Molecular and Morphological Redescriptions of Enteric Myxozoans, *Enteromyxum leei* (formerly *Myxidium* sp. TP) and *Enteromyxum fugu* comb. n. (syn. *Myxidium fugu*) from Cultured Tiger Puffer

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(Received March 19, 2004)

ABSTRACT—The phylogenies of two enteric myxozoans, *Myxidium fugu* and *Myxidium* sp. TP, from cultured tiger puffer *Takifugu rubripes* were re-evaluated based on their small subunit ribosomal RNA (SSU rRNA) gene sequence and spore morphology. Phylogenetic analysis shows that *M. fugu* and *Myxidium* sp. TP are grouped with *Enteromyxum* species as a monophyletic clade, not with other *Myxidium* spp. It was also illustrated that spores of *M. fugu* satisfy the criteria for the genus *Enteromyxum* for spore morphology. SSU rRNA gene sequence of *Myxidium* sp. TP shared 99.6% identity to that of *Enteromyxum leei*. Experimental transmission of *Myxidium* sp. TP to red sea bream *Pagrus major* induced the formation of mature spores, which were indistinguishable from those of *E. leei* in morphology and morphometrics. *Myxidium* sp. TP is identified as *E. leei*, and *M. fugu* is reassigned as *Enteromyxum fugu* comb. n.

Key words: *Enteromyxum*, *Enteromyxum leei*, *Myxidium*, SSU rRNA, *Takifugu rubripes*, emaciation disease, *Pagrus major*

The emaciation disease has emerged since the mid 1990s in the Japanese culture fields of tiger puffer *Takifugu rubripes*. Affected fish showed clinical signs of severe emaciation including sunken eyes, bony ridges on the head and a tapered body (Ogawa and Yokoyama, 2001). In the intestine of the infected fish, two new myxozoans, *Myxidium fugu* and *Leptotheca fugu*, and one unidentified myxozoan tentatively designated as *Myxidium* sp. TP (Tin Tun *et al.*, 2000) were found. Histopathological studies indicated that *Myxidium* sp. TP and *L. fugu*, histozoic parasites of the intestinal epithelium, were highly pathogenic and responsible for the emaciation disease (Ogawa and Yokoyama, 2001; Tin Tun *et al.*, 2002).

Myxidium sp. TP was first placed in the genus

Myxidium according to the morphology of immature spores (Tin Tun *et al.*, 2000) but has not been identified to the species level so far because of the absence of mature spore formation. It is unknown why *Myxidium* sp. TP could not fully mature to the spore stage. We speculate that tiger puffer may be an “abnormal host” for this parasite. *Myxidium* sp. TP appears to be closely related to the myxozoans belonging to the genus *Enteromyxum*, due to their marine, histozoic and enteric characteristics. *Enteromyxum leei* is pathogenic and infects the intestine of mainly sparid fishes including gilt-head sea bream *Sparus aurata* and red sea bream *Pagrus major*, in which the parasite forms numerous spores (Diamant, 1997). This parasite was first described as *Myxidium leei* (Diamant *et al.*, 1994) but Palenzuela *et al.* (2002) recognized that *M. leei* did not properly fit into the genus *Myxidium* according to the morphological and molecular analyses, and conse-

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quently transferred *M. leei* to the genus *Enteromyxum*.

In the present study, we conducted the SSU rRNA gene sequence analysis of *M. fugu* and *Myxidium* sp. TP to re-evaluated their taxonomic positions. Additionally, a transmission experiment to red sea bream was attempted to produce mature spores of *Myxidium* sp. TP for the morphological examination of this parasite.

Materials and Methods

Molecular analysis

Parasites: The enteric myxozoans were obtained from the intestinal tissues of heavily infected tiger puffer, and then preserved in 80% ethanol. The sample of *M. fugu* was collected from cage-cultured tiger puffer located in Kumamoto Prefecture, while *Myxidium* sp. TP was provided from a tiger puffer farmer in Oita Prefecture. Previous examinations showed that the fish from Kumamoto was infected only with *M. fugu*, whereas the fish from Oita was infected with both *M. fugu* and *Myxidium* sp. TP.

DNA cloning and sequencing: Parasite DNA was extracted from the suspension of scraped intestinal mucosa, using QIAamp DNA Mini Kit (Qiagen Inc., Germany). Myxozoan nuclear SSU rDNA was amplified by PCR using the primers MM18Sf (ctggttgattctgccagtgtgc) and MM18Sr (cggtagtagcgacgggagc) which have been used for the amplification of SSU rDNA of some marine enteric myxozoans (Palenzuela *et al.*, 2002). For *M. fugu*, the products were ligated into a vector (pT7Blue T-Vector, Novagen, USA), which was used to transform competent *Escherichia coli* cells (JM109, TaKaRa, Japan). On the other hand, the PCR Cloning⁺ Kit (Qiagen) was used for the PCR products from the template containing both *M. fugu* and *Myxidium* sp. TP DNA. Transformants were selected by the blue/white screening method on plates containing X-Gal and the presence of the inserts of the expected size was confirmed by PCR using commercial primers which amplify the insert regions. Both strands of the DNA were sequenced from at least 3 different clones containing each myxozoan gene, using an ABI PRISM 310 sequencer (Applied Biosystems, USA). Because of the coinfection of two myxozoans, SSU rRNA gene sequence of *M. fugu* was firstly determined and then clones containing a gene different from *M. fugu* were used for *Myxidium* sp. TP. Consensus sequence assemblages were aided with Genetyx Mac 10.0 and Genetyx Mac ATSQ 3.0 (Software Development, Japan).

Phylogenetic inference: Sequences were aligned using Genetyx Mac 10.0 and ClustalX1.8 (Thompson *et al.*, 1997) with the homologous sequences from other myxozoans available on public databases. Unambiguously aligned positions were used for phylogenetic inference performed by PAUP v.4.0b4 (Swofford, 1999) using the neighbor-joining (NJ) method applied to dis-

tances corrected according to Kimura's 2-parameter model (Kimura, 1980). Missing-data sites, including gaps, were ignored. Support for the internal branches of the tree was assessed by 100 bootstrap resamplings of the data set (Felsenstein, 1985). PKX organism (U70623) was selected as an outgroup, as it is a member of the Malacosporea, another class in the phylum Myxozoa, which has been demonstrated to be an outlier from other myxosporeans according to the analysis of SSU rRNA gene sequences (Kent and Palenzuela, 2002). GenBank accession numbers of additional sequences utilized in this analysis are shown in Fig. 1.

Experimental transmission to red sea bream

Fish: A stock of tiger puffer coinfecting with *M. fugu* and *Myxidium* sp. TP was kept as the donor fish for the experimental infection in the facilities of Kumamoto Prefectural Fisheries Research Center. The infection cycle was maintained *in vivo* by cohabitation of the infected donor fish and uninfected tiger puffer until required. Specific-myxozoan-free red sea bream were provided from a commercial hatchery in Kumamoto Prefecture and held in aquaria using filtered seawater in the Research Center.

Transmission experiment by feeding: From 10 infected donor tiger puffer described above, the intestines were freshly collected and chopped. A group of 21 naïve red sea bream (average body weight, 140 g) was fed pieces of the gut tissues. Subsequently, the fish were placed in a 500 L tank supplied with flow-through-filtrated seawater maintained at 25°C. At time intervals of 3, 6 and 9 weeks post-feeding, 7 fish were randomly selected from the tank and examined for *Myxidium* infections as described below. Twenty-one control fish were held in another tank under equivalent conditions and examined for the parasites.

Parasitological examination: Following the dissection of test fish, the digestive tracts were opened and gut contents were removed by washing with filtered seawater. Imprint slides of the intestinal mucosa were prepared for each individual fish, air-dried and stained with Diff-Quik (International Reagents Corp., Japan) for microscopical observation. Pieces of the gut tissues were fixed in 10% formalin and processed by routine histology. Paraffin sections cut at 5 µm were stained with Uvitex 2B (Ciba Geigy, Switzerland) and hematoxylin-eosin and observed under a light microscope (Yokoyama *et al.*, 1996). For comparison, the intestinal tissue sections of sharpnose sea bream *Diplodus puntazzo* infected with *Enteromyxum leei* and of turbot *Scophthalmus maximus* infected with *Enteromyxum scophthalmi* were prepared for Uvitex 2B-stainability. For spore morphology, the frozen intestines of tiger puffer and red sea bream were thawed and scrapings of the intestinal mucosa were suspended in phosphate buffered saline. Drops of the suspensions were placed

on the glass slides, and the spore morphology was examined in wet mounts under a phase-contrast microscope. Digital spore images were taken and measurements were made for 20 spores from the multiple images.

Results

Analysis of SSU rRNA gene sequence

SSU rRNA gene sequences of 1,590 bp and 1,588 bp were obtained from *M. fugu* and *Myxidium* sp. TP, respectively. The sequence of *Myxidium* sp. TP shared a 99.6% identity to that of *E. leei*. A phylogenetic tree constructed with NJ method revealed that *M. fugu* and *Myxidium* sp. TP grouped with *Enteromyxum leei* and *Enteromyxum scopthalmi* as a monophyletic clade, with maximum bootstrap support (Fig. 1). This clade was also distinct from that containing all the species from the

genus *Myxidium* available on the database. The sequence data determined in the current study have been deposited in GenBank as follows: *Enteromyxum fugu* (syn. *Myxidium fugu*); accession number AY520573; *Enteromyxum leei* (= *Myxidium* sp. TP); AY520574.

Experimental transmission to red sea bream

The developmental stages of *M. fugu* and *Myxidium* sp. TP were first detected in the intestine of experimentally infected fish at 6 weeks post-feeding of infected materials (Table 1). Prevalence of infection with *M. fugu* reached 100% (7 infected fish out of 7 examined fish) by 6 weeks, while that of *Myxidium* sp. TP was 57% (4 out of 7) and 50% (2 out of 4) by 6 and 9 weeks, respectively. At week 9, some mature spores of *Myxidium* sp. TP were observed in the intestine of red sea bream. No clinical signs of the emaciation disease were exhibited during the experimental period.

Fig. 1. Phylogenetic tree inferred from neighbor-joining analysis of the SSU rRNA gene from *Enteromyxum leei* (= *Myxidium* sp. TP), *Enteromyxum fugu* and related myxozoans. Bootstrap values obtained out of 1000 samplings are shown at nodes.

Table 1. Experimental transmission of *Myxidium fugu* and *Myxidium* sp. TP to red sea bream by feeding infected gut tissue of tiger puffer

Weeks post-feeding	Control			Fed infected tissue		
	Average body weight (g)	<i>M. fugu</i>	<i>Myxidium</i> sp. TP	Average body weight (g)	<i>M. fugu</i>	<i>Myxidium</i> sp. TP
3	157	0/7*	0/7	144	0/7	0/7
6	167	0/7	0/7	200	7/7	4/7
9	174	0/7	0/7	131	6/7	2/4

*: Number of infected fish / number of examined fish

Fig. 2. Gut epithelium of red sea bream experimentally infected with *Enteromyxum leei* (arrows). Uvitex 2B-H & E stains. Dual exposure with UV and normal light. Bar = 30 μ m.

Histologically, it was shown that *Myxidium* sp. TP developed intercellularly in the gut epithelium and that the intestinal epithelial lining undulated in places (Fig. 2). Plasmodia of *Myxidium* sp. TP in red sea bream were found to be strongly stained by Uvitex 2B (Fig. 2), as documented in the tiger puffer (Tin Tun *et al.*, 2000). *Enteromyxum leei* in the sharpnose sea bream also stained positive with Uvitex 2B, while *E. scophthalmi* was negative (data not shown).

Spore morphology

Spores of *Myxidium* sp. TP from the experimentally infected red sea bream were identical in shape to those of *Enteromyxum leei*. The spores were slightly crescent in shape and polar capsules were large, elongated, tapering distally and opening at the ends of the spore (Figs. 3a and b). Measurements of spores are

Fig. 3. Fresh spores of *Enteromyxum leei* (= *Myxidium* sp. TP) from red sea bream. Phase-contrast microscopy. **a:** top view. **b:** frontal view. Bars = 5 μ m.

Table 2. Comparison of the spore dimensions (mean $\pm$ s. d. and range in parentheses) between *Enteromyxum leei* and *Myxidium* sp. TP.

Myxozoan	Spore (μm)		Polar capsule (μm)		Reference
	Host	Length	Width	Length	
<i>Enteromyxum leei</i>					
<i>Sparus aurata</i>	14.7 (13.2–15.2)	6.9 (5.6–7.8)	74 (6.2–8.8)	3.2 (2.8–3.8)	Diamant <i>et al.</i> (1994)*
<i>Sparus aurata</i>	15 – 18	7 – 8	7	3	Diamant (1992)*
<i>Sparus aurata</i>	16.7±1 (15–18)	9.7±1 (8–11)	8.5±1.3 (8–9)	2.7±0.7 (2.5–4)	Sakiti <i>et al.</i> (1996)
<i>Sparus aurata</i>	17.7±0.6 (16.7–18.4)	8.2±0.4 (7.5–8.1)	7.7±0.3 (7.5–8.1)	2.9±0.4 (2.3–3.5)	Padros <i>et al.</i> (2001)**
<i>Sparus aurata</i>	16.2 (15.2–17.7)	7.8 (7.5–8.6)	8.2 (6.9–9.8)	3.6 (3.1–4.3)	Diamant (1998)
<i>Diplodus pundazzo</i> & <i>Pagrus major</i>	15–19	5–7	6.5–9	2.5–4	Le Breton & Marques (1995)
<i>Myxidium</i> sp. TP					
<i>Pagrus major</i>	17.5±0.9 (15.9–19.1)	9.2±2.4 (7.9–11.0)	6.4±0.4 (6.1–7.2)	3.0±0.6 (2.1–3.6)	This study

* Materials fixed in formalin

** Material fixed in ethanol and re-hydrated in HBSS

given in Table 2. Polar filaments and sutural line were not clearly visible.

The spore morphology of *Myxidium fugu* from tiger puffer satisfied the criteria for the genus *Enteromyxum* previously described by Palenzuela *et al.* (2002), i.e., slightly crescent shaped, large and elongated polar capsules tapering to their distal side and opening at the end

of the spore, but discharging in opposite directions relative to a longitudinal plane bisecting the spore in top or bottom view (Figs. 4a and 4b). It was also illustrated that spores developed in disporic sporoblasts (Fig. 4c).

Discussion

SSU rRNA gene analysis in this study strongly suggests that *Myxidium* sp. TP and *E. leei* are the same species. Molecular data alone is considered inadequate to identify myxozoan species but has been widely utilized to support the validity of morphological evidence. Yokoyama *et al.* (2004a) demonstrated that SSU rRNA gene sequences shared 100% identity between *Myxobolus buri* from yellowtail *Seriola quinqueradiata* and *Myxobolus acanthogobii* from yellowfin goby *Acanthogobius flavimanus*, concluding the synonymy of the 2 parasites. In contrast, Schlegel *et al.* (1996) documented a 2% variation within *Myxidium lieberkuehni* SSU rRNA gene sequences that were derived from various isolates of the same species. Yokoyama *et al.* (2004b) also found a difference of 0.9% between Japanese and Canadian isolates of *Kudoa thyrsites*. It is highly likely that the minor difference (0.4%) in the present study between the SSU rRNA sequences of *Myxidium* sp. TP and *E. leei* is within intra-specific variation.

An experimental transmission of *Myxidium* sp. TP to red sea bream, for the first time, induced the formation of mature spores, allowing us to observe the spore morphology of this parasite. Morphological comparison between spores of *Myxidium* sp. TP from red sea bream and those of *E. leei* from different references also supports the two myxozoans being the same organism. The spore morphometry of *Myxidium* sp. TP was consistent with that of *E. leei* and distinct from the only other current member of the genus, *E. scopthalmi*, which is considerably larger than *E. leei*. Although minor morphometric differences were detected between *Myxidium* sp. TP and various isolates of *E. leei*, the variability might be caused by different preparations. It is possible that somewhat small sizes of spore dimensions given by Diamant (1992) and Diamant *et al.* (1994) are due to shrinkage of spores fixed in formalin, whereas the measurements in the present study were taken from thawed spores. Otherwise, it may be explained by the host difference, which could possibly affect the final size of myxozoan spores. Diamant (1998) proved that experimental transmission of *E. leei* between gilt-head sea bream and red drum *Sciaenops ocellatus* produced different sizes of the spores.

One of the most unique characters of *Enteromyxum* species is fish-to-fish transmission of the parasites (Diamant, 1997; Redondo *et al.*, 2002). *Myxidium* sp. TP and *Myxidium fugu* were also demonstrated to trans-

Fig. 4. *Enteromyxum fugu*. a, b: fresh spores on top (a) and front (b) views. Bars = 5 μ m. Phase-contrast microscopy. c: intestinal imprint stained with Diff-Quik. A disporous plasmodium is observed. Bar = 10 μ m.

mit directly from fish to fish (Yasuda *et al.*, 2002). In the present study, experimental infection of *Myxidium* spp. from tiger puffer to red sea bream was achieved, suggesting that the direct transmission is a common biological feature for *Enteromyxum* spp., as well as the broad host range (Padros *et al.*, 2001). Positive stainability of *E. leei* for Uvitex 2B indicates that developmental stages of the parasite possess a chitin component, which was also reported for *Myxidium* sp. TP (Tin Tun *et al.*, 2000). Potential roles and functions of chitin in the parasites remain unknown, but Uvitex 2B stainability may become complementary criteria for a diagnostic feature for *E. leei*.

Molecular and morphological evidence of *M. fugu* revealed the close affinity with the genus *Enteromyxum*. One inconsistency is that *M. fugu* is a coelozoic parasite, while all the other species of *Enteromyxum* are histozoic. However, this genus was established very recently and only two species (*E. leei* and *E. scopthalmi*) have been included so far. Thus, we now propose to change the diagnosis of the *Enteromyxum* to accommodate a coelozoic parasite in the digestive system of marine fish and to transfer *M. fugu* to *Enteromyxum fugu* comb. n.

It has recently been of great concern that exotic pathogens may be translocated by the importation of culture seedlings (Wakabayashi, 1996). Chronologically, Diamant (1992) documented that the first outbreak of *E. leei* was in 1991–1992 in Cyprus, followed by expansion into Mediterranean countries, whereas in Japan, the emaciation disease of tiger puffer was first noticed in 1996. This time lag implies that the origin of this parasite is from European countries, though we have no convincing evidence of the introduction of fish seedlings harboring the parasite. Alternatively, a report by Kent (1999) may give us another view for the origin. He found a myxozoan resembling *E. leei* from the anemone fish *Amphiprion frenatus* reared in the aquarium in San Diego, California, whose brood stock was captured from the Indo-Pacific Ocean. Although this parasite has not yet been identified, *E. leei* may be originally a parasite of tropical fishes that have been transferred to Japan in aquarium fish. To solve these questions, further molecular studies using a more variable region of the gene (the ITS region) derived from wide geographical and different host samples are required (Andree *et al.*, 1999).

In conclusion, we reexamined the 2 enteric myxozoans from cultured tiger puffer based on the molecular and morphological studies, and identified *Myxidium* sp. TP as *Enteromyxum leei* and reassigned *M. fugu* as *Enteromyxum fugu* comb. n.

Acknowledgements

We are thankful to Dr. Oswaldo Palenzuela, Instituto de Acuicultura de Torre la Sal, for kindly sending paraffin

embedded samples of *Enteromyxum leei*-infected and *Enteromyxum scopthalmi*-infected tissues and to Dr. Mark A. Freeman, the University of Tokyo, for reviewing this manuscript.

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