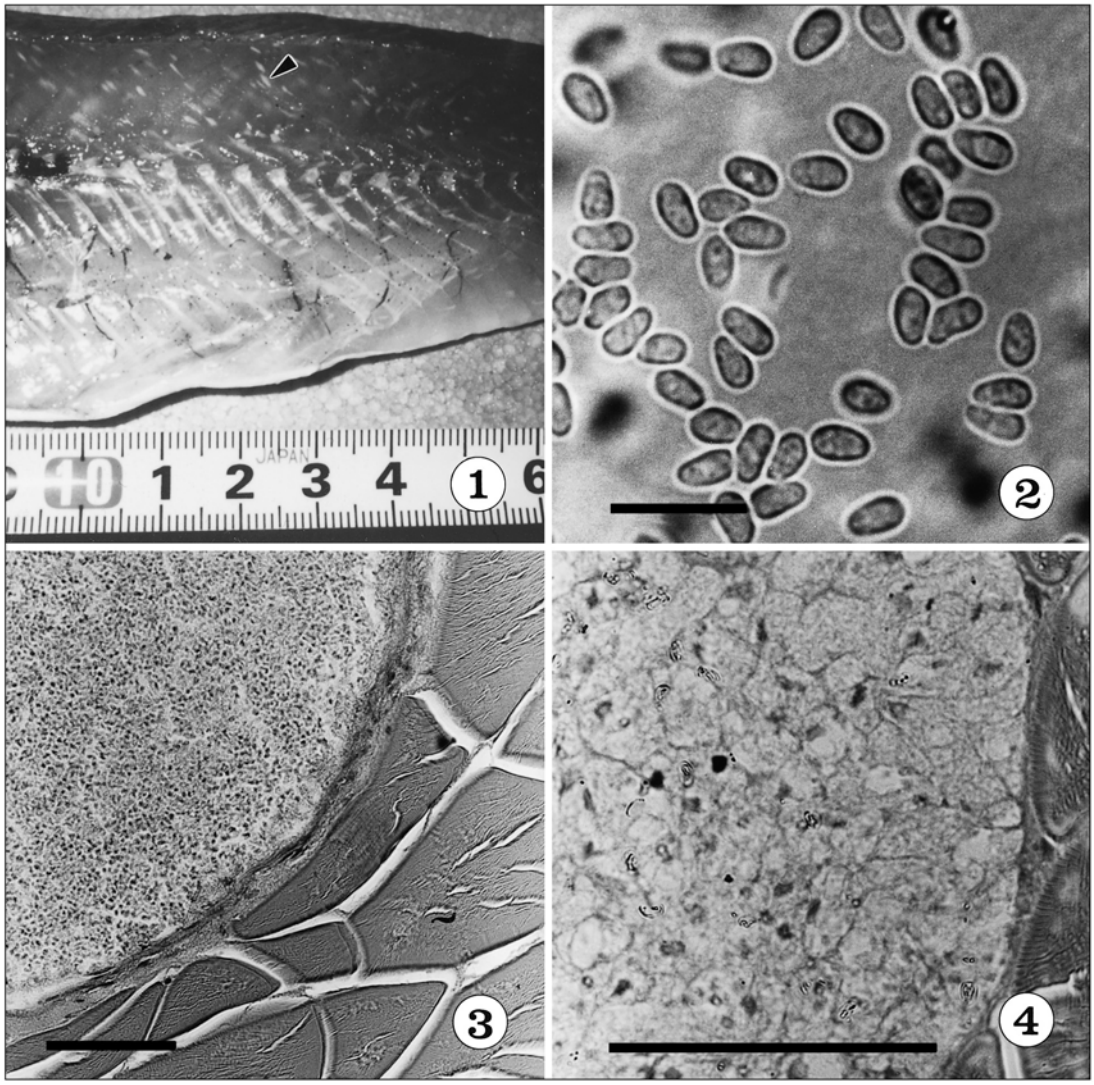
Numerous macroscopic, whitish, spindle-shaped foci of infection, up to 3-4 mm in size, were observed throughout the skeletal muscle (Fig. 1). In wet mounts, fresh spores were mostly ovoid to pyriform in shape, 4.1 (3.7-4.8) µm × 2.2 (2.1-2.7) µm in size, and possessed a large vacuole at the posterior end (Fig. 2). Histological observations showed that parasite foci were each encapsulated by a host-produced fibrous membrane (Fig. 3). Pre-sporogonic developmental stages were not found. Neither free spores nor spores ingested by host phagocytes were observed around the peripheries of lesions. No liquefaction of muscle fibres was observed. The presence of sporophorous vesicles (SPVs) was not clearly demonstrated in the advanced infections observed that comprised entirely of mature spores (Fig. 4). TEM revealed that spores possessed 13-15 coils to their isofilar polar tube (Fig. 5). Transverse sections showed no furrows, corresponding to raised mosaic-like fields, on the surface of the exospore.

The partial rDNA (530f-580r) sequence of *Microsporidium cypselurus* was 1348 bp in length. Percentage sequence identities between *M. cypselurus* and other related microsporidians are given in Table 1. *M. cypselurus* demonstrates 84% sequence identity with both *Glugea anomala* and *Pleistophora typicalis* across 1383 and 1358 bp respectively, but only 68.4% identity with *M. seriolae* (1428 bp). All phylogenetic analyses showed *M. cypselurus* to be distinct from *M. seriolae* and the other seabream muscle-infecting microsporidians, as well as from *M. prosopium* (Figs. 6, 7). Analyses

Table 1. Percentage identities between equivalent microsporidian rDNA sequences. Below the diagonal is the number of bases compared and above the diagonal is the percentage identity.

Species	1	2	3	4	5	6	7
1. *Microsporidium cypselurus*	-	82.6	68.4	84.0	84.0	79.4	53.8
2. *Microsporidium prosopium*	953	-	76.4	77.0	82.4	78.9	59.3
3. *Microsporidium seriolae*	1428	937	-	67.7	73.1	68.1	64.2
4. *Glugea anomala*	1383	966	1483	-	83.5	82.0	57.4
5. *Pleistophora typicalis*	1358	929	1487	1461	-	82.1	54.6
6. *Loma salmonae*	1279	927	1440	1415	1398	-	55.0
7. *Nucleospora salmonis*	1436	923	1436	1479	1477	1472	-

Accession numbers of the other microsporidian sequences used are provided in Materials and Methods.



Figs. 1-4. *Microsporidium cypselurus* sp. n. in the flying fish *Cypselurus pinnatibarbatus japonicus*. **Fig. 1.** Infected musculature; gross appearance. Note numerous white streaks of parasite foci (arrowhead) in the musculature. **Fig. 2.** Fresh spores in wet mount preparation. **Fig. 3.** Parasite mass encapsulated by host collagen fibres and connective tissue; a histological section stained with Uvitex 2B and H&E. **Fig. 4.** Degenerating parasite mass; a histological section stained with Uvitex 2B and H&E. Note spores scattered throughout the matrix of the lesion. Scale bars: Fig. 2 = 10 μm; Figs. 3, 4 = 50μm.

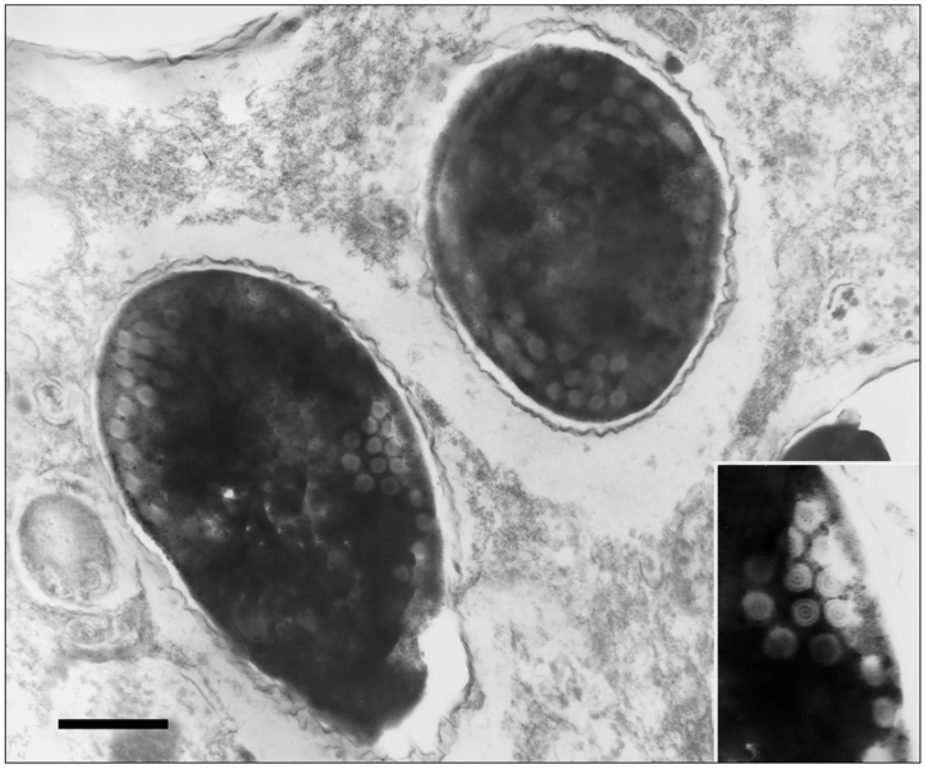


Fig. 5. Spores of *Microsporidium cypselurus* sp. n.; transmission electron micrograph. Note 13-15 coils to the isofilar polar tube in the transverse section (inset). Scale bar = 500 nm.

based on 530f-580r rDNA sequences (c. 1400 bp), extending from within the SSU, through the ITS region and into the LSU, revealed *M. cypselurus* to be most closely related to the clade incorporating the *Pleistophora* and *Glugea* spp. (Fig. 6, neighbour-joining algorithm) or to actually be a member of this clade (Fig. 6, maximum likelihood algorithm). Cladograms constructed from approximately 900 bp of the 3' end of the SSU indicated a similar relationship, with *M. cypselurus* comprising a sister taxon to that including the Pleistophoridae and *Glugea* spp. (Fig. 7, neighbour-joining; Fig. 8, neighbour-joining indicating branch lengths), or to position within this grouping (Fig 7, maximum likelihood). All analyses show *M. cypselurus* to be more closely related to the Pleistophoridae than either the *Loma/Ichthyosporidium* clade or *M. prosopium*.

T y p e h o s t : Flying fish, *Cypselurus pinnatibarbatus japonicus* (Franz) (Teleostei: Exocoetidae).

T y p e l o c a l i t y : Coast of Yakushima, Kagoshima Prefecture, Japan (30°20'N, 130º30'E).

S i t e o f i n f e c t i o n : Trunk musculature.

P r e v a l e n c e : Not determined. Only one infected fish examined.

T y p e m a t e r i a l : Syntype specimens deposited in the collection of the National Science Museum, Tokyo, Japan, accession number NSMT-Pr-175. Histological preparation stained with Uvitex 2B and H&E.

E t y m o l o g y : The specific name refers to the generic name of the type host.

DISCUSSION

Although the taxonomic position of the microsporidium described in the current study is uncertain, the morphological and molecular analyses enabled it to be discriminated from *Microsporidium seriolae*. No microsporidia infections in flying fish have been reported previously and existing descriptions of microsporidia that exhibit the same clinical signs and morphology are not known. Consequently, the present microsporidium is thought to represent a new species. Analyses based on ribosomal DNA sequence data suggest an affinity between this parasite and a clade that incorporates members of the genera *Pleistophora* and *Glugea*. Nilsen et al. (1998) suggested that the genus *Pleistophora* was not monophyletic, with several species more closely related to *Glugea* than to what he referred to as the "core" *Pleistophora*. The microsporidium described here demonstrated neither evidence of xenoma formation, one of characteristics of the genus *Glugea*, nor sporophorous vesicles (SPVs), one of the definitive characteristics of the genus *Pleistophora*.

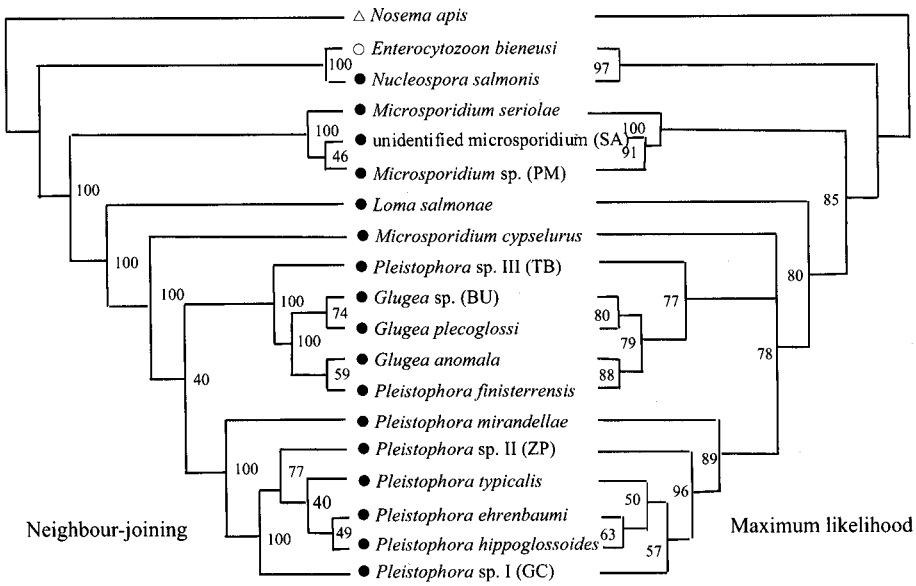


Fig. 6. Cladograms of 19 microsporidia based on the complete 530f-580r rDNA region (of approximately 1400 bp). Tree constructions used neighbour-joining or maximum likelihood algorithms. Figures at nodes represent percentage of tree topologies. Symbols represent: ● fish host, ○ mammalian host, Δ insect host. Abbreviations represent the hosts: SA, *Sparus aurata*; PM, *Pagrus major*; TB, *Taurulus bubalis*; BU, *Byturus unicolor*; ZP, *Zeugopterus punctatus*; GC, *Glyptocephalus cynoglossus*.

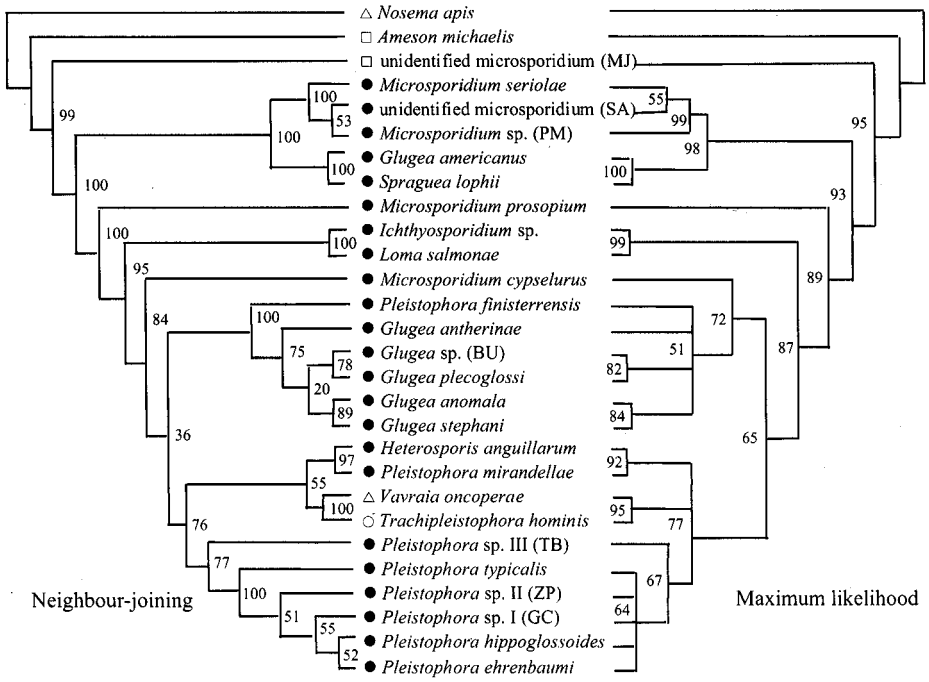


Fig. 7. Cladograms of 28 microsporidia based on approximately 900 bp of SSU rDNA sequence data. Tree constructions used neighbour-joining or maximum likelihood algorithms. Figures at nodes represent percentage of tree topologies. Symbols represent: ● fish host, ○ mammalian host, Δ insect host, □ crustacean host. Abbreviations represent the hosts: MJ, *Metapenaeus japonicus*; SA, *Sparus aurata*; PM, *Pagrus major*; BU, *Byturus unicolor*; TB, *Taurulus bubalis*; ZP, *Zeugopterus punctatus*; GC, *Glyptocephalus cynoglossus*.

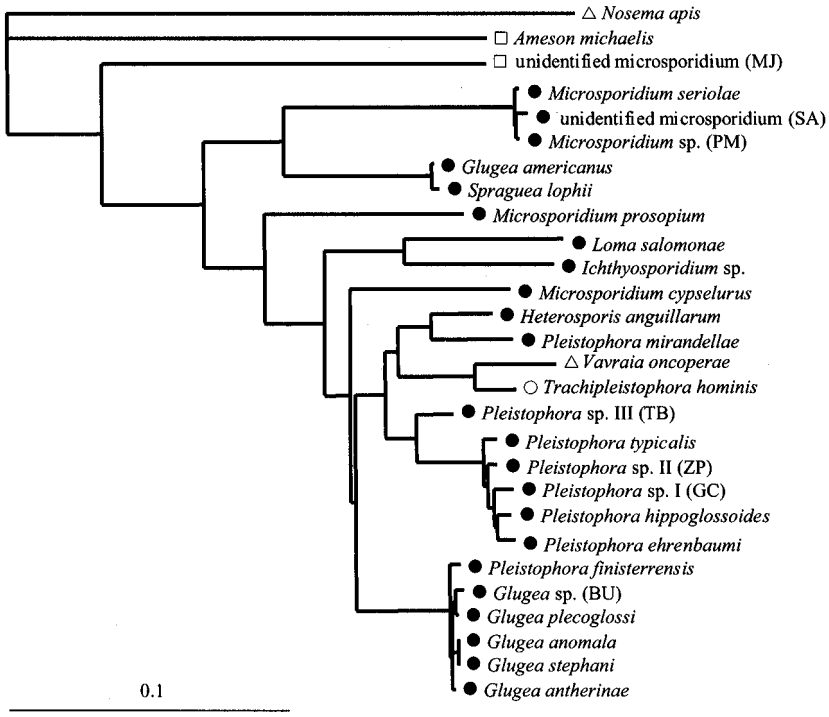


Fig. 8. Cladogram of 28 microsporidia based on approximately 900 bp of SSU rDNA sequence data. Tree construction used the neighbour-joining algorithm and indicates branch lengths. Symbols represent: ● fish host, ○ mammalian host, Δ insect host, □ crustacean host. Abbreviations represent the hosts: MJ, *Metapenaeus japonicus*; SA, *Sparus aurata*; PM, *Pagrus major*; TB, *Taurulus bubalis*; ZP, *Zeugopterus punctatus*; GC, *Glyptocephalus cynoglossus*; BU, *Byturus unicolor*.

SPVs typically persist long after spore maturation (Canning and Lom 1986). Nevertheless, Dyková (1995) reported that pleistophoran SPV walls disappeared within the "cysts" and that a thick wall of connective tissue built up around sporophorocysts in latter stage infections. It is possible that the sample examined in the current study might have contained only advanced infections. However, further justification is required to verify the formation of SPVs during the development of this microsporidian.

Recently, Lom et al. (1999) created a new genus *Kabataia* for a microsporidium infecting the musculature of sutchi catfish *Pangasius sutchi*. This genus name was subsequently revised to *Kabatana* (Lom et al. 2000, see also Dyková and Lom 2000). The present microsporidium shared a number of similarities with this newly established genus. However, *Kabatana* has no connective tissue capsules around the spore masses and SPVs were absent. Indeed, Lom et al. (1999) suggested that *Microsporidium seriolae* should, subsequent to further confirmation, be included in this genus. Further ultrastructural examinations of *M. seriolae* and sequence data for the type species *Kabatana arthuri* would be required to confirm the relationship with the flying fish microsporidium. Kent et al. (1999) described a new species of microsporidium from the musculature of mountain whitefish *Prosopium williamsoni* in British Columbia. These parasite foci occurred within the connective tissue of the endomysium. Their ribosomal DNA sequence data suggested that it belongs to the *Ichthyosporidium* "group" as designated by Baker et al. (1995), although percentage sequence identities showed it to be discrete from any of the genera incorporated into this grouping. Due to the absence of pre-spore developmental stages, Kent et al. (1999) were unable to assign taxonomic status to the microsporidium and it was provisionally placed in the collective genus *Microsporidium* as *M. prosopium*. The lack of morphological data for the early life-stages of the microsporidian described in the current study similarly necessitates its placement within the *Microsporidium*. Nevertheless, the rDNA analyses indicate that the systematic placement of the flying fish microsporidium is distant to that of *M. prosopium* and that both are likely to represent new genera of fish-infecting microsporidians.

Microsporidian taxonomy has traditionally been based on the morphology of spores and pre-spore developmental stages, principally at the ultrastructural level. Although transmission electron microscopy is still essential to microsporidia systematics, a number of discrepancies between phylogenies based on ultrastructural and molecular analyses have been revealed (Nilsen 2000). The future reorganisation of

microsporidia classification will require a combination of both morphological and molecular data. Generic designation of the microsporidium isolated from flying fish in the current study awaits characterisation of pre-spore developmental stages.

Acknowledgements. The authors would like to thank Mr. Iwao Takemaru of Kagoshima Prefectural Fisheries Experimental Station for his provision of the infected flying fish material.

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Received 23 January 2001 Accepted 13 June 2001