■ **BRIEF COMMUNICATION**

Kudoa ogawai (Myxosporea: Kudoidae) Infection in Cultured Olive Flounder *Paralichthys olivaceus*

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Abstract: Since *Kudoa septempuntata* was identified as a causative agent of food poisoning associated with raw olive flounder *Paralichthys olivaceus*, interest and concern regarding the parasite have increased. However, there have been no investigations or reports of other *Kudoa* species infecting the fish (except for *K. paralichthys*, which infects the brain) in Korea. We found cysts filled with myxospores of *Kudoa* species in muscles of cultured olive flounder specimens and identified these to the species level. Mature spores were quadrate, measuring 8.7 ± 0.5 μm in length, 9.2 ± 0.4 μm in thickness, and 12.9 ± 0.6 μm in width. The spores containing 4 polar capsules had a length of 2.1 ± 0.2 μm and a width of 1.8 ± 0.3 μm. The partial 18S and 28S rDNA of isolates showed 99-100% similarities with *K. ogawai*. Using these morphological and molecular analyses, the species was identified as *K. ogawai*. This study is the first report of *K. ogawai* infection in cultured olive flounder in Korea.

Key words: *Kudoa ogawai*, olive flounder, identification, foodborne disease

Myxozoans are parasites that complete a life cycle through vertebrate (mainly fish) and invertebrate (mainly annelid) hosts [1]. The genus *Kudoa* contains myxosporeans with 4 or more shall valves and polar capsules, and the parasites infect various fish species [2,3]. Some *Kudoa* spp. reduce the commercial value of fish by causing post-mortem liquefaction (such as *K. thyrsites, K. lateolabracis,* and *K. neothunni*) [3-7], cyst formation in muscles (*K. iwatai* and *K. amamiensis*) [8,9], and spinal deformation (*K. yasunagai*) [2,10,11]. In addition, previous studies have reported the relationship between *Kudoa* spp. and food-poisoning [12-14]. These findings have led to increased interest and concern regarding *Kudoa* infection in commercially important fish. Olive flounder, *Paralichthys olivaceus*, is an important fish species cultured in Korea, and there have been several reports of myxosporean infections (*Enteromyxum leei, Parvicapsula anisocaudata, P. curvatura,* and *Sinuolinea capsularis*), including *Kudoa* spp. (*K. septempunctata* and *K. paralichthys*), in the fish [15-19]. The Fish Vaccine Research Center has been monitoring the parasitic infections of olive flounder cultured in Jeju island and has isolated another *Kudoa* sp. from olive flounder muscle. The aim of the present study was to identify the *Kudoa* sp. using morphological and molecular analyses and to reveal differences between the present isolate and other *Kudoa* spp. known to infect olive flounder. In addition, the information obtained from this study will be applied to the inspection and identification of *Kudoa* spp.

Olive flounder samples (n = 2, 56.4 ± 4.3 cm) were obtained from an olive flounder farm situated on Jeju island. The fish samples were examined microscopically to detect *K. septempunctata* spores in squash preparations, and cysts filled with another *Kudoa* sp. were found. The cysts were collected using forceps and were squashed on a glass slide. The spores were wet mounted and observed under a light microscope and photographed at 400× or 1,000× magnification. Measurements of myxospores were obtain from 20 spores using the ImageJ image processing software (available at http://rsb.info.nih.gov/ij/) according to Lom and Arthur's criteria (1989) [20].

The myxospores were collected and passed through a 40 μm cell strainer. DNA was extracted from partially purified parasites using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) following the manufacturer's instructions. Portions of 18S and 28S rDNA were amplified by PCR using a combination of primers that we (28S-KO2000F: 5′-CTGTCCGTACCGAATCCG-3′) and other groups (18S-18e: 5′

-CTGGTTGATCCTGCCAGT-3′, 18S-MyxospecF: 5′-TTCT-GCCCTATCAACTWGTTG-3′, 18S-MyxospecR: 5′-GGTTTCN CDGRGGGMCCAAC-3′, 18S-ERIB10: 5′-CTTCCGCAG-GTTCACCTA-3′, 28S-Kt28SF: 5′-CAAGACTACCTGCTGAAC-3′, 28S-NLF1050: 5′-AATCGAACCATCTAGTAGCTGG-3′, 28S-NLR1270: 5′-TTCATCCCGCATCGCCAGTTC-3′, 28S-NLR1694(mo): 5′-GTTAGGCAATGGCTTAGGACC-3′, 28S-3R: 5′-GAGCACTGGGCAGAAATC-3′, 28S-NLR3113(mo): 5′-GTC-TAAACCCAGCTCACGTTC-3′, and 28S-NLR3284: 5′-TTCT-GACTTAGAGGCGTTCAG-3′) have designed [2,16,21-25]. PCR products were treated with the AccuPrep Genomic PCR Purifi-cation Kit (Bioneer) to remove excess primers and dNTPs, and were directly sequenced using BigDyeTM Terminator v3.1 in an ABI 3730xl Sequencer. The 18S and 28S rDNA sequences of isolates were compared in the GenBank database using the Basic Local Alignment Search Tool (BLAST) search engine to find sequences with a high degree of similarity. Multiple align-ments of 18S rDNA sequences were made using Clustal X 2.0 [26] with 7 homologous sequences from *Kudoa* spp. and one sequence from *E. leei* (outgroup) reported in olive flounder (except *K. lateolabracis*). The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7 [27].

Oval cysts 1 mm in length approximately, were observed in the squashed muscle sample (Fig. 1A). Cysts were shorter in length and thicker than the pseudocysts of *K. septempunctata* that are usually detected, and the myxospores were also differ-ent in form from those of *K. septempunctata* (Fig. 1B, C). The myxospores were quadrate in the apical view and had 4 equal spore valves, each containing one polar capsule with a round-ed peripheral edge. In addition, the appearance of the 4 polar capsules was similar to a 4-leaf clover (Fig. 1D). In side view, spores were rounded triangle-shaped, and prominent apical projections were observed (Fig. 1E). The spores measured 8.7±0.5 μm (8.3-9.6 μm) in length, 9.2±0.4 μm (8.4-10.0 μm) in thickness, and 12.9±0.6 μm (11.5-13.9 μm) in width. The polar capsule length was 2.1±0.2 μm (1.7-2.5 μm) and polar capsule width was 1.8±0.3 μm (1.4-2.5 μm) (Table 1). The species isolated in the present study was then compared with 8 *Kudoa* spp. that have been previously reported in olive flounder. *Kudoa ogawai* was the most similar to the isolated species in terms of measurements and shape of spores, num-ber of valves per spore, apical projection, and infection site. *Kudoa paralichthys* and *K. shiomitsui* showed similarities to the isolate in terms of spore shape and number of valves per spore. However, they are smaller than the isolate and have a different infection site (brain and heart vs muscle). In addi-tion, they have small projections that are not easily observed, while the isolate has distinct apical projections. Although *K. lateolabracis* and *K. thyrsites* had similarities number of valves per spore and share the same infection site as the isolate, they have distinct spore shapes with one large polar capsule. *Kudoa septempunctata* and *K. igami* have similar spore measurements and the same infection site as the isolate. However, they have a different spore shape (stellate vs. quadrate) as well as a differ-
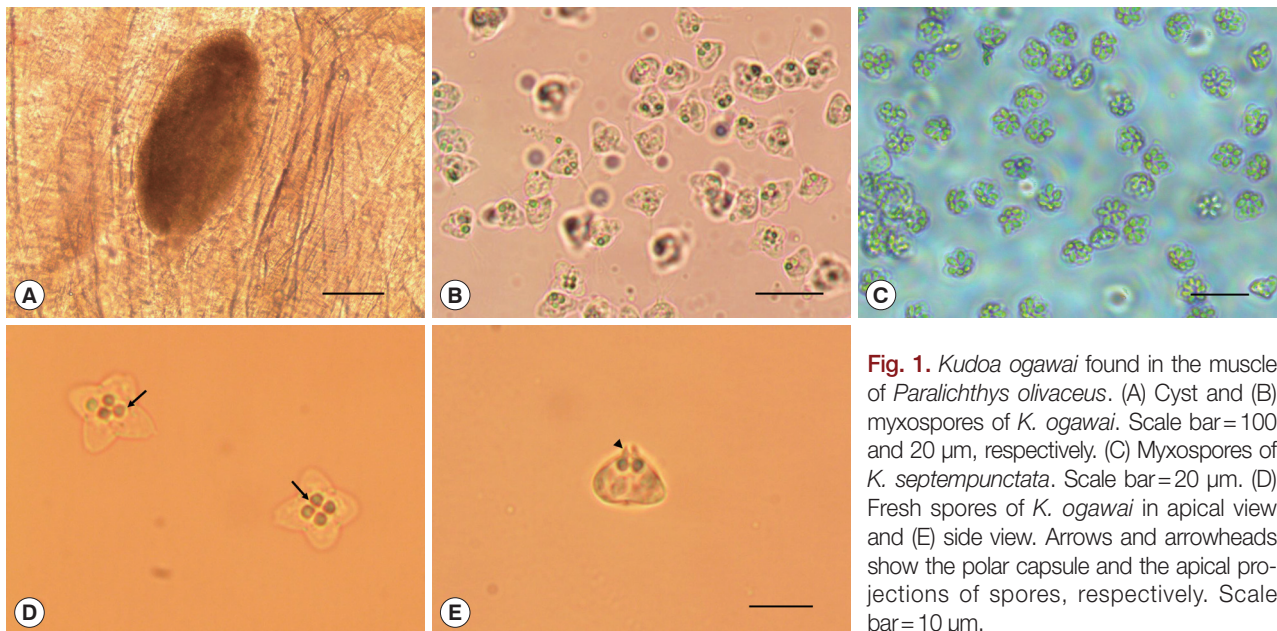

Fig. 1. *Kudoa ogawai* found in the muscle of *Paralichthys olivaceus*. (A) Cyst and (B) myxospores of *K. ogawai*. Scale bar = 100 and 20 μm, respectively. (C) Myxospores of *K. septempunctata*. Scale bar = 20 μm. (D) Fresh spores of *K. ogawai* in apical view and (E) side view. Arrows and arrowheads show the polar capsule and the apical pro-jections of spores, respectively. Scale bar = 10 μm.

Table 1. Comparison of *Kudoa* sp. (present isolate) with the other *Kudoa* spp. reported in olive flounders *Paralichthys olivaceus*

Species	Spore length	Spore thickness	Spore width	Polar capsule length	Polar capsule width	Spore shape (Np. of valves per spore)	Apical projections	Infection site	References
Kudoa sp.	8.7±0.5 (8.3-9.6)	9.2±0.4 (8.4-10.0)	12.9±0.6 (11.5-13.9)	2.1±0.2 (1.7-2.5)	1.8±0.3 (1.4-2.5)	Quadrate (4)	+	Muscle	Present study
K. ogawai	9.5±0.5	9.9±0.7	12.3±0.7	-	-	Quadrate (4)	+	Muscle	[28]
K. septempunctata	7.9-8.9	8.9-10.0	11.1-13.1	3.7-5.3	2.2-2.8	Stellate (6/7)	Tiny projections	Muscle	[29]
K. lateolabracis[a]	5.4-6.9	9.9-12.9	8.4-9.9 (Max) 5.9-6.9 (Min)	4.0-5.9 (Large) 3.0-4.0 (Small)	2.5-3.5 (Large) 1.5-2.0 (Small)	Quadrate with unequal polar capsule (4)	-	Muscle	[4]
K. thyrsites	6.9-8.9	12.9-17.8	7.9-11.9 (Max) 6.9-7.9 (Min)	4.0-5.9 (Large) 2.0-4.0 (Large)	1.5-3.0 (Large) 1.5-2.5 Large)	Quadrate with unequal polar capsule (4)	-	Muscle	[4]
K. igami	7.0±0.3	9.6±0.6	10.8±0.6	-	-	Stellate (6)	-	Muscle	[28]
K. paralichthys	4.5-6.0	6.3-7.7	7.4-8.9	1.9-2.7	1.0-1.4	Quadrate (4)	Small projections	Brain	[19]
K. yasunagai[b]	4.3-7.3	7.1-10.2	9.4-13.9	3.4-4.3	2.2-2.9	Stellate (7)	-	Brain	[11]
K. shiomitsui[c]	6.2-7.4	7.4-8.7	8.8-10.3	1.9-3.9	1.3-1.7	Quadrate (4)	Small projections	Heart	[30]

[a]Data provided from *K. lateolabracis* isolated from *Lateolabrax* sp. at the original description (Yokoyama et al., 2004 [4]).
[b]Data provided from *K. yasunagai* isolated from *Lateolabrax japonicus* (Hsieh et al., 1984 [11]).
[c]Data provided from *K. shiomitsui* isolated from *Stephanolepis cirrhifer* (Kasai et al., 2016 [30]).

ent number of valves per spore (6 or 7 vs 4). In addition, their apical projections are not prominent as those of the isolate (Table 1) [4,11,19,28-30].

Partial sequences of the 18S rDNA (1,650 bp) and 28S rDNA (3,395 bp) were obtained from the isolate and had 100 and 99.9% similarities with the 18S (KX163082; 1,631/1,631) and 28S rDNA (KX163083; 3,318/3,323) sequences of *K. ogawai* isolated from olive flounder in Japan, respectively. Unfortunately, there is no 18S rDNA sequence of *K. paralichthys* available on GenBank. In addition, the 18S rDNA sequence of *K. lateolabracis* reported from olive flounder could not be found, thus the sequence of *K. lateolabracis* isolated from Chinese sea bass was used to construct the phylogenetic tree. The phylogenetic tree was divided into 4 groups and the isolate was clustered with *K. ogawai* (Fig. 2). Based on the morphological and molecular comparisons, the myxospores were identified as *K. ogawai* and the sequences were deposited in GenBank (accession numbers MK850343 and MK850346, respectively).

Korea and Japan have a food culture that involves eating raw fish, which increase the risk of food poisoning. *Kudoa septempunctata* has been reported as a causative agent of food poisoning after ingesting raw olive flounder [12,31]. Kawai et al. [12] reported that oral administration of *K. septempunctata* spores elicited accumulation of fluid in the intestine and produced watery diarrhea in suckling mice. They also showed that the direct feeding of olive flounder meat containing *K. septempunctata* spores as well as purified spores cause vomitting in house musk shrews. Ohnish et al. [31] revealed that *K. septempunctata* sporoplasm plays an important role in mediating the toxicity of *K. septempunctata*. In addition, previous studies have investigated the epidemiological relationship between *K. septempunctata* ingestion and food poisoning in Korea and Japan [13,32]. Iwashita et al. [32] reported 3 clinical cases related to *K. septempunctata* ingestion in the Mie prefecture of Japan, and Kim et al. [13] also reported on the epidemiologic characteristics of 16 outbreaks of foodborne illnesses caused by *K. septempunctata* in the Gyeonggi Province of Korea. However, some studies have suggested that there is no relationship between food poisoning and ingesting olive flounder infected by *K. septempunctata* [33-35]. Jang et al. [33] showed that suckling mice inoculated with $5 \times 10^{6\text{-}7}$ *K. septempunctata* spores did not show symptoms of diarrhea, and no pathological change in intestine of the mice. Ahn et al. [34] came to similar results in study using adult mice. In addition, they exhibited that *K. septem-*
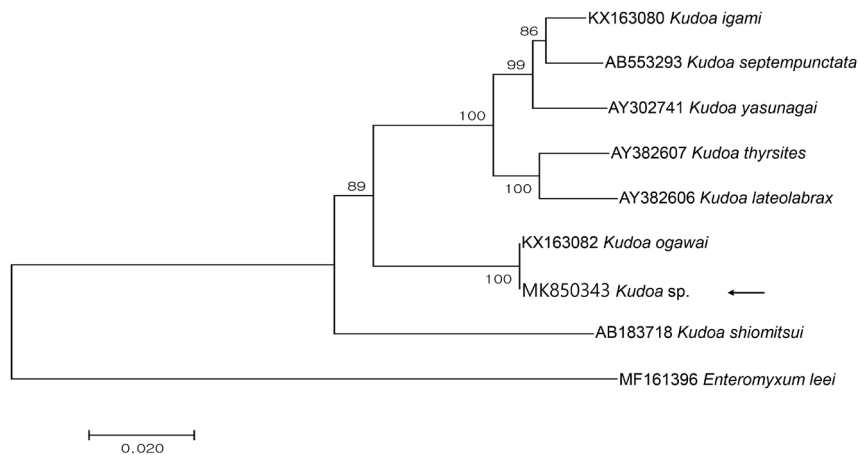
Fig. 2. Phylogenetic tree generated by MEGA 7, based on the aligned partial 18S rDNA sequences of *Kudoa* sp. newly obtained from the present study and *Kudoa* spp. previously reported in olive flounder. *Enteromyxum leei* was set as the outgroup and the bootstrap values (1,000 replicates) are shown at the nodes on the neighbor-joining tree. The present species is indicated by an arrow and is clustering with *K. ogawai*.

punctata spores did not cause inflammation on Caco-2 cells [35]. It is therefore up for discussion whether *K. septempunctata* causes food poisoning or not, however a few cultured olive flounder are undeniably infected by the parasite.

In the present study, *Kudoa* sp. was discovered to infect the muscle of cultured olive flounder, and the isolate was identified as *K. ogawai* based on morphological and molecular comparisons. There have been no studies revealing the relationship between *K. ogawai* and food poisoning, however, there have been several reports that other myxosporeans (not *K. septempunctata*) may cause foodborne diseases [14,36,37]. Kawase et al. [36] reported that *K. hexapunctata* genes were detected in the feces of a patient exhibiting diarrhea and vomiting after eating raw juvenile Pacific bluefin tuna, *Thunnus orientalis*, and Ohnishi et al. [14] also detected the genes and spores of *K. iwatai* from Rock bream (barred knifejaw), *Oplegnathus fasciatus*, and Blackhead seabream, *Acanthopagrus schlegelii*, associated with gastrointestinal disease. In addition, *Unicapsula seriolae* is also suspected to be a parasite associated with an unidentified foodborne disease of caused by ingestion of Greater amberjack, *Seriola dumerili* [37]. Screening for *K. septempumnctata* is carried out on cultured olive flounder by the Korean government, however other myxosporeans containing other *Kudoa* spp. that infect the fish are not investigated. Because *Kudoa* spp. have a low host specificity [38], there is a possibility that cultured olive flounders are infected by other *Kudoa* spp. (or myxosporeans) related with foodborne illness. In addition, most consumers do not want to eat fish infected by any parasites. Thus, more studies investigating other myxosporeans (especially parasites that infect muscle) and developing control methods against parasite infections should be conducted.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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