Molecular characterization of kudoid parasites (Myxozoa: Multivalvulida) from somatic muscles of Pacific bluefin (Thunnus orientalis) and yellowfin (T. albacores) tuna

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Abstract
The public health importance of Kudoa infection in fish remains unclear. Recently in Japan a Kudoa species, K. septempunctata, was newly implicated as a causative agent of unidentified food poisoning related to the consumption of raw olive flounder. Other marine fishery products are also suspected as causative raw foods of unidentified food poisoning. For this study, we detected kudoid parasites from sliced raw muscle tissues of a young Pacific bluefin and an adult yellowfin tuna. No cyst or pseudocyst was evident in muscles macroscopically, but pseudocysts were detected in both samples histologically. One substitution (within 1100 bp overlap) and ten substitutions (within 753 bp overlap) were found respectively between the partial sequences of 18S and 28S rDNAs from both isolates. Nucleotide sequence similarity searching of 18S and 28S rDNAs from both isolates showed the highest identity with those of K. neothunni from tuna. Based on the spore morphology, the mode of parasitism, and the nucleotide sequence similarity, these isolates from a Pacific bluefin and a yellowfin tuna were identified as K. neothunni. Phylogenetic analysis of the 28S rDNA sequence revealed that K. neothunni is classifiable into two genotypes: one from Pacific bluefin and the other from yellowfin tuna. Recently, an unidentified kudoid parasite morphologically and genetically similar K. neothunni were detected from stocked tuna samples in unidentified food poisoning cases in Japan. The possibility exists that K. neothunni, especially from the Pacific bluefin tuna, causes food poisoning, as does K. septempunctata.

Keywords
Kudoa neothunni, Pacific bluefin tuna, yellowfin tuna, phylogenetic analysis

The genus Kudoa includes myxosporean parasites that infect various marine teleosts. They are characterized by spores with four or more shell valves and polar capsules in each valve (Moran et al. 1999; Lom and Dyková 2006). Several Kudoa species have been of economic concern in aquaculture and commercial marine fisheries because of negative visual impact on product quality related to macroscopic cysts in somatic muscles and because of post-mortem myoliquefaction, commonly known as “jellied meat”. In Japan, cases of unidentified food poisoning putatively related to raw fish consumption have increased since around 12 years ago (Yunokawa 2012). Recently, a new species, K. septempunctata, was detected in somatic muscle of an aquacultured olive flounder (Paralichthys olivaceus) imported from Korea (Matsukane et al. 2010). That species was identified as a causative agent of food poisoning (Kawai et al. 2012). However, marine fishery products other than olive flounder are also implicated as causative raw foods related to unidentified food poisoning (Yunokawa 2012). Tuna (Thunnus spp.) is a widely consumed fish in Japan. Most Japanese people eat this fish raw in dishes such as sashimi. Reportedly, food poisoning from unidentified causes is often related to the consumption of tuna (Suzuki

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At present, five Kudoa species that are histozoic in somatic muscles (K. crumena, K. nova, K. neothunni, K. thunni, and K. shiomitsu) have been found in tuna (Arai and Matsumoto 1953; Moran et al. 1999; Fiala and Bartosova 2010; Zhang et al. 2010; Matsukané et al. 2011), but it remains unclear whether these species cause food poisoning or not. Furthermore, the host specificity of K. septempunctata found in olive flounder remains unclear. Therefore, it is important to identify the Kudoa species found in tuna to investigate the cause of unidentified food poisoning related to tuna consumption. For this study, we detected kudoid parasites from 2 of 15 tuna samples and identified them using molecular and morphological examinations.

For the examination of kudoid parasites, 15 tuna samples were provided during July 2011–February 2012: 6 Pacific bluefin tuna (Thunnus orientalis), 7 yellowfin tuna (T. albacores), and 2 bigeye tuna (T. obesus). All samples, collected at markets in Osaka City and Osaka Municipal Central Wholesale Market, were sliced raw fish for raw consumption as sashimi. Three blocks per sample, approximately 1 cm × 1 cm × 0.5 cm, were taken from different sites of somatic muscle using disposable scissors. They were minced on a disposable culture dish using the same scissors. DNA was extracted and purified from the 0.1 g of minced muscle using DNeasy Blood and Tissue Kits (Qiagen Inc., Germany) according to the manufacturer’s instructions. The 18S ribosomal RNA gene (18S rDNA) and the 28S ribosomal RNA gene (28S rDNA) regions were amplified respectively using primer pairs of Kud6f (Whipps et al. 2003-18gM (Freeman et al. 2008) and Kt28S1f-28S1R (Burger and Adlard 2010). The PCR products were sequenced in both directions using an automated sequencer (ABI 3130; Applied Biosystems). The obtained sequences were aligned with the representative nucleotide sequence of Kudoa species using ClustalX2 (http://www.clustal.org/clustal2/) with initial fixed parameter values. Subsequent phylogenetic analysis of these sequences was performed using MEGA 5 (http://www.mega-software.net/). The obtained nucleotide sequences are available in the International Nucleotide Sequence Database (INSD, GenBank/DDBJ/EMBL) under accession numbers AB698884, AB698885, AB710384, and AB710385. The samples found to be Kudoa-positive by PCR were also examined using light microscopy. The remainder of the minced muscle was sieved through a 100 µm nylon sheet after adding phosphate buffered saline (PBS). The resulting homogenate was passed through a 100 µm cell strainer (BD Biosciences, USA) into a 50 ml disposable centrifuge tube to remove the debris. The homogenate was then centrifuged at 1500 rpm for 10 min (H-500FR; Kokusan Corp., Japan). After centrifugation, the supernatant was discarded and a part of pellet was provided for light microscope observations by both wet mount and staining (Giemsa) preparations. All measurements are given in micrometers (µm). For histological examination, a quantity of Kudoa-positive sliced muscle tissue was fixed in 10% formalin. Then the fixed tissues were processed using the standard histological method. Histological sections (5 µm) were stained with haematoxylin and eosin (HE) and were observed using light microscopy.

Two tuna samples, a young Pacific bluefin tuna landed in Nagasaki Prefecture and an adult yellowfin tuna landed in Shizuoka Prefecture, showed positive amplification for 18S and 28S rDNAs by PCR, but the other 13 samples were negative for both by PCR. One substitution (within 1100-bp overlap) and 10 substitutions (within 753-bp overlap) were found respectively between the partial sequences of 18S and 28S rDNAs from both isolates. Nucleotide sequence similarity searching of the 18S rDNA from the isolates of a Pacific bluefin and a yellowfin tuna showed 100% identity, respectively, with those of K. neothunni from a Pacific bluefin (AB693042) and a yellowfin tuna (AB693049). Sim-
Similarly, the sequence of the 28S rDNA (753-bp) from the present isolates of a Pacific bluefin and a yellowfin tuna showed 100% identity with those of *K. neothunni* isolates from tuna samples described above. No morphological differences of spores were observed between isolates. Spores from both isolates were stellate with six equal shell valves. Each valve had a single polar capsule (Fig. 1). Spores (n = 30 per sample) were of 10.2 (9.0–12.0) or 10.3 (9.0–12.0) width, with 6.8 (6.0–7.5) thickness in both isolates. No cyst or pseudocyst was evident in sliced muscles macroscopically, but pseudocysts were observed histologically in both samples (Fig. 2). Spore morphology and the mode of parasitism in somatic muscle of both isolates were similar to those of *K. neothunni*. Therefore we identified both isolates as *K. neothunni*. *Kudoa neothunni*, previously designated as *Hexacapsula neothunni* and recently reassigned to *Kudoa* (Whipps et al. 2004), was reported for the first time in somatic muscle from a yellowfin tuna in Japan (Arai and Matsumoto 1953). Since then, only four reports in the relevant literature have described *K. neothunni* (Arai and Matsumoto 1953; Konagaya 1982; Whipps et al. 2004; Adlard et al. 2005), although it has been recognized as a causative agent of myoliquefaction (Arai and Matsumoto 1953; Konagaya 1982). Recently, the nucleotide sequence data of ribosomal DNA of *K. neothunni* from a Pacific bluefin and a yellowfin tuna were published. We compared them with sequence data obtained from the present isolates to reveal the molecular characteristics of *K. neothunni*. Based on the phylogenetic analysis of 18S rDNA, *K. neothunni* was classified into two genetically different types: Pacific bluefin and yellowfin tuna types. Also, *K. scomberomori* was grouped with the yellowfin tuna type (Fig. 3a). However, both types in *K. neothunni* were mutually distinct and also distinct from their genetically close *K. grammatorcyni* by phylogenetic analysis of 28S rDNA (nucleotide sequence data of 28S rDNA of *K. scomberomori* are not available in INSD). We inferred that *K. neothunni* might comprise at least two genotypes (Pacific bluefin and yellowfin tuna genotypes). Recently, consumption of raw Pacific bluefin tuna has been suspected as a cause of food poisoning in Japan (Suzuki 2012). In those cases, kudoid parasites similar to *K. neothunni* morphologically and closely related with *K. grammatorcyni* and *K. scomberomori*, but distinct from other kudoid species found in somatic muscles of tuna in the phylogenetic analysis of 18S rDNA as shown in Fig. 3a, were detected from stocked tuna samples in food poisoning cases of unknown etiology. Although the sequence data from their samples have not been published, we infer that the kudoid parasites detected from the raw tuna samples in unidentified food poisoning cases are *K. neothunni*. As shown in the present phylogenetic analysis, *K. neothunni* are clearly classifiable into two genotypes. We speculate that the Pacific bluefin tuna genotype in *K. neothunni* might have pathogenic potential for humans. Additional epidemiological and experimental studies must be undertaken to confirm our speculation. Moreover, further molecular identification of *K. neothunni* from tuna samples is necessary to confirm the host specificity of at least the two genotypes found in this study.

![Fig. 2. Histological section of muscle from a yellowfin tuna infected with *K. neothunni*. Arrow-head indicates pseudocysts in myofibril. HE stain](image)
Fig. 3. Phylogenetic relations of the present Kudoa isolates from a Pacific bluefin and a yellowfin tuna to the representative Kudoa species based on the 18S (a) and 28S (b) rDNA sequences. Distances were estimated using the Tamura–Nei model with pairwise deletion of missing data. The trees were constructed using the neighbour-joining method. Bootstrap support (>50% for 1000 replicates) is shown at each node. Arrows indicate the present Kudoa isolates.
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References


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