

Microsporidian Infection in the Trunk Muscle of Hatchery-bred Juvenile Spotted Halibut *Verasper variegatus*

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ABSTRACT—Recently, a microsporidian infection was found in the trunk muscle of hatchery-bred juvenile spotted halibut *Verasper variegatus*. The disease occurred from mid-July to the end of September 2007 and the cumulative mortality reached approximately 20%. Infected fish showed the external sign of a concave body surface on the eyed side, and the microsporidian parasite formed numerous ‘cysts’ in the muscle. These characteristics resembled ‘beko’ disease caused by *Microsporidium seriolae* from cultured yellowtail *Seriola quinqueradiata*, *Microsporidium* sp. from red sea bream *Pagrus major* (RSB) and *Microsporidium* sp. from gilthead sea bream *Sparus aurata* (GSB), but several differences were observed in associated pathological findings such as internal hemorrhage around the ‘cysts’. Average dimensions of spores from spotted halibut (SH) were $3.07 \times 2.13 \mu\text{m}$, which were relatively smaller than those of *M. seriolae*, *Microsporidium* sp. RSB and *Microsporidium* sp. GSB, but the ranges of measurements overlapped among them. Molecular analysis of rDNA sequences suggested that the present parasite, provisionally named as *Microsporidium* sp. SH, was distinct from the other known species.

Key words: Microsporidia, *Verasper variegatus*, *Microsporidium seriolae*, beko disease, spotted halibut, *Seriola quinqueradiata*

Spotted halibut *Verasper variegatus* is one of new candidate species for Japanese marine aquaculture due to its high commercial value and its importance to coastal fisheries. In Nagasaki Prefecture, the Kyushu region of Japan, the seed production of spotted halibut started in 2003, and trials of aquaculture in land-based tanks have been conducted on an experimental scale. Recently, a microsporidian infection was found in the trunk muscle of hatchery-reared juvenile spotted halibut in Nagasaki Prefecture. The clinical signs of infected spotted halibut resembled ‘beko’ disease caused by *Microsporidium seriolae* in cultured yellowtail *Seriola quinqueradiata* and *Microsporidium* sp. in red sea bream *Pagrus major* (Egusa, 1982; Egusa *et al.*, 1988). It has been known that epizootics of ‘beko’ disease frequently occurred in cultured yellowtail in Kyushu (Sano *et al.*,

1998). Although the host range for *M. seriolae* and *Microsporidium* sp. from red sea bream is unknown, the geographical distribution, infection site and gross appearance are similar in the three hosts. In addition, another unidentified microsporidian was reported from the musculature of cultured gilthead sea bream *Sparus aurata* in Malta (Abela *et al.*, 1996). The microsporidian from gilthead sea bream also produced whitish nodules in the skeletal muscle, and spore morphology was similar to the above species. Based on these findings, the four microsporidians were considered to be closely related. Thus, the microsporidian species from red sea bream (RSB), gilthead sea bream (GSB), and spotted halibut (SH) are hereinafter referred to as *Microsporidium* sp. RSB, *Microsporidium* sp. GSB, and *Microsporidium* sp. SH, respectively. The aim of the present study is to clarify whether *Microsporidium* sp. SH is conspecific with the other related muscle-infecting microsporidians.

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Materials and Methods

Fish samplings

Juvenile spotted halibut (0-year old) were obtained from a land-based tank supplied with non-filtered sea water at a culture farm in Nagasaki Prefecture. The samples were randomly collected on 20 July, 2007 (average body length, 9.7 cm; average body weight, 13.8 g; $n = 10$) and 20 September, 2007 (average body length, 12.3 cm; average body weight, 30.0 g; $n = 10$). Fish were filleted and visually observed for microsporidian 'cysts' in the trunk muscle. Prevalence of infection was defined as the percentage of fish with 'cysts' in the trunk muscle among the fish examined. The number of 'cysts' in the trunk muscle was also counted. Water temperature in the tank was recorded throughout the rearing period using a temperature data logger. Numbers of dead fish showing the typical signs of the disease were determined as the cumulative mortality.

Morphological and histological observation

Fresh spore preparations were examined with a compound microscope and photographed by a digital camera (DP20-5, Olympus). Spore dimensions ($n = 30$) were measured from multiple digital images. To induce extrusion of the polar tubes, the following treatments were tried; hydrogen peroxide, potassium hydroxide, drying, and heating. Dissected 'cysts' were fixed in 10% buffered formalin, dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned at 5 μm . The sections were stained with haematoxylin and eosin (H & E) and Uvitex 2B (Yokoyama *et al.*, 1996). Sections were examined with a fluorescence microscope utilizing a 100 W mercury bulb (ultra violet excitation: 330–380 nm).

Molecular analysis

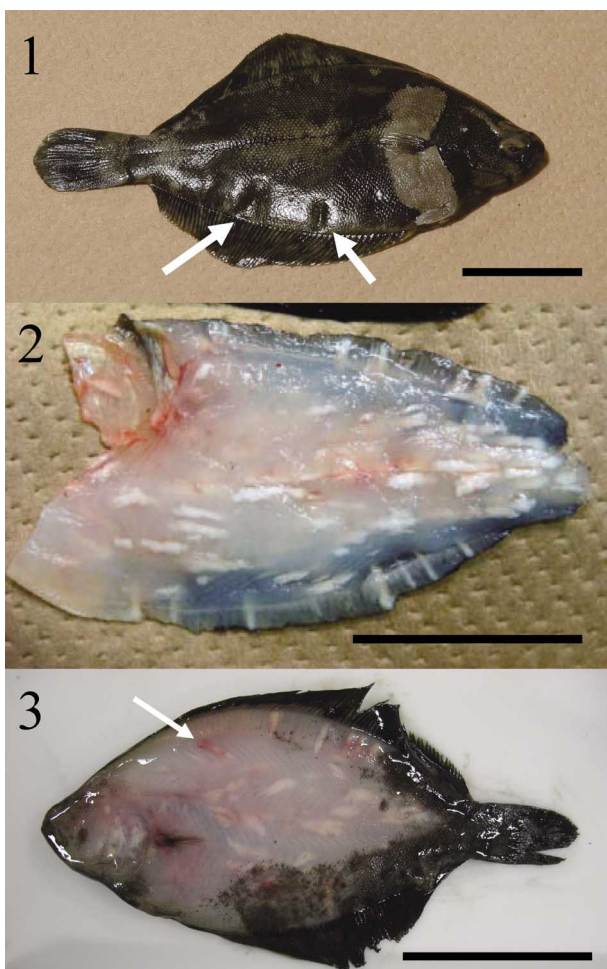
Isolation and purification of spores were conducted according to the procedures of Bell *et al.* (2001). Genomic DNA was extracted from freshly harvested spores using QIAamp DNA mini kit (Qiagen Inc., Germany). The targeted DNA (approximately 1450–1465 bp), which includes the ITS region, the LSU and the SSU, was amplified using the PCR primers 530f (5'-GTGCCATCCAGCCGCGG-3') and 580r (5'-GGTCCGTGTTTCAAGACGG-3'). PCR reactions were carried out in total volume of 20 μL . Each PCR mixture contained 0.5 μL of Takara Ex TaqTM HS (Takara Inc., Japan), 2 μL of 10 \times Ex Taq Buffer, 1.6 μL of dNTP Mixture (2.5 mM each), 0.3 μL of each primer (100 μM) and 1.0 μL of extracted DNA suspension. PCR reactions were performed in iCycler (Bio-Rad, USA) and carried out as described by Bell *et al.* (2001). Amplified target products were purified by QIAquick Gel Extraction Kit (Qiagen). Purified products were sent to Takara

Inc. for sequencing. Both forward and reverse PCR primers, as well as designed "walking" primers were used for dye termination PCR sequencing. The obtained SSU rDNA (1463 bp) was edited and compared with sequences in GenBank using BLAST 2.0 and all related sequences selected and aligned with Clustal X (Thompson *et al.*, 1997) using default settings. Phylogenetic analyses of aligned sequences, excluding the gaps, were conducted by neighbor-joining (NJ) method which was implemented in the MEGA3 computer package (Kumar *et al.*, 2004) and maximum likelihood (ML) method which was conducted by PHYML online web server (Guindon *et al.*, 2005), respectively. The Kimura's two-parameter model was used to NJ tree and substitution model of HKY to ML tree. The sequence of *Microsporidium* sp. SH determined in the current study was submitted to GenBank and assigned the accession number: EU871680. Additional sequences (with GenBank accession numbers) utilised in the analyses are: *Microsporidium seriolae* (AJ296332), *Microsporidium* sp. RSB (AJ295323), *Microsporidium* sp. GSB (AJ295324), *Microsporidium cypselurus* (AJ300706), *Microsporidium prosopium* (AF151529), *Microsporidium* sp. STF (AY140647), *Microsporidium* sp. JES2002G (AJ438962), *Heterosporis anguillarum* (AF387331), *Heterosporis* sp. PF (AF356225), *Kabatana takedai* (AF356222), *Kabatana newberryi* (EF202572), *Pleistophora typicalis* (AF044387), *Pleistophora hippoglossoides* (AF044388 and AJ252953), *Pleistophora ehrenbaumi* (AF044392), *Pleistophora mulleri* (AJ438985), *Pleistophora finsterrensis* (AF044393), *Pleistophora ovariae* (AJ252955), *Pleistophora* sp. 1 (AF044394), *Pleistophora* sp. 2 (AF044389), *Pleistophora* sp. 3 (AF044390), *Pleistophora* sp. PA (AJ252958), *Pleistophora* sp. TB (AJ252957), *Ovipleistophora mirandellae* (AF356223 and AJ252954), *Trachipleistophora hominis* (AJ002605), *Glugea plecoglossi* (AJ295326), *Glugea americanus* (AF056014), *Glugea anomala* (AF044391), *Glugea atherinae* (GAU15987), *Glugea stephani* (AF056015), *Glugea* sp. GS1 ex Scottish sticklebacks (AJ295325), *Glugea* sp. (AY090038), *Loma acerinae* (AJ252951), *Loma embiotocia* (AF320310), *Loma salmonae* (U78736), *Pseudoloma neurophilia* (AF322654), *Myosporidium merluccius* (AY530532), *Microgemma caulleryi* (AY033054), *Microgemma* sp. (AJ252952), *Tetramicra brevifilum* (AF364303), *Spraguea lophii* (AF056013), *Spraguea americana* (AY465876), Unidentified microsporidian S1 (AJ295328), Unidentified microsporidian MYX1 (AJ295329), *Vavraia culicis* (AJ252961), *Thelohania butleri* isolate 1 (DQ417114), and *Thelohania butleri* isolate 2 (DQ417115).

Results

Gross observation

Infection of *Microsporidium* sp. SH first appeared on 13 July 2007, when the water temperature was 23°C. Mortality occurred chronically from mid-July to



Figs. 1–3. Gross observations of spotted halibut infected with *Microsporidium* sp. SH. Bars = 3 cm. Note the concave body surface (arrows) on the eyed side (**Fig. 1**), numerous 'cysts' in the trunk muscle (**Fig. 2**), and internal hemorrhages (arrow) around the 'cysts' (**Fig. 3**).

the end of September 2007, and the cumulative mortality reached approximately 20%. Infected fish showed the external sign of a concave body surface particularly on the eyed side (**Fig. 1**). Numerous macroscopic, whitish, spindle-shaped 'cysts', up to 2–5 mm in length, were observed throughout the skeletal muscle (**Fig. 2**). Hemorrhagic lesions were occasionally observed around 'cysts' (**Fig. 3**). Prevalence of infection was 100% and 60% on 20 July and 20 September 2007, respectively. Average number of 'cysts' in the trunk muscle was 24.5 on 20 July 2007. On 20 September 2007, average number of 'cysts' was 6.8, though maximum number of 'cysts' reached at 60 in one fish.

Morphology and histopathology

Spores were oval to pyriform in shape and possessed a conspicuous posterior vacuole (**Fig. 4**). Average (and range) dimensions of 30 spores were $3.07 (2.8\text{--}3.8) \times 2.13 (1.8\text{--}2.3) \mu\text{m}$ (**Table 1**). No extrusion of the polar tubes was observed in any treatment tested. Sporophorous vesicles (SPVs) during the developmental cycle were not observed. Histological examination revealed developing 'cysts' containing presporogonic and sporogonic stages of the parasite in the trunk muscle of spotted halibut sampled on 20 July 2007. No remarkable host responses were observed, whereas hemorrhages were seen around the parasite

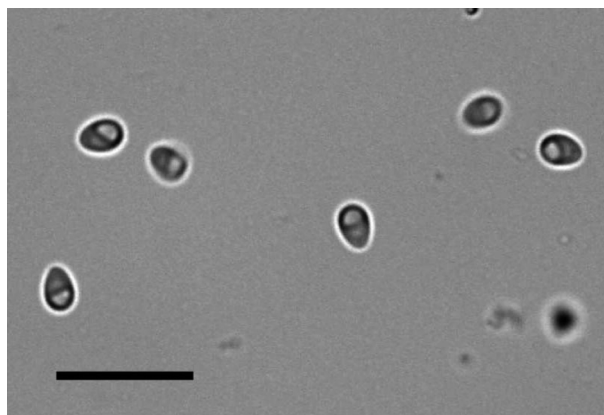


Fig. 4. Fresh spores of *Microsporidium* sp. SH. Bar = 10 μm .

Table 1. Comparison between spore dimensions (in μm , mean and range in parentheses) of *Microsporidium* sp. SH and related species

Species	Spore length	Spore width	Polar tube length	Host	Locality	Reference
<i>Microsporidium</i> sp. SH	3.07 (2.8–3.8)	2.13 (1.8–2.3)	ND*	<i>Verasper variegatus</i>	Japan	The present study
<i>Microsporidium seriolae</i>	3.34 (2.9–3.7)	2.22 (1.9–2.4)	49 (44–52)	<i>Seriola quinqueradiata</i>	Japan	Egusa (1982) Egusa <i>et al.</i> (1988)
<i>Microsporidium</i> sp. RSB	3.47 (2.9–3.9)	2.29 (1.9–2.6)	38.4 (30.5–50.5)	<i>Pagrus major</i>	Japan	Egusa <i>et al.</i> (1988)
<i>Microsporidium</i> sp. GSB	3.24	3.0	ND	<i>Sparus aurata</i>	Malta	Abela <i>et al.</i> (1996)

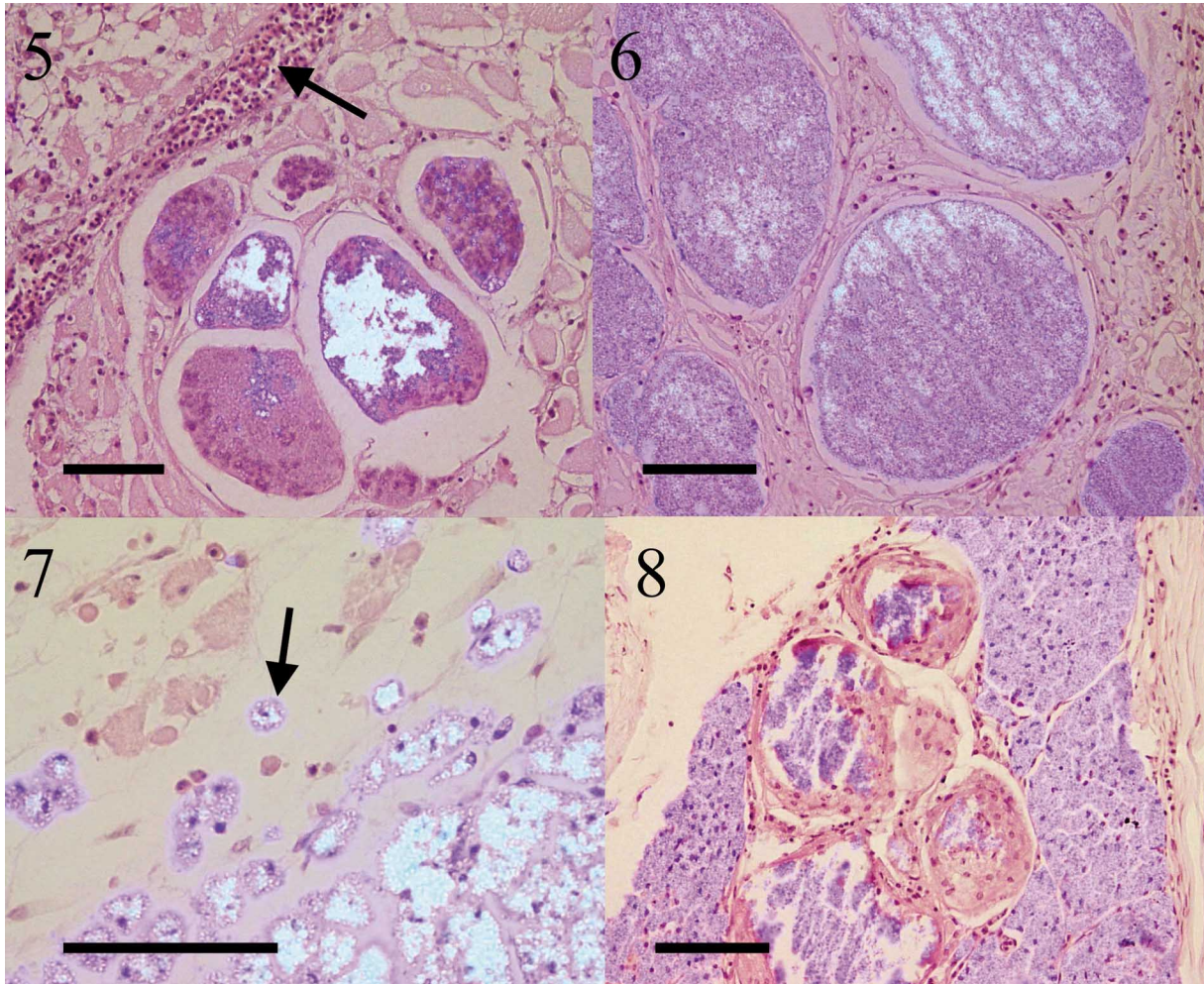
*ND: not determined.

masses (Fig. 5). Muscle tissue around 'cysts' showed extensive liquefaction and atrophy. In the samples on 20 September 2007, 'cysts' were fully packed with mature spores, while some were disrupted (Fig. 6). Parasite foci were infiltrated by inflammatory cells, followed by spore phagocytosis (Fig. 7). Necrosis and degeneration of the peripheral tissues were also evident. Encapsulation of parasites by host's fibrous tissues occurred, resulting in macrophage centers (Fig. 8).

Molecular analysis

Partial 530f-580r rDNA sequence of 1463 bp was obtained from *Microsporidium* sp. SH. Percentage sequence identities between *Microsporidium* sp. SH and other related microsporidians are given in Table 2, demonstrating 97.8%, 98.0% and 97.1% with *M. seriolae*, *Microsporidium* sp. RSB and *Microsporidium* sp. GSB,

respectively. Phylogenetic analysis of 530f-580r rDNA region (of approximately 1400 bp) using the ML method showed that *Microsporidium* sp. SH grouped with the other related muscle-infecting species, suggesting that they belong to the same genus (Fig. 9). Additional phylograms based on about 900 bp of SSU rDNA of 44 species from at least 13 genera are also provided to enable relationships between large numbers of microsporidian species and genera to be compared. The maximum likelihood analysis of Fig. 10 placed the four microsporidians from the musculature of marine fishes in a common clade with *Kabatana takedai* and *K. newberryi*, which infect the musculature of salmonid fishes and tidewater goby *Eucyclogobius newberryi*, respectively. The NJ analysis also yielded a similar tree topology (data not shown).



Figs. 5–8. Development of *Microsporidium* sp. SH in the musculature of spotted halibut and associated host responses. Histological sections stained by Uvitex 2B-H & E. Bar = 50 μ m. **Fig. 5:** Developing 'cysts' containing immature (not fluoresced) and mature (fluoresced) spore stages. Hemorrhagic lesions are observed (arrow). **Fig. 6:** Fully developed 'cysts' packed with mature spores. **Fig. 7:** Degenerated 'cysts' and phagocytosed spores (arrow). **Fig. 8:** Formation of macrophage centers.

Species	1	2	3	4
1. <i>Microsporidium</i> sp. SH	—	97.8	98.0	97.1
2. <i>Microsporidium seriolae</i>	1463	—	99.4	98.2
3. <i>Microsporidium</i> sp. RSB	1463	1463	—	98.3
4. <i>Microsporidium</i> sp. GSB	1453	1453	1453	—

Phylogenetic tree showing relationships between various microsporidian species. The tree is rooted at the bottom with *Thelohania butleri* isolates. Bootstrap values are indicated at the nodes. The tree shows several major clades, including a large group of *Microsporidium* species and a group containing *Pleistophora*, *Glugea*, and *Heterosporis* species.

Species and bootstrap values (from top to bottom):

- Pleistophora ehrenbaumi* (70)
- Pleistophora hippoglossoideos* (90)
- Pleistophora* sp.1 (70)
- Pleistophora typicalis* (100)
- Pleistophora mulleri* (87)
- Pleistophora* sp.2 (100)
- Heterosporis anguillarum*
- Glugea plecoglossi* (87)
- Glugea* sp. GS1 (95)
- Glugea anomala* (95)
- Pleistophora finisterrensis* (100)
- Pleistophora* sp.3 (97)
- Microsporidium cypselurus*
- Microsporidium* sp. STF
- Loma salmonae* (100)
- Loma embiotocia* (97)
- Unidentified microsporidian MYX1 (100)
- Pseudoloma neurophilia*
- Microsporidium seriola*
- Microsporidium* sp. GSB
- Microsporidium* sp. SH** (100)
- Microsporidium* sp. RSB (91)
- Unidentified microsporidian S1
- Microsporidium* sp. JES2002G
- Thelohania butleri* isolate 2
- Thelohania butleri* isolate 1

Discussion

cessful extrusion of the polar tubes in *Microsporidium* sp. SH is unknown. Egusa (1982) also found that neither hydrogen peroxide nor potassium hydroxide induced the polar tube extrusion, but only a few (less than 1%) spores extruded the polar tube immediately after drying by heat. However, in the present study, no extrusion of the polar tubes of *Microsporidium* sp. SH was observed even by the same method. Rare occurrence of polar tube extrusion may be one of characteristics for this group of microsporidians.

Molecular analysis of rDNA strongly suggested that *Microsporidium* sp. SH was distinct from the other known

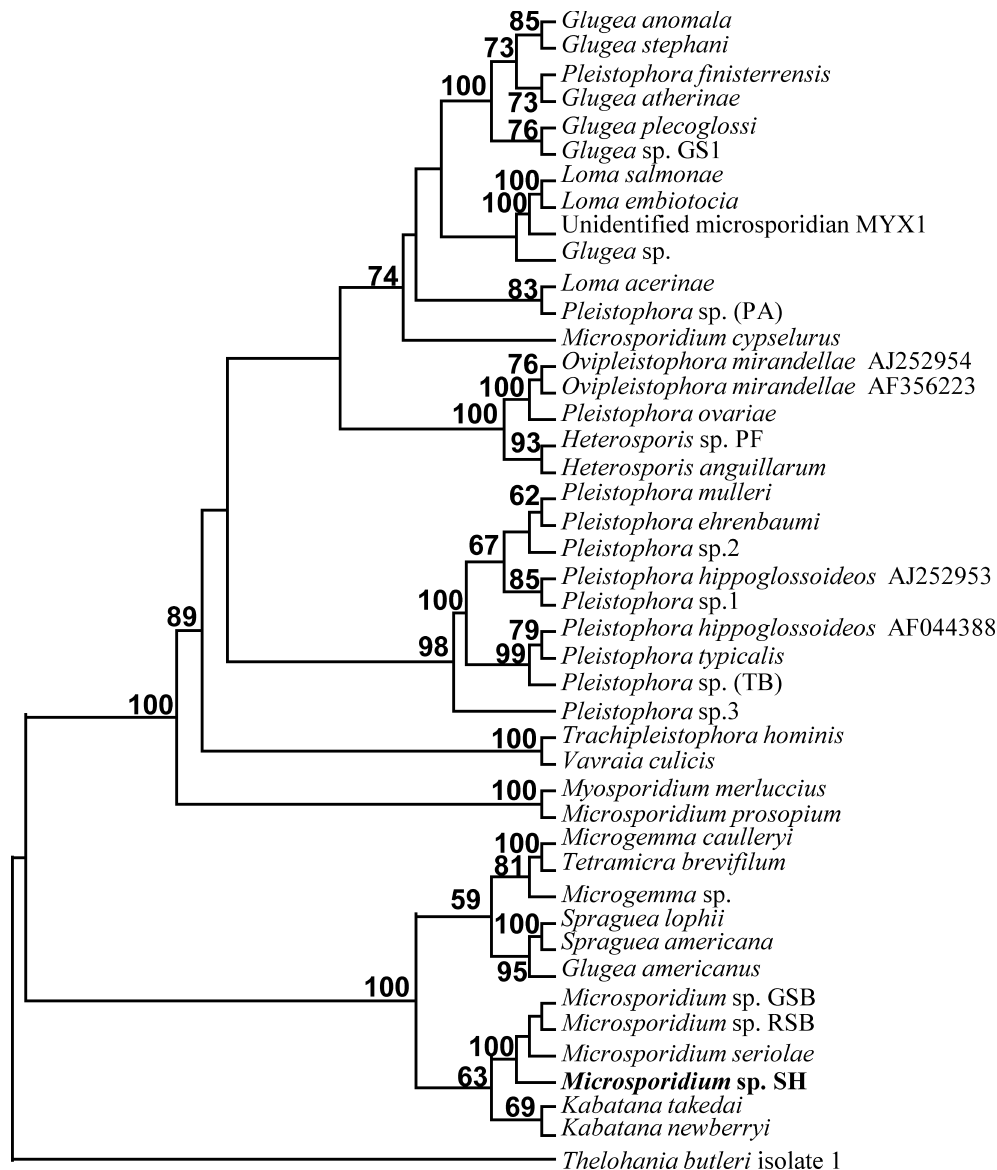


Fig. 10. Phylogram of 44 microsporidians based on approximately 900 bp of SSU rDNA sequence by maximum likelihood algorithm. Figures at nodes represent percentages of 500 bootstrap replicates and only the values above 50 are displayed.

species. The 530f-580r portion of rDNA examined in the current study, which includes the ITS region, the LSU and the SSU, is of considerable utility for phylogenetic studies of Microspora, containing moderately and highly variables, as well as conserved sequences (Bell *et al.*, 2001). The differences in percentage sequence identities (97.1–98.0%) between *Microsporidium* sp. SH and the other related species are represented by more than 20 bases transitions, insertions or transversions in consistent positions.

To solve the question whether the three microsporidians (*Microsporidium* sp. SH, *Microsporidium* sp. RSB, and *M. seriolae*) are conspecific, experimental infection trials will lead to a decisive results. However, artificial transmission using *M. seriolae* spores has failed, suggesting involvement of the intermediate

host in the life cycle (Sano *et al.*, 1998). Instead, exposure of uninfected fish to the enzootic waters for each microsporidian may be designed as an alternative experimental method. A preliminary experiment showed that no microsporidian infection was found in red sea bream which were exposed to the infective waters of *M. seriolae* (unpublished data by I. Takami and F. Yokoyama). Nevertheless, further controlled exposure tests are required.

It is worthwhile to refer to Lom *et al.* (1999) who established a new genus *Kabatana* (afterwards changed to *Kabatana*) for *Microsporidium arthuri*, a microsporidian developing in the trunk muscle of sutchi catfish *Pangasius sutchi*. They also mentioned that *M. seriolae* and *Microsporidium* sp. RSB may belong to the genus. The close relationship of the fish muscle-infect-

ing species in the present study with *K. takedai* and *K. newberryi* suggests that *Microsporidium* sp. SH also is a member of the same genus. However, whether they belong to *Kabatana* requires ultrastructural examinations of the parasites. In addition, molecular analysis of the type species *Kabatana arthuri* is now under investigation.

Several noticeable differences were observed in associated pathological findings between *Microsporidium* sp. SH and the other muscle-infecting microsporidians. Internal hemorrhages around the 'cysts' and the high mortality attributed to the parasite have not been documented in infections with *M. seriolae*, *Microsporidium* sp. RSB or *Microsporidium* sp. GSB. Usually, most of juvenile yellowtail infected with *M. seriolae* has been known to recover from the disease (Egusa, 1982). Thus, fish farmers of yellowtail are worried about the unsightly appearance due to visible 'cysts' remaining in the fillets rather than fish mortality. It is likely that spotted halibut is highly vulnerable to *Microsporidium* sp. SH. This parasite is considered to be a threat not only to the marketability but also to the productivity of cultured spotted halibut with potential economic losses.

Acknowledgements

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