Kudoa hexapunctata* n. sp. (Myxozoa: Multivalvulida) from the somatic muscle of Pacific bluefin tuna *Thunnus orientalis* and re-description of *K. neothunni* in yellowfin tuna *T. albacares

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ABSTRACT

Since *Kudoa septempunctata* in olive flounder (*Paralichthys olivaceus*) was indicated to cause food poisoning in humans, other *Kudoa* species are suspected to have pathogenic potential. Recently, a myxosporean possibly associated with food poisoning in humans consuming raw Pacific bluefin tuna, *Thunnus orientalis*, was identified as *K. neothunni*. This is a known causative myxosporean of post-harvest myoliquefaction in yellowfin tuna *T. albacares*. Regardless of the significant differences in the 28S rDNA sequence and the pathological character (with/without myoliquefaction) between the two *T. orientalis* and *T. albacares* isolates, they were considered intraspecific variants of *K. neothunni*. However, the light and low-vacuum electron microscopic observations in the present study revealed that there were two morphotypes; pointed- and round-type spores, which were significantly differentiated by the ratio of suture width to spore width. Furthermore, the two morphotypes were genetically distinguishable by the 28S rDNA sequence analysis. This morphological and molecular evidence validates that the two *Kudoa* types are separate species, and thus the pointed- and round-types are referred to as *K. neothunni* and *K. hexapunctata* n. sp., respectively. *K. neothunni* was detected solely from *T. albacares*, whereas *K. hexapunctata* n. sp. was found mainly in *T. orientalis* but also from *T. albacares*.

Keywords: *Kudoa hexapunctata*; *Kudoa neothunni*; *Thunnus orientalis*; *Thunnus albacares*; Pacific bluefin tuna; food poisoning

1. Introduction

Multivalvulid myxosporeans comprise more than 80 species predominantly found in marine fishes [1]. Among them, kudoid myxosporeans contain economically important species. Several muscle-infecting *Kudoa* species either produce macroscopic cysts in the musculature, e.g., *K. amamiensis* in yellowtail *Seriola quinqueradiata*, or are associated with post-mortem myoliquefaction, e.g., *K. neothunni* in the yellowfin tuna (YFT) *Thunnus albacares*. These signs lower the commercial value of the infected fish [2].

In recent years, *K. septempunctata* from the olive flounder, *Paralichthys olivaceus*, has been recognized as a causative food poisoning agent in humans [3]. Subsequently, the potential effects of other *Kudoa* species on human health were suspected to be similar [4]. More recently, a myxosporean isolated from raw Pacific bluefin tuna (PBT) *Thunnus orientalis*, which have been associated with cases of food poisoning, was identified as *K. neothunni* based on its 18S rDNA sequence and spore morphology [5, 6]. Regardless of the notable differences in 28S rDNA sequence (22 base pair differences across 2,245 bp length) and pathological character (with/without myoliquefaction) between the two isolates from PBT and YFT, Lin et al. [6] considered them to be intraspecific variants of *K. neothunni*. Abe and Maehara [5] also noted 10 substitutions within a 753 bp 28S rDNA overlap between PBT and YFT isolates and proposed that they represented two *K. neothunni* genotypes.

However, because only a few *Kudoa* isolates were analyzed in both studies, the evidence for this species identification seems insufficient. In the present study, therefore, we aimed to solve the question on the classification of *K. neothunni* based on detailed morphological and molecular studies of a larger data set.

2. Materials and methods

2.1 Fish samples

Three specimens of juvenile wild PBT were collected from Nagasaki, Kochi, and Aomori Prefectures, Japan, in December 2013, February 2013, and July 2012, respectively. Four specimens of juvenile cultured PBT (0-1 year old) were obtained from anonymous fish farmers in western Japan between February and July 2012. Seven specimens of sliced raw fish (sashimi) from adult wild YFT were purchased at local markets between June and September 2012. One sample of YFT muscle block (36 kg in body weight) showing typical post-harvest myoliquefaction was provided by Okinawa Prefectural Institute of Health and Environment, in November 2011. The latter fish was captured off the Philippines in October 2011 and landed at Naha, Okinawa Prefecture in November 2011. The precise capture locality of YFT bought at markets is not known, and thus the location described in the price tag was used as the origin of each sample. All samples were frozen at -20°C or -80°C until spore collection.

After thawing of the samples, several pieces of muscle tissue were taken from different parts of the samples. As for the YFT with myoliquefaction, four pieces of muscle tissue were collected, two from the liquefied and two from the non-liquefied part of the same fish. The tissue was then flattened between two glass plates; pseudocysts were then examined under a dissecting microscope with transmitted light. To avoid cross-contamination of multiple *Kudoa* species, pseudocysts were individually excised from each specimen with fine forceps and squashed on a glass

slide with a few drops of phosphate buffered saline (PBS). After the spores had been photographed under a light microscope at 1000× magnification, the remaining spore suspensions were placed in a 1.5 ml tube and frozen at -20°C or -80°C for molecular analysis.

2.2 Morphological examination

For each specimen, spore measurements were taken from 20 spores, when available, on a computer using ImageJ (image-processing program available at: <http://rsb.info.nih.gov/ij/>). The spores were measured following the methods of Adlard et al. [7]. To express the extent of the pointed valves quantitatively, the ratio of suture width to spore width were calculated. The statistical significance of difference in the extent of the pointed valves between the two morphotypes was tested with a t-test using JMP version 10 (SAS Institute).

2.3 Low-vacuum scanning electron microscopy

To collect *Kudoa* spores, samples of fish muscle were minced with surgical scissors in 5× volume of 1/15 mol/l, pH 7.2 phosphate buffer (PB), passed through a cell strainer (40 µm diameter) (Becton, Dickinson and Company Bioscience Falcon, NJ, USA) and centrifuged at 600 g for 5 min. After the *Kudoa* spores in crude suspension were examined microscopically at a magnification of 400×, the spores were purified using Percoll density gradient centrifugation. Part of the spore sample was fixed in 2% glutaraldehyde in PB for 2 hours and used for low-vacuum scanning electron

microscopy (LV-SEM). Following three PB washes with an average pore diameter of 1.2 μm SEMPore[®] (JEOL Ltd Datum, Tokyo, Japan); the spore sample was dehydrated through a graded ethanol series, immersed in warmed *t*-butylalcohol, and frozen for 40 s in liquid nitrogen. The frozen sample on the SEMPore[®] was mounted on the specimen stage of an LV-SEM (model JSM-5600LV, JEOL, Tokyo, Japan). The specimen chamber was evacuated to 60 Pa and slowly sublimated for 60 min. The spore sample was examined at an accelerating voltage of 20 kV.

2.4 Polymerase chain reaction (PCR) and sequence analysis

The DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) from the 50 mg fish samples. The DNA samples positive for *Kudoa* 18S rDNA by the real-time PCR assay described below were amplified using two primer sets (Ku18F1/Ku18R1 and Ku18F2/Ku18R2; Table 1) for the analysis of the *Kudoa* 18S rDNA gene and three primer sets (Myxo28S1F/Ku28R1, Ku28F2/Myxo28S3R and Ku28F3/Ku28R3; Table 1) for the *Kudoa* 28S rDNA gene (Myxo28S1F and Myxo28S3R primers previously reported by Whipps et al.) [8]. PCR amplification was performed in a reaction mixture (50 μl) containing 5 μl of DNA template, 1.0 U of Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 0.4 μM of each primer, and 0.25 mM of each deoxynucleoside triphosphate (dNTP). The following cycling parameters were used: (1) Taq activation at 94°C for 3 min; (2) 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min; and (3) a final extension at 72°C for 5 min.

The PCR products of the *Kudoa* 18S and 28S rDNA from the fish samples were

sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI PRISM 3130 Genetic Analyzer.

2.5 Real-time PCR assay

The *Kudoa* 18S rDNA sequence was targeted for the real-time PCR assay. According to Abe and Maehara [5], the PBT and YFT genotypes were analyzed separately. The PBT genotype primers and probe set (PBT-F, PBT-R and PBT-P; Table 1) were designed based on the PBT genotype sequence, whereas those (YFT-F, YFT-R and YFT-P; Table 1) for the YFT genotype were designed using Primer Express v 2.0 (Applied Biosystems, CA, USA) and the Taqman[®] minor groove binding (MGB) probe. Real-time PCR was performed in a final volume of 50 µl containing Universal Master Mix (Applied Biosystems), 900 nM of the two primers sets (PBT-F and PBT-R, YFT-F and YFT-R), 250 nM Taqman MGB probes (PBT-P, YFT-P), and 4 µl DNA template. PCR reactions were analyzed with a 7900HT Fast Real Time PCR System (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The gene copy quantity was determined using the standard curve method provided with a plasmid DNA control template.

2.6 Phylogenetic analysis

The multiple alignments and phylogenetic analysis of the obtained sequences of the

Kudoa spp. 28S rDNA gene (2671 bp) were performed using Clustal W [9]. Maximum likelihood (ML) and Bayesian analyses were conducted by PHYML version 2.4.5 software [10] and MrBayes version 3.1.2. [11], respectively. The ML method and a general time-reversible (GTR) model were used to calculate genetic distances. Statistical support was evaluated using 1,000 bootstrap replicates for ML. In Bayesian analysis, we ran four simultaneous chains (nchain = 4), 1,000,000 generations, and an initial burn-in of 1,250, at which point the likelihood values had stabilized. The GTR model with a proportion of invariant bases and four categories of among-site rate variation were used; the trees were sampled every 100 generations. The ML tree and Bayesian tree data files were visualized using MEGA version 4.0.2 [12]. The GenBank accession numbers and names of each *Kudoa* spp. strain used in the phylogenetic analysis of the 28S rDNA gene are shown in Fig. 5.

3. Results

3.1 Spore morphology

Spores from all of the *Kudoa* isolates examined in this study possessed six equal-sized spore valves and polar capsules. The number of spore valves and polar capsules was almost consistently six (>99%) and very rarely five or seven. Spore dimensions almost overlapped or differed only slightly among all isolates (Table 2).

However, there were two distinct morphotypes in terms of spore shape. The extent of pointed spore valves differed giving the round- and pointed-types (Fig. 1). In side view, particularly, the pointed-type spores had a prominent valve extension (Fig.

1H). The mean ratio of suture width to spore width of the round-type spores was greater than 70%, whereas those of the pointed-type spores were less than 70% (Fig. 2). All seven PBT isolates were classified as the round-type, whereas three and five YFT isolates were classified as round- and pointed-types, respectively (Fig. 2, Table 2).

LV-SEM also revealed that PBT and YFT *Kudoa* spores had six spore valves, which were equal in size and shared the central point (Fig. 3). Two morphotypes were observed as well as under light microscopy. The suture lines of the round- type spores were clearly visible, compared to those of pointed- type spores.

3.2 Molecular analysis of *Kudoa* spp.

The 18S and 28S rDNA sequences (1,653 bp and 3,234 bp, respectively) of round-type *Kudoa* spores were obtained from seven PBT and four YFT samples, which were all 100% identical among the *Kudoa* isolates and deposited in GenBank (Accession nos. AB902954, AB902956 for 18S rDNA, and AB902955, AB902957 for 28S rDNA). Similarly, five pointed-type spore 18S and 28S rDNA (1,653 bp and 2,337 bp, respectively) sequences were identical to five YFT samples, these were also deposited in GenBank (Accession nos. AB902958, AB902060 for 18S rDNA, and AB902959, AB902961 for 28S rDNA). The round- and pointed-type spore 18S rDNA sequences were 99.9% similar (2 bp differences in 1,653 bp), whereas the 28S rDNA were 99.3% similar (22 bp differences in 3,237 bp).

Round- and pointed-type spore 28S rDNA nucleotide sequences exhibited 100% identity with those of *K. neothunni* isolated from PBT (a 2,143 bp overlap with AB693042) and YFT (a 2,337 bp overlap with AB693049), respectively, as described

by Lin et al. [6] (Fig. 4).

3.3 *Kudoa* spp. real-time PCR assay

The real-time PCR assay of 15 fish specimens revealed that the PBT-genotype was detected in seven PBT and four YFT specimens (Pacific Ocean, Indian Ocean A and B, and Philippines) (Table 3). The YFT-genotype was detected in five YFT specimens (Vanuatu, South Korea, Philippines, Fiji, and unknown locality). Only YFT specimen with myoliquefaction (Philippines) had both genotypes. The two morphotypes, round- and pointed-type, corresponded perfectly to the two genotypes, PBT- and YFT-type, respectively (Table 3). The YFT-type distributed uniformly within the somatic muscle of the YFT specimen from Philippines, as the 18S rDNA copy numbers (per gram) were almost similar among the four different parts of the muscle (Table 4). In contrast, the 18S rDNA copy numbers (per gram) of the PBT-type varied widely among the different parts of the muscles (Table 4).

From here on, the *Kudoa* YFT genotype isolates with pointed-type spores and PBT genotype with round-type spores are referred to as *K. neothunni* and *K. hexapunctata* n. sp., respectively.

3.4 Phylogenetic analysis

In the phylogenetic tree based on the 28S rDNA, the eleven *K. hexapunctata* n. sp. isolates and the five *K. neothunni* isolates were divided into two distinct clades, and

grouped with *K. neothunni* from PBT (AB693042) and *K. neothunni* from YFT (AB693049), respectively (Fig. 5). The *K. neothunni* isolates identified by Abe and Maehara [5] were closely related in the 28S rDNA comparison, but the sequences they deposited were considerably shorter (678 and 687 bp). Therefore, the isolates were excluded from the phylogenetic analysis.

3.5 Descriptions

3.5.1 *Kudoa hexapunctata* n. sp.

The spores are hexaradiate in apical view, with six equal spore valves each containing one polar capsule (Fig. 6). The peripheral edge of the spore valve is rounded. The polar capsules are convergent, pyriform in shape. The polar filament is coiled in two turns within the polar capsule. In side view, the spores are pyramidal in shape without extensions. The spore measurements are summarized in Table 2.

Taxonomic summary

Host type: *Thunnus orientalis*, Pacific bluefin tuna (Teleostei, Scombridae).

Additional host: *Thunnus albacares*, yellowfin tuna (Teleostei, Scombridae).

Sample location: Nagasaki Prefecture, Japan

Additional locations for *T. albacares*: Pacific Ocean, Indian Ocean

Site of infection: Somatic muscle. Pseudocysts in the myofibers are usually invisible to the naked eye (subclinical).

Materials: Syntype specimens (Methylene blue stained smear of spores), accession numbers, MPM Coll. No. 20912, Meguro Parasitological Museum, Tokyo, Japan;

NSMT-Pr 354, National Museum of Nature and Science, Tokyo, Japan.

Etymology: The species name is derived from the possession of the six polar capsules.

Japanese name: Mutsuboshi-Kudoa (Mutsuboshi means six stars in Japanese.) In addition, Nanahoshi-Kudoa meaning *Kudoa* with seven stars in Japanese is proposed as a new Japanese name for *K. septempunctata* Matsukane, Sato, Tanaka, Kamata and Sugita-Konishi, 2010.

3.5.2 *Kudoa neothunni* (Arai and Matsumoto, 1953)

The spores are hexaradiate in apical view, with six equal spore valves each containing one polar capsule (Fig. 6). The peripheral edge of the spore valve is rather pointed, compared to those of *K. hexapunctata* n. sp. The polar capsules are convergent, pyriform in shape. The polar filament is coiled in two turns within the polar capsule. In side view, the spores are pyramidal in shape with prominent extensions. The spore measurements are summarized in Table 2.

Taxonomic summary

Host: *Thunnus albacares*, yellowfin tuna (Teleostei, Scombridae)

Additional hosts: *T. obesus* and *T. orientalis* have been reported [13] but re-examination is required.

Sample location: Banda Sea

Additional locations: For *T. albacares*, off Taiwan [13], Vanuatu, Philippines (landed in Okinawa), Fiji, and one unknown locality (landed in South Korea)

Site of infection: Somatic muscle. Infected muscle often exhibits liquefaction after the host's death.

Materials: Neotype specimens (Methylene blue stained smear of spores). Accession numbers for Vanuatu specimen, MPM Coll. No. 20913, Meguro Parasitological Museum, Tokyo, Japan; NSMT-Pr 355, National Museum of Nature and Science, Tokyo, Japan. Accession numbers for Philippines specimen, MPM Coll. No. 20914, Meguro Parasitological Museum, Tokyo, Japan; NSMT-Pr 356, National Museum of Nature and Science, Tokyo, Japan.

Japanese name: Kihada-Kudoa (Kihada means yellowfin tuna in Japanese.)

3.6 Remarks

K. hexapunctata n. sp. and *K. neothunni* are indistinguishable by both the morphometric parameters usually measured and by the 18S rDNA sequence. However, *K. hexapunctata* n. sp. is differentiated from *K. neothunni* by spore shape. The spore valves of *K. hexapunctata* n. sp. are rather rounded, whereas those of *K. neothunni* are pointed. The difference in the ratio of suture width to spore width is statistically significant (Fig. 2). *K. hexapunctata* n. sp. and *K. neothunni* can be separated by mean ratio of suture width to spore width being above and below approximately 70%, respectively. When the ratio of suture width to spore width are calculated for the other *K. neothunni* isolates using the spore dimensions previously reported, *K. neothunni* in the original description of Arai and Matsumoto [14] is 64.5%, as expected. However, that of *K. neothunni* described from PBT by Lin et al. [6] is 76.4%.

Furthermore, the 28S rDNA sequences of the two species were different at the invariable positions (22 bp differences in 3,237 bp) (Fig. 4). In addition, *K. neothunni* causes myoliquefaction in fish hosts, whereas *K. hexapunctata* n. sp. does not.

Combined with the morphological, molecular, and pathological differences, *K. hexapunctata* n. sp. should be considered a different species from *K. neothunni*.

4. Discussion

K. neothunni, first designated *Hexacapsula neothunni* and later reassigned to *Kudoa* [8], was originally described from YFT with post-harvest myoliquefaction. Since then, PBT and the bigeye tuna (*Thunnus obesus*) have also been documented as additional hosts based on superficial similarities in spore morphology [5, 6, 8]. Despite differences in 28S rDNA between *Kudoa* isolates from YFT and PBT being noted by Lin et al. [6] and Abe and Maehara [5], neither research group could assign them as separate species. Nonetheless, in the present study, detailed morphological and molecular comparisons with multiple isolates revealed the existence of two distinct morphotypes, which corresponded perfectly to two genotypes in the 28S rDNA sequence. This evidence validates the separation of the two types of *Kudoa* into two distinct species. Of the two types, it seems reasonable that the YFT genotype with pointed-type spores is referred to as *K. neothunni*, because of its host and pathological character (myoliquefaction). Thus, we have created a new species for the PBT genotype with round-type spores; *K. hexapunctata* n. sp. In addition, the present study suggests that *K. neothunni* (AB693042) from PBT described by Lin et al. [6] is synonymized with *K. hexapunctata* n. sp., because of the host specificity, high genetic affinity, and morphological similarity (> 70% suture width/spore width).

Based on the 18S rDNA sequence, only one (a 1,100 bp overlap [5]) or two substitutions (a 1,720 bp overlap [6]) were found between *K. hexapunctata* n. sp. and *K. neothunni*. However, many other *Kudoa* species differ from closely related species by only a few bases of the 18S rDNA sequence. For example, there is a 2 bp difference between *K. rosenbuschi* and *K. alliaria* [15], and between *K. chaetodoni* and *K. yasunagai* [16], and a 1 bp difference between *K. paraquadricornis* and *K. quadricornis* [17]. Therefore, to classify *Kudoa* species, analysis of the 28S rDNA has greater resolving power than the 18S rDNA. Although *K. chaetodoni* differs from *K. yasunagai* in the 18S rDNA by only 0.19%, their 28S rDNA differs by 0.6–1.3% [16]. Recent identification of myxosporeans has depended on multiple criteria, i.e. a combination of genetic, morphological, and other biological data [18]. Considering the recent trends, the notable differences in spore morphology, 28S rDNA, and pathological character give support to *K. hexapunctata* n. sp. being a new species.

Separation of the *Kudoa* isolates examined in this study into two distinct species implies that *K. neothunni* is not pathogenic in humans, because food poisoning in association with consumption of tuna has only occurred with juvenile PBT [4]. Subclinical infection with *K. hexapunctata* n. sp. suggests that sashimi containing spores could be eaten and not noticed by consumers. This also applies to *K. septempunctata* in olive flounder [19]. Thus, accurate diagnosis of *K. hexapunctata* n. sp. infection is important for public health, by avoiding risks for consumers. The real-time PCR diagnostic assay to detect and differentiate *K. hexapunctata* n. sp. and *K. neothunni* developed in this study will be useful for identifying the causes of food poisoning cases and for monitoring of *K. hexapunctata* n. sp. in tuna from fish markets. Further studies will be required to elucidate the pathogenicity of *K. hexapunctata* n. sp.

to humans by animal experiments or Caco-2 cells as used for *K. septe mpunctata* in olive flounder [3, 20].

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Figure legends

Fig. 1: Fresh *Kudoa hexapunctata* n. sp. spores. A-C: top view, D: side view. Fresh *K. neothunni* spores. E-G: top view, H: side view. Scale bars: 10 µm.

Fig. 2: Box plot of the extent of the pointed valves (suture width/spore width) for 15 *Kudoa* isolates from Pacific bluefin tuna (white box) and yellowfin tuna (shaded box). Origins of tuna specimens are shown on X-axis.

Fig. 3: *Kudoa hexapunctata* n. sp. (A-C) and *K. neothunni* (D-F) spores by low-vacuum scanning electron microscopy. Scale bars: 2 µm.

Fig. 4: 28S rDNA sequences of round-type *Kudoa* from PBT (the present study), *K. neothunni* from PBT (AB693042), pointed-type *Kudoa* from YFT (the present study), and *K. neothunni* from YFT (AB693049).

Fig. 5: Phylogenetic relationships obtained by ML analysis among 28S rDNA gene sequences of *Kudoa hexapunctata* n. sp. and *Kudoa neothunni* isolates and reference strains. The ML tree was derived with a GTR model using estimates of the proportion of

446 invariable site parameters of 0.478. Significant bootstrap support from ML by 1,000
447 replicates and Bayesian analysis are shown above the node in the order ML/BI. The
448 scale bar represents the distance in substitutions per nucleotide. GenBank accession
449 numbers are given in parentheses.

450 **Fig. 6:** Schematic diagrams of *K. hexapunctata* n. sp. (above) and *K. neothunni* (below)
451 spores. W: width, T: thickness, S: suture width, L: length, PCL: polar capsule length,
452 PCW: polar capsule width.

453

Fig. 1

Fig. 2

Fig. 3

Fig. 5

Fig. 6

Table 1. Oligonucleotide primers used for PCR assays in the present study

Primer name		Primer sequence (5' to 3')	Position	Accession no.
PBT-F	(forward)	GGCTAGTGAAAAGCCAACTTATGG	1587-1610	AB693042
PBT-R	(reverse)	TTCGCGGATTCCAACCTATT	1614-1630	AB693042
PBT-P	(probe)	CACTTGTGTGGCTAAAT	1633-1652	AB693042
YFT-F	(forward)	GCCAACTTATGGTCGCACTGT	1599-1619	AB693049
YFT-R	(reverse)	TTCCCTTTCGCGGATTCC	1621-1637	AB693049
YFT-P	(probe)	TGTGGCTGGATATAGGT	1639-1656	AB693049
Ku18F1	(forward)	AGCCATGCAAGTCTAAGTTCA	26-46	AB553293
Ku18R1	(reverse)	TTTCACTGAGGGCCCAACCTGGATT	1012-1036	AB553293
Ku18F2	(forward)	ATTAAAAGGGACATTTGAGGG	814-839	AB553293
Ku18R2	(reverse)	TACGGAAACCTTGTTACGACT	1680-1700	AB553293
Myxo28S1F	(forward)	AGTAACTGCGAGTGAAGCG	80-98	FJ417058
Ku28R1	(reverse)	TCACGCATAGTTCACCATCT	946-965	FJ417058
Ku28F2	(forward)	AGTGAAAGCGAGAGATGAGA	845-864	FJ417058
Myxo28S3R	(reverse)	GAGCACTGGGCAGAAATC	2506-2523	FJ417058
Ku28F3	(forward)	ATTAAAACAAAGCATTGCGAT	2459-2479	FJ417058
Ku28R3	(reverse)	GCATTGCATCACTGGCCTAT	3504-3523	FJ417058

Table 2. Comparison of spore dimensions (μm , mean $\pm$ SD, range in parentheses) of *Kudoa hexapunctata* n.sp. and *K. neothunni* from Pacific bluefin tuna (PBT) and yellowfin tuna (YFT)

Host	Origin	Spore					Polar capsule		Morphotype*	Species
		Width	Length	Thickness	Suture Width	Suture width / Width (%)	Length	Width		
PBT (This study)	Farmed A	8.7 $\pm$ 0.4 (8.0-9.2)	5.9 $\pm$ 0.4 (5.0-6.4)	7.5 $\pm$ 0.3 (7.0-8.2)	6.6 $\pm$ 0.4 (5.6-7.4)	76.4 $\pm$ 4.2 (67.0-82.6)	2.7 $\pm$ 0.4 (2.2-3.1)	1.5 $\pm$ 0.2 (1.3-1.8)	Round	<i>K. hexapunctata</i> n. sp.
	Farmed B	10.6 $\pm$ 0.4 (10.1-11.2)	7.2 $\pm$ 0.4 (6.4-7.9)	9.3 $\pm$ 0.3 (8.6-10.0)	8.4 $\pm$ 0.4 (7.7-9.0)	79.6 $\pm$ 4.2 (71.0-87.3)	3.4 $\pm$ 0.4 (2.7-4.5)	2.0 $\pm$ 0.2 (1.7-2.2)	Round	<i>K. hexapunctata</i> n. sp.
	Farmed C	10.1 $\pm$ 0.4 (9.2-10.9)	6.8 $\pm$ 0.4 (6.2-7.4)	8.6 $\pm$ 0.3 (7.9-9.0)	7.4 $\pm$ 0.3 (6.9-7.8)	73.7 $\pm$ 2.9 (69.0-80.0)	3.4 $\pm$ 0.4 (2.5-4.0)	1.8 $\pm$ 0.2 (1.4-2.0)	Round	<i>K. hexapunctata</i> n. sp.
	Farmed D	8.7 $\pm$ 0.4 (8.0-9.2)	5.9 $\pm$ 0.3 (5.4-6.3)	7.4 $\pm$ 0.2 (7.0-7.8)	6.5 $\pm$ 0.2 (6.0-6.8)	75.0 $\pm$ 3.3 (67.8-83.3)	2.9 $\pm$ 0.4 (2.2-3.4)	1.6 $\pm$ 0.1 (1.3-1.9)	Round	<i>K. hexapunctata</i> n. sp.
	Nagasaki	10.4 $\pm$ 0.5 (9.1-11.4)	7.5 $\pm$ 0.5 (6.6-8.2)	9.1 $\pm$ 0.5 (8.4-9.9)	7.8 $\pm$ 0.5 (6.5-8.5)	75.3 $\pm$ 3.4 (67.3-80.7)	3.2 $\pm$ 0.5 (2.0-3.9)	1.7 $\pm$ 0.2 (1.4-2.0)	Round	<i>K. hexapunctata</i> n. sp.
	Kochi	10.3 $\pm$ 0.7 (8.9-11.4)	7.3 $\pm$ 0.3 (6.7-7.9)	9.0 $\pm$ 0.6 (7.9-10.2)	7.7 $\pm$ 0.5 (6.9-8.8)	75.3 $\pm$ 5.4 (66.5-82.9)	3.3 $\pm$ 0.4 (2.7-4.0)	1.8 $\pm$ 0.2 (1.5-2.3)	Round	<i>K. hexapunctata</i> n. sp.
	Aomori	10.7 $\pm$ 0.5 (9.4-11.8)	7.7 $\pm$ 0.5 (6.9-8.4)	9.2 $\pm$ 0.5 (8.0-10.1)	8.2 $\pm$ 0.4 (7.3-8.9)	76.7 $\pm$ 4.5 (67.3-85.7)	3.4 $\pm$ 0.4 (2.8-4.0)	1.8 $\pm$ 0.2 (1.3-2.1)	Round	<i>K. hexapunctata</i> n. sp.
YFT (This study)	Pacific Ocean	10.2 $\pm$ 0.4 (9.3-11.0)	7.1 $\pm$ 0.5 (6.4-8.1)	9.0 $\pm$ 0.3 (8.4-9.4)	8.0 $\pm$ 0.3 (7.3-8.4)	78.0 $\pm$ 3.3 (72.1-84.8)	3.2 $\pm$ 0.6 (2.3-4.1)	1.9 $\pm$ 0.2 (1.6-2.2)	Round	<i>K. hexapunctata</i> n. sp.
	Indian Ocean A	9.7 $\pm$ 0.3 (9.3-10.4)	7.0 $\pm$ 0.4 (6.0-7.5)	8.5 $\pm$ 0.3 (7.9-9.0)	7.3 $\pm$ 0.3 (6.7-7.7)	75.5 $\pm$ 2.8 (67.9-79.9)	3.6 $\pm$ 0.5 (2.3-4.1)	1.9 $\pm$ 0.2 (1.5-2.1)	Round	<i>K. hexapunctata</i> n. sp.
	Indian Ocean B	9.8 $\pm$ 0.4 (9.1-10.7)	7.2 $\pm$ 0.3 (6.7-7.4)	8.7 $\pm$ 0.6 (7.8-10.3)	7.8 $\pm$ 0.6 (7.0-9.3)	79.2 $\pm$ 4.0 (72.6-87.6)	3.6 $\pm$ 0.4 (2.8-4.1)	1.9 $\pm$ 0.2 (1.5-2.3)	Round	<i>K. hexapunctata</i> n. sp.
	Vanuatu	11.7 $\pm$ 0.7 (10.7-12.9)	7.0 $\pm$ 0.4 (6.3-7.5)	9.7 $\pm$ 0.5 (8.6-10.5)	7.4 $\pm$ 0.3 (6.8-7.9)	63.4 $\pm$ 4.0 (57.1-72.7)	3.5 $\pm$ 0.7 (2.4-4.5)	2.0 $\pm$ 0.1 (1.8-2.2)	Pointed	<i>K. neothunni</i>
	South Korea	11.0 $\pm$ 0.5 (9.7-11.5)	6.6 $\pm$ 0.4 (5.9-7.4)	9.2 $\pm$ 0.5 (8.4-10.0)	7.3 $\pm$ 0.4 (6.4-7.8)	63.4 $\pm$ 4.0 (57.1-72.7)	3.5 $\pm$ 0.4 (2.4-4.5)	1.9 $\pm$ 0.1 (1.7-2.2)	Pointed	<i>K. neothunni</i>
	Philippines	11.8 $\pm$ 0.7 (11.1-12.7)	NA	10.4 $\pm$ 0.4 (9.7-10.8)	7.9 $\pm$ 0.6 (6.9-8.5)	66.9 $\pm$ 4.0 (61.4-70.0)	NA	1.7 $\pm$ 0.1 (1.6-1.9)	Pointed	<i>K. neothunni</i>
	Fiji	12.0 $\pm$ 0.5 (11.0-12.7)	6.5 $\pm$ 0.4 (5.9-7.4)	10.1 $\pm$ 0.5 (9.3-11.0)	7.4 $\pm$ 0.4 (6.9-8.5)	62.0 $\pm$ 3.5 (57.2-72.3)	3.5 $\pm$ 0.4 (2.8-4.1)	2.1 $\pm$ 0.1 (1.8-2.3)	Pointed	<i>K. neothunni</i>
	Unknown	11.5 $\pm$ 0.6 (10.6-12.1)	6.8 $\pm$ 0.3 (6.3-7.7)	9.7 $\pm$ 0.6 (9.1-10.5)	7.6 $\pm$ 0.2 (7.4-7.9)	66.0 $\pm$ 2.2 (64.3-69.6)	3.9 $\pm$ 0.4 (2.9-4.3)	2.0 $\pm$ 0.2 (1.7-2.1)	Pointed	<i>K. neothunni</i>
YFT (Arai and Matsumoto, 1953)	Banda Sea	11.0 (9.1-13.0)	6.2 (5.3-7.3)	NA	7.1 (5.9-8.7)	64.5	2.5 (2.0-3.1)	1.6 (1.3-2.1)	Pointed	<i>K. neothunni</i>
YFT or PBT (Abe and Maehara, 2013)	Unknown	10.2 or 10.3 (9.0-12.0)	NA	6.8 (6.0-7.5)	NA	NA	NA	NA	?	?
PBT (Li et al., 2013)	Unknown	10.6 (9.5-11.4)	7.6 (7.3-7.7)	9.7 (8.9-10.9)	8.1 (7.3-8.6)	76.4	3.8 (3.6-4.1)	2.0 (1.8-2.3)	Round	<i>K. hexapunctata</i> n. sp.

*Round- and pointed-types were classified above or below 70% in suture width/spore width, respectively.

Table 3. The relationship between morphotypes and genotypes of *Kudoa* spp. from Pacific bluefin tuna (PBT) and yellowfin tuna (YFT)

Host	Origin	Morphotype	Genotype	Species	Detection of YFT-type		Detection of PBT-type	
					Ct values	Copy numbers/g	Ct values	Copy numbers/g
PBT	Farmed A	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	16.8	9.5×10^9
	Farmed B	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	20.8	6.1×10^8
	Farmed C	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	16.2	1.4×10^{10}
	Farmed D	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	20.6	6.7×10^8
	Nagasaki	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	19.2	1.9×10^9
	Kochi	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	15.4	3.5×10^{10}
	Aomori	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	15.9	3.1×10^{10}
YFT	Pacific Ocean	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	29.8	2.2×10^6
	Indian Ocean A	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	20.6	6.8×10^8
	Indian Ocean B	Round	PBT	<i>K. hexapunctata</i> n.sp.	-	-	22.0	2.8×10^8
	Vanuatu	Pointed	YFT	<i>K. neothunni</i>	26.1	8.9×10^7	-	-
	South Korea	Pointed	YFT	<i>K. neothunni</i>	23.2	5.9×10^8	-	-
	Philippines	Round / Pointed	PBT/YFT	<i>K. hexapunctata</i> n. sp. / <i>K. neothunni</i>	23.5	5.2×10^8	23.9	7.0×10^8
	Fiji	Pointed	YFT	<i>K. neothunni</i>	25.0	1.9×10^8	-	-
	Unknown	Pointed	YFT	<i>K. neothunni</i>	25.5	1.3×10^8	-	-

Table 4. Ct values in real-time PCR and estimated copy numbers per gram-muscle by detection for PBT- and YFT-type *Kudoa* from liquefied and non-liquefied parts of yellowfin tuna (YFT)

Part of somatic muscle YFT	Detection of YFT-type		Detection of PBT-type	
	Ct values	copy numbers/g	Ct values	copy numbers/g
Liquefied part 1	23.0	6.6×10^8	27.6	5.9×10^6
Liquefied part 2	23.9	3.7×10^8	25.9	1.8×10^7
Normal part 1	23.0	6.8×10^8	23.4	9.9×10^7
Normal part 2	24.0	3.6×10^8	18.6	2.7×10^9
Average	23.5	5.2×10^8	23.9	7.0×10^8
Ave. liquefied part	23.5	5.1×10^8	26.7	1.2×10^7
Ave. normal part	23.5	5.2×10^8	21.0	1.4×10^9

Kudoa hexapunctata n. sp.
(Round-type spores)

Kudoa neothunni
(Pointed-type spores)

K. hexapunctata n. sp. and *K. neothunni* were morphologically and molecularly distinguishable.

Extent of pointed valves for 15 *Kudoa* isolates from Pacific bluefin tuna (white box) and yellowfin tuna (shaded box)