



Morphological and molecular genetic characterization of *Kudoa konishiae* n. sp. (Myxosporaea: Multivalvulida) in the muscle of Japanese Spanish mackerel (*Scomberomorus niphonius*)

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Abstract

The Japanese Spanish mackerel (*Scomberomorus niphonius*; Perciformes: Scombridae) is widely distributed in the continental shelves of the northwestern Pacific Ocean around Japan, Sea of Japan, and East China Sea. In the present study, small, spindle-shaped myxosporean plasmodia (0.15–0.53 mm by 0.04–0.13 mm) were frequently encountered in the myofiber of trunk muscles of two Japanese Spanish mackerels; one fished in the Sea of Japan off western Japan and the other in the northwestern Pacific Ocean off southern Japan in the autumn of 2016. Isolated myxospores of *Kudoa konishiae* n. sp. (Myxosporaea: Multivalvulida) from these two fish were stellate with six equal shell valves and polar capsules, 8.1–9.7 µm in width, 7.1–8.8 µm in thickness, and 7.1–8.8 µm in length. The polar capsules were teardrop-shaped, 2.7–4.7 µm by 1.2–2.5 µm. The lateral view of spores revealed a drawstring-pouch shape. The nucleotide sequences of the 18S and 28S ribosomal RNA gene (rDNA) were distinct from any recorded species. Phylogenetic trees demonstrated a close relationship of the present new species with *Kudoa* spp. with stellate spores with five or more shell valves/polar capsules, recorded in scombrid fishes. To clarify the phylogenetic relationships between three closely related species, i.e., *Kudoa konishiae* n. sp., *Kudoa hexapunctata*, and *Kudoa neothunni*, three mitochondrial DNA genes (cytochrome *c* oxidase subunit 1 gene (*cox-1*) and the small and large subunits of the ribosomal RNA gene (*rns-rnl*)) of two isolates of the new species, six isolates of *K. hexapunctata*, and 13 isolates of *K. neothunni* were sequenced. The interspecific and intraspecific variations of the newly obtained *cox-1* and *rns-rnl* nucleotide sequences of *K. hexapunctata*, *K. neothunni*, and *K. konishiae* n. sp. were clarified for the first time.

Keywords *Kudoa konishiae* n. sp. · Myxosporaea · Multivalvulida · *Scomberomorus niphonius* · Japan · Morphology · rDNA · Mitochondrial gene · *Kudoa hexapunctata* · *Kudoa neothunni*

Introduction

Kudoa Meglitsch, 1947, is the largest genus in the order Multivalvulida (Myxozoa: Myxosporaea), with more than 100 nominal species recorded to date (Moran et al. 1999; Lom and Dyková 2006; Eiras et al. 2014b; Sato and Kasai 2016). The

genus is defined as myxosporans with four or more shell valves (SV) and polar capsules (PC) which are equal in number (Whipps et al. 2003a, 2004; Lom and Dyková 2006). During the last two decades, introducing molecular genetic analyses into the process of specifically differentiating kudoids and other myxosporans has enabled a more precise understanding of their biodiversity and biogeography. As discussed in a recent article from our laboratory (Kasai et al. 2017a), although a group of *Kudoa* spp. characterized by myxospores of unequal quadrangular pyramid shape and containing one large and three smaller SV/PC showed fairly close morphological resemblance to each other, at least nine species were differentiated by nucleotide sequencing of the ribosomal RNA gene (rDNA), i.e., *K. thyrssites*, *K. minithyrssites*, *K. lateolabracis*, *K. megacapsula*, *K. whippsi*, *K. gunterae*, *K. cheilodipteri*, *K. parathyrssites*, and *K. akihittoi* (Whipps et al. 2003b; Yokoyama et al. 2004; Yokoyama and

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Itoh 2005; Whipps and Kent 2006; Burger and Adlard 2010a; Heiniger et al. 2013; Kasai et al. 2016, 2017a). Despite the fact that different extents of nucleotide variation of the rDNA within a species can complicate the specific identification, phylogenetic critique of the phenotypical variation of a species facilitates our understanding of the real biodiversity, as previously demonstrated by numerous studies on a wide range of myxosporeans (Diamant et al. 2005; Burger et al. 2008; Burger and Adlard 2010b, 2011; Miller and Adlard 2012, 2013; Azevedo et al. 2014; Bartošová-Sojtková et al. 2014; Fiala et al. 2015; Alama-Bermejo et al. 2016; Sekiya et al. 2016; Kato et al. 2017).

Up until the end of 2016, 25 *Kudoa* spp. and two *Uncapsula* spp. had been recorded from edible marine fish species in the natural seawater around Japan or popular imported marine fish consumed daily by Japanese people (Sato and Kasai 2016). This recent survey, however, highlights the need to record more myxosporeans from daily consumed marine fish. In the present study, we characterize morphologically and genetically a new kudoid species exhibiting stellate spores with six equal SV/PC, *Kudoa konishiae* n. sp., from the Japanese Spanish mackerel, *Scomberomorus niphonius* (Cuvier, 1832), purchased from fish markets in western Japan.

Materials and methods

Fish samples and parasitological examination

Whole bodies of eight individuals of the fish species Japanese Spanish mackerel were purchased from local fish markets in western Japan: two individuals fished on October 17, 2016, in the Sea of Japan off Abu-cho, Yamaguchi Prefecture, Japan, and six individuals fished on November 8 and 29, 2016, in the northwestern Pacific Ocean off Kagoshima Prefecture, Japan. The standard lengths and body weights of these eight individuals were 42–50 (46) cm and 0.78–1.02 (0.86) kg, respectively (range with the average in parentheses). Following transportation of the samples on ice, fish were cut open, and their gills and viscera were removed and examined under a dissection microscope. Filleted fish meats were examined on the day of arrival or frozen until examination. Thin slices of muscle fillets were pressed between two glass plates and examined under a dissection microscope to detect the presence of myxosporean cysts or pseudocysts.

When myxosporean plasmodia were detected, muscle slices were placed in physiological saline, and parasitized myofibers were carefully isolated with fine forceps. The release of myxospores from a pseudocyst in the myofiber was executed with fine forceps. Myxospores were observed using a microscope equipped with differential interference contrast imaging, photographed at a magnification of $\times 800$, then transformed into photographs with Adobe® Photoshop® ver. 11.0

(Adobe Systems, San Jose, CA, USA). Photographs were then printed at a high magnification. Measurements were conducted on multiple printed photographs following the guidelines of Lom and Arthur (1989). All measurements are expressed in μm unless otherwise stated. Ranges with the means in parentheses are presented. Following removal of a portion of the myxospores for DNA extraction, the parasite was fixed in 10% neutral-buffered formalin solution and 70% ethanol solution. Specimens collected in the present work were deposited in the Meguro Parasitological Museum, Tokyo, Japan, under collection nos. 21377 and 21378.

For scanning electron microscopy, a portion of the formalin-fixed myxospores was washed three times in 0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ solution (PB), pH 7.8, and immersed in 2.5% glutaraldehyde in PB overnight. Following three washes in PB, the sample was post-fixed in 1% (w/v) osmium tetroxide in PB for 1 h. After washing three times in PB, the sample was dehydrated through a graded ethanol series, immersed in warmed *t*-butyl-alcohol, and cooled at 4 °C for 2 h. All processing was conducted using SEMpore® with 0.6- μm pore size (JEOL, Akishima, Tokyo, Japan) to reduce myxospore loss. The polycarbonate membrane of SEMpore® was then freeze-dried (model JFD-300; JEOL), mounted on stubs, and sputter-coated with gold-palladium at 200 Å (model JFC-1500; JEOL). Samples were examined using a scanning electron microscope (model JSM-6100; JEOL) at an accelerating voltage of 15 kV.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Parasite DNA was extracted from kudoid plasmodia using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the instructions of the manufacturer. PCR amplification of overlapping fragments of the rDNA was performed in a 20- μl volume containing a DNA polymerase, Blend Taq-Plus- (TOYOBO, Dojima Hama, Osaka, Japan), and primers as described previously (Li et al. 2013; Kasai et al. 2015). The PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly. When direct sequencing was not satisfactory, the purified PCR products were cloned into the plasmid vector pTA2 (Target Clone™; TOYOBO) and transformed into *Escherichia coli* JM109 (TOYOBO) according to the instructions of the manufacturer. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.), and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

Further, molecular genetic characterization of kudoid isolates was conducted on the mitochondrial DNA, i.e., cytochrome *c* oxidase subunit 1 gene (*cox-1*) and the small and

large subunits of the ribosomal RNA gene (*rns-rnl*). To this end, five plasmodia of two isolates (ABU and KGS) of the kudoid from two Japanese Spanish mackerel individuals and 22 plasmodia of ten yellowfin tunas (*Thunnus albacares*), six longtail tunas (*Thunnus tonggol*), and two Pacific bluefin tunas (*Thunnus orientalis*) were individually analyzed (Table 1). Detailed information on the tuna samples has been documented in a previous report (Kasai et al. 2017b). Amplification of two of the mitochondrial genes was attempted by using the primer pairs described by Takeuchi et al. (2016) for *Kudoa septempunctata* (*cox1*-F1 and *cox1*-R1 for the first round of PCR, followed by *cox1*-F2 and *cox1*-R3 for the second round of PCR; and similarly *rnl*-F1 and *rnl*-R1, followed by *rnl*-F2 and *rnl*-R2); however, this proved unsuccessful. Consequently, new primer pairs were designed using the online software Primer3web ver. 4.0.0 (Untergasser et al. 2012) and referring to a complete mitochondrial genome of *K. hexapunctata* (DDBJ/EMBL/GenBank accession no. LC009437; Takeuchi et al. 2015). The PCR cycling protocol was 3 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 60 s at 72 °C, followed by a final extension at 72 °C for 7 min. Primer pairs of *Kudoa_Cox1*-F3 and *Kudoa_Cox1*-R3, *Kudoa_RnL*-F3 and *Kudoa_RnL*-R3, and *Kudoa_RnL*-F4 and *Kudoa_RnL*-R4 (Table 2) were used to amplify long mitochondrial DNA sequences (the first pair for *cox1* and the second and third pairs for *rns-rnl*). When necessary, a second round of PCR using the primer pair *Kudoa_Cox1*-F4 and *Kudoa_Cox1*-R4 (Table 2) was conducted after the first round of PCR. Two primers, *Kudoa_RnL*-F5 and *Kudoa_RnL*-R5, were used only for sequencing, whereas the other primers mentioned above were used for both PCR amplification and amplicon sequencing.

The nucleotide sequences obtained in the present study are available from the DDBJ/EMBL/GenBank databases under the accession nos. LC316965 and LC316966 (rDNA), LC316989–LC316996 (*cox1*), and LC316997–LC317003 (*rns-rnl*).

Phylogenetic analysis

For phylogenetic analysis, the newly obtained rDNA nucleotide sequences of the Japanese Spanish mackerel *Kudoa* sp. in the present study and related *Kudoa* sequences retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson et al. 1994), with subsequent manual adjustment. The accession numbers of the sequences analyzed in the present study are given in the figures showing phylogenetic trees. Regions judged to be poorly aligned and characters with a gap in any sequence were excluded from subsequent analyses; 1371 characters, of which 368 were variable, remained for subsequent analysis for the 18S rDNA of the majority of kudoids forming either cysts or pseudocysts (70 taxa), 1401 characters,

of which 169 were variable, for the 18S rDNA of mainly pseudocyst-forming kudoids (42 taxa), and 564 characters, of which 243 were variable, for the 28S rDNA of mainly pseudocyst-forming kudoids (46 taxa). Maximum likelihood (ML) analysis was performed with the program PhyML as described previously (Matsukane et al. 2010; Li et al. 2013). Three *Unicapsula* spp. (Myxosporea: Multivalvulida; DDBJ/EMBL/GenBank accession nos. AB971675, AB971677, and AB971679) recorded in Japan, Vietnam, and Australia (Miller and Adlard 2013; Tomochi et al. 2014) were used as an outgroup for the construction of the ML phylogenetic tree based on the 18S rDNA of the majority of kudoids. To increase the resolution of the ML phylogenetic trees, kudoid species forming cysts for plasmodia, which usually position near the root of, but form separate clades from, the vast majority of *Kudoa* spp. in phylogenetic trees based on the rDNA, were omitted from the analyses, and selected species forming pseudocysts were phylogenetically analyzed.

Similarly, partial mitochondrial DNA sequences of *cox1* and *rnl* were aligned; 435 characters, of which 106 were variable, and 709 characters, of which 137 were variable, remained for subsequent analyses for *cox1* and *rnl*, respectively. ML phylogenetic trees were constructed with multiple *K. septempunctata* sequences (three *cox1* haplotypes and two *rnl* haplotypes; Takeuchi et al. 2016) as an outgroup for each gene.

Results

Incidence of kudoid infection in the Japanese Spanish mackerel

Two Japanese Spanish mackerels, one from the Sea of Japan off Abu-cho, Yamaguchi Prefecture, and the other from the northwestern Pacific Ocean off Kagoshima Prefecture, were infected with a myxosporean species forming pseudocysts in the myofiber of trunk muscles. Stellate myxospores isolated from the plasmodia had six equal SV/PC which were radially arranged in apical view and drawstring-pouch-shaped in lateral view with teardrop-shaped PC (Figs. 1e–h, and 2). Since the isolated myxospores are unique in morphology and distinct from any of the known *Kudoa* spp. as shown later in the present study, a new species, *Kudoa konishiae* n. sp., is erected for this parasite as follows.

Description

Kudoa konishiae n. sp. (Myxosporea: Multivalvulida)

(Figs. 1 and 2, and Table 3)

Small, club-shaped plasmodia with blunt ends or spindle-shaped plasmodia, 0.15–0.42 (0.28) mm by 0.07–0.12 (0.09)

Table 1 Samples used for the analyses of mitochondrial genes of *Kudoa* spp

Species	Host fish	Locality ^a	Isolate	DDBJ/EMBL/GenBank accession no. ^b		Reference ^c	
				mtDNA (<i>cox-1</i>)	mtDNA (<i>rnl</i>)		rDNA
<i>Kudoa konishiae</i> n. sp.							
<i>Scomberomorus niphonius</i>		Sea of Japan off Yamaguchi, Japan	ABU1, ABU2, ABU3	LC316996	LC317003	LC316965/LC316966	Present study
<i>Scomberomorus niphonius</i>		NWPO off Kagoshima, Japan	KGS1, KGS2	LC316996	LC317003	LC316965/LC316966	Present study
<i>Kudoa hexapunctata</i>							
<i>Thunnus orientalis</i>		Japan	2012.6.3	LC009437	LC009437	–	Takeuchi et al. 2015
<i>Thunnus orientalis</i>		Japan	Meji 111209	LC316989	LC316998	LC200462; LC200463	Kasai et al. 2017b
<i>Thunnus orientalis</i>		Japan	Meji1 20607	LC316989	LC316997	LC200464; LC200465	Kasai et al. 2017b
<i>Thunnus albacares</i>		WPO (unknown)	TZK120119	LC316990	LC316999	LC200466/LC200467	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaD14	LC316991	LC316999	LC200469/LC200470	Kasai et al. 2017b
<i>Thunnus tonggol</i>		East China Sea off Nagasaki, Japan	KNG-4	LC316992	LC317000	LC200477	Kasai et al. 2017b
<i>Kudoa neoithunni</i>							
<i>Thunnus albacares</i>		CWPO off Philippines	NSMT:Pr321	LC316993	LC317001	AB693049	Li et al. 2013
<i>Thunnus albacares</i>		CWPO off Philippines	LeaD4	LC316993	LC317001	LC200478	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaD8	LC316994	LC317001	LC200479/LC200480	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaD10	LC316994	LC317001	LC200481	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaD12	LC316994	LC317001	LC200482	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaT2	LC316993	LC317001	LC200484	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaT19	LC316993	LC317001	LC200485/LC200486	Kasai et al. 2017b
<i>Thunnus albacares</i>		CWPO off Philippines	LeaT20	LC316993	LC317001	LC200487	Kasai et al. 2017b
<i>Thunnus tonggol</i>		East China Sea off Nagasaki, Japan	KNG-1; KNG-2; KNG-3; KNG-5; KNG-6	LC316995	LC317002	LC200497-LC200501	Kasai et al. 2017b
<i>Kudoa septempunctata</i>							
<i>Paralichthys olivaceus</i>		Japan	0904	AB731753	AB731753	–	Takeuchi et al. 2015
<i>Paralichthys olivaceus</i>		Japan	201,204	LC009436	LC009436	–	Takeuchi et al. 2015
<i>Paralichthys olivaceus</i>		Japan	0904	AB915830	AB915832	–	Takeuchi et al. 2016
<i>Paralichthys olivaceus</i>		Japan	20,120,426	AB915831	AB915833	–	Takeuchi et al. 2016
<i>Paralichthys olivaceus</i>		Korea	Ken_eki_66200104-3	LC014799	AB915832	–	Takeuchi et al. 2016

^a Abbreviation of locality: CWPO, Central Western Pacific Ocean; NWPO, Northwestern Pacific Ocean; and WPO, Western Pacific Ocean

^b Bars indicate no available data. Except for nucleotide sequences of *K. septempunctata* and *K. neoithunni*, all other nucleotide sequences were newly obtained in the present study

^c Original records for individual kudooids are shown as reference

Table 2 Primers used to amplify and sequence nucleotides of the *cox-1* and *rns-rnl* genes of *Kudoa* spp

Primer name	Direction	Nucleotide sequence	Position of 5'-terminus ^a
<i>cox-1</i>			
Kudoa_Cox1-F3	Forward	5'-TTGCTGCTGKYTGTTTCATYGGCACAATTC-3'	551
Kudoa_Cox1-R3	Reverse	5'-GCCTAAATAGCCTGGAAYRAGGAAWC-3'	1317
Kudoa_Cox1-F4	Forward	5'-TATGGYAARGAAGGWCTGATTTC-3'	655
Kudoa_Cox1-R4	Reverse	5'-TCTAGGGATTCCWMRRAGACCTGCT-3'	1260
<i>rns-rnl</i>			
Kudoa_RnL-F3	Forward	5'-CTGATGTGGTGRGGTTGTGAGGAA-3'	5
Kudoa_RnL-R3	Reverse	5'-CCATRTTTCACGAGGCCCTT-3'	1418
Kudoa_RnL-F4	Forward	5'-TGCATGKCCCAATTCTCCTCAAGTT-3'	346
Kudoa_RnL-R4	Reverse	5'-CTTGCCCTATTGCCCTTARTTCCTT-3'	1620
Kudoa_RnL-F5	Forward	5'-AGGAAAAGGTTGGGTGGAGT-3'	1108
Kudoa_RnL-R5	Reverse	5'-ACTGTTCACCTTGGTCCCAT-3'	495

^a Relative position of the 5'-terminus of each primer to the 5'-terminus of *cox-1* (9259th–10623rd nucleotides) and *rns-rnl* (11389th–13264th nucleotides) sequences of *Kudoa hexapunctata* (DDBJ/EMBL/GenBank accession no. LC009437)

mm ($n = 8$) or 0.22–0.53 (0.34) mm by 0.04–0.13 (0.08) mm ($n = 8$) in the two host fish, forming pseudocysts in the myofiber of trunk muscles, polysporic and synchronized spore development. Myxospores stellate with six equal SV/PC in apical view, without SV ornamentation and apical projection, demonstrated by scanning electron microscopy round peripheral expansion of SV with distinct suture lines between them in apical view. In lateral view, myxospores drawstring-pouch in shape, PC teardrop-like, were occupying apical half of myxospores. Coils of polar filament were not seen in wet preparations. The spores were having dimensions of ($n = 20$): width 8.1–9.7 (9.0), thickness 7.2–8.8 (8.2), sutural thickness 6.7–7.5 (7.1), length 7.1–7.9 (7.5), PC length 2.7–4.2 (3.6), and PC width 1.2–1.8 (1.5).

Nucleotide sequences of the 18S and 28S rDNA, 1561, and 743 bp in length, respectively, were obtained for five plasmodia from two fish samples fished in the Sea of Japan (three plasmodia of ABU isolate) and northwestern Pacific Ocean (two plasmodia of KGS isolate). The nucleotide sequences of each region were identical for all plasmodia (DDBJ/EMBL/GenBank accession nos. LC316965 and LC316966). Partial nucleotide sequences of the mitochondrial genes *cox-1* (437 bp) and *rns-rnl* (1367 bp) were similarly obtained for each isolate and were absolutely identical to each other (DDBJ/EMBL/GenBank accession nos. LC316996 and LC317003).

Taxonomic summary

Host *Scomberomorus niphonius* (Cuvier, 1832), Japanese Spanish mackerel (Actinopterygii: Perciformes: Scombridae).

Locality Sea of Japan, off Abu-cho, Yamaguchi Prefecture, Japan. In addition, northwestern Pacific Ocean off Kagoshima Prefecture, Japan.

Site of infection Pseudocysts in the myofiber of somatic muscles.

Materials deposited Hapantotype no. 21377, Meguro Parasitological Museum, Tokyo, Japan (ABU isolate). Paratype no. 21378 in the same museum (KGS isolate).

Prevalence One of two individuals fished in the Sea of Japan, or one of six individuals in the northwestern Pacific Ocean in the autumn of 2016. In each fish individual, 13–28 (15.8) pseudocysts/g muscle or 4–7 (4.7) pseudocysts/g muscle.

Etymology The species is named in honor of Prof. Yoshiko Sugita-Konishi, Azabu University, Kanagawa, Japan, who is a former director of the Division of Microbiology, National Institute of Health Sciences, Japan, and played a crucial role in identifying the cause of food poisoning due to unknown cause(s) after consumption of fresh fish fillets, currently known as “*Kudoa* food poisoning.”

Remarks

Four *Kudoa* spp. have previously been described from *Scomberomorus* spp., i.e., *Kudoa crumena* in Atlantic Spanish mackerel (*S. maculatus*) (Iversen and Van Meter 1967), *K. permulticapsula* and *K. scomberomori* in narrow-barred Spanish mackerel (*S. commerson*) (Whipps et al. 2003a; Adlard et al. 2005), and *Kudoa* sp. in Serra Spanish

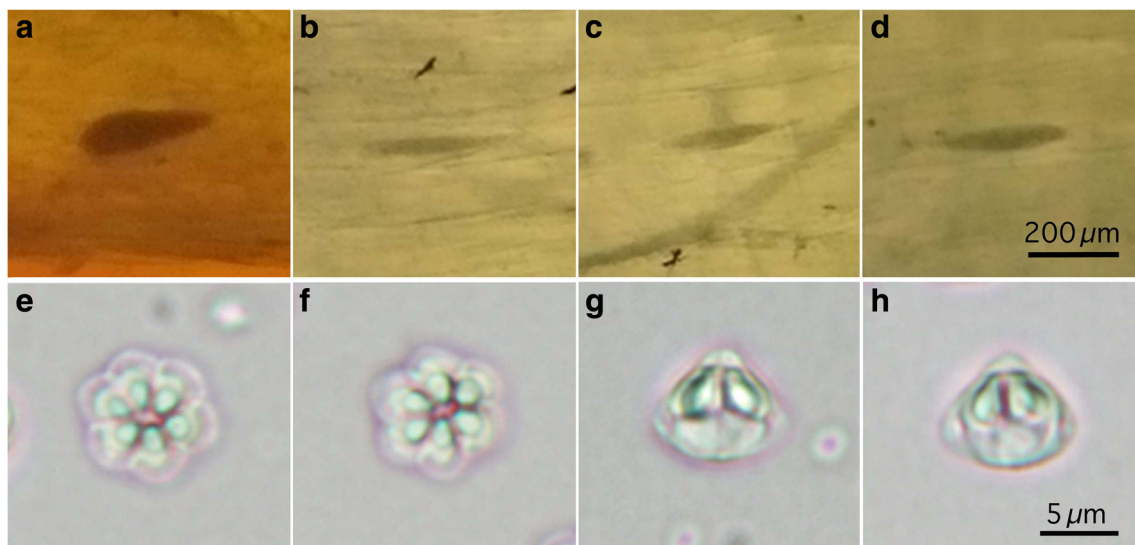


Fig. 1 Photographs of plasmidia (**a–d**) and fresh myxospores (**e–h**) of *Kudoa konishiae* n. sp. from *Scomberomorus nipponius*. Myxospores are shown in apical (**e, f**) and lateral (**g, h**) view. All photographs of each set

(**a–d** or **e–h**) are at the same magnification, with the scale shown on the rightmost photograph of each line (**d, h**)

mackerel (*S. brasiliensis*) (Eiras et al. 2014a). Except for *K. scomberomori* which has stellate myxospores with six equal SV/PC, the three other species are distinct from the present new species in myxospore morphology, having four or 13 SV/PC (Iversen and Van Meter 1967; Whipps et al. 2003a; Eiras et al. 2014a). Although myxospores of *K. scomberomori*, *K. neothunni*, *K. hexapunctata*, and *K. grammatorcyni* are stellate with six SV/PC, those of *K. konishiae* n. sp. are morphologically different in terms of spore dimensions (see Table 3) and the degree of peripheral SV expansion (rounded edge vs. pointed edge). Furthermore, scanning electron microscopy revealed a greater relative spore height (spore length against spore thickness or width) compared with the other kudoids displaying stellate myxospores with six SV/PC (Fig. 2). The morphological uniqueness of the present new species from known kudoids is supported by

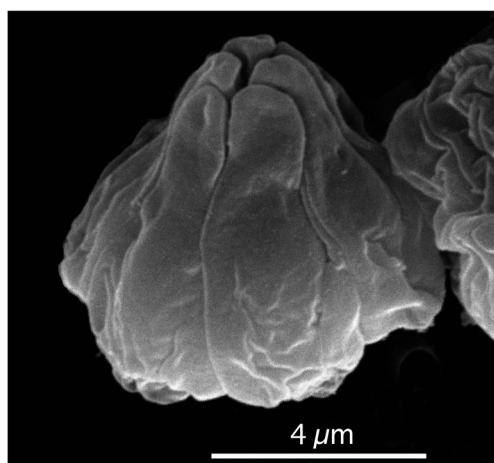


Fig. 2 Lateral view of a *Kudoa konishiae* n. sp. myxospore by scanning electron microscopy

molecular genetic analyses of the 18S and 28S rDNA and mitochondrial DNA genes (*cox-1* and *rnl*) as discussed below.

Phylogenetic analyses of the rDNA

Nucleotide sequences of the 18S and 28S rDNA, 1561 and 743 bp long, respectively, were obtained for *K. konishiae* n. sp. The closest 18S rDNA nucleotide sequence was *K. permulticapsula* (AY078429) with 99.23% (1548/1560) identity and one nucleotide insertion/deletion (indel), followed by *K. neothunni*, *K. hexapunctata*, *K. grammatorcyni*, and *K. scomberomori* with 99.04% (1546/1561) or 98.98% (1545/1561) identity. Other *Kudoa* spp. had 98.78% (1541/1560) or lower identities with frequent nucleotide indels. The closest 28S rDNA nucleotide sequence was *K. neothunni* with 95.81% (709/740) identity and three indels, followed by *K. hexapunctata* with 94.73% (701/740) identity and three indels. The 28S rDNA nucleotide sequences of other *Kudoa* spp. showed frequent indels and lower identities with that of *K. konishiae* n. sp. Regarding molecular rDNA homology with *Kudoa* spp. with deposited nucleotide sequences, *K. konishiae* n. sp. was positioned at the root or near the root of clades of *Kudoa* spp. with stellate myxospores with five to seven (mainly six) SV/PC from scombrid fish (confined to the genera *Grammatorcynus*, *Scomberomorus*, and *Thunnus* at present) in the phylogenetic trees based on the 18S and 28S rDNA nucleotide sequences (Figs. 3 and 4).

Phylogenetic analyses of the mitochondrial DNA genes (*cox-1* and *rnl*)

Partial *cox-1* nucleotide sequences of two isolates of *K. konishiae* n. sp. were successfully obtained by nested

Table 3 Morphological characteristics of *Kudoa* spp. with six or seven shell valves/polar capsules (SV/PC) from different origins

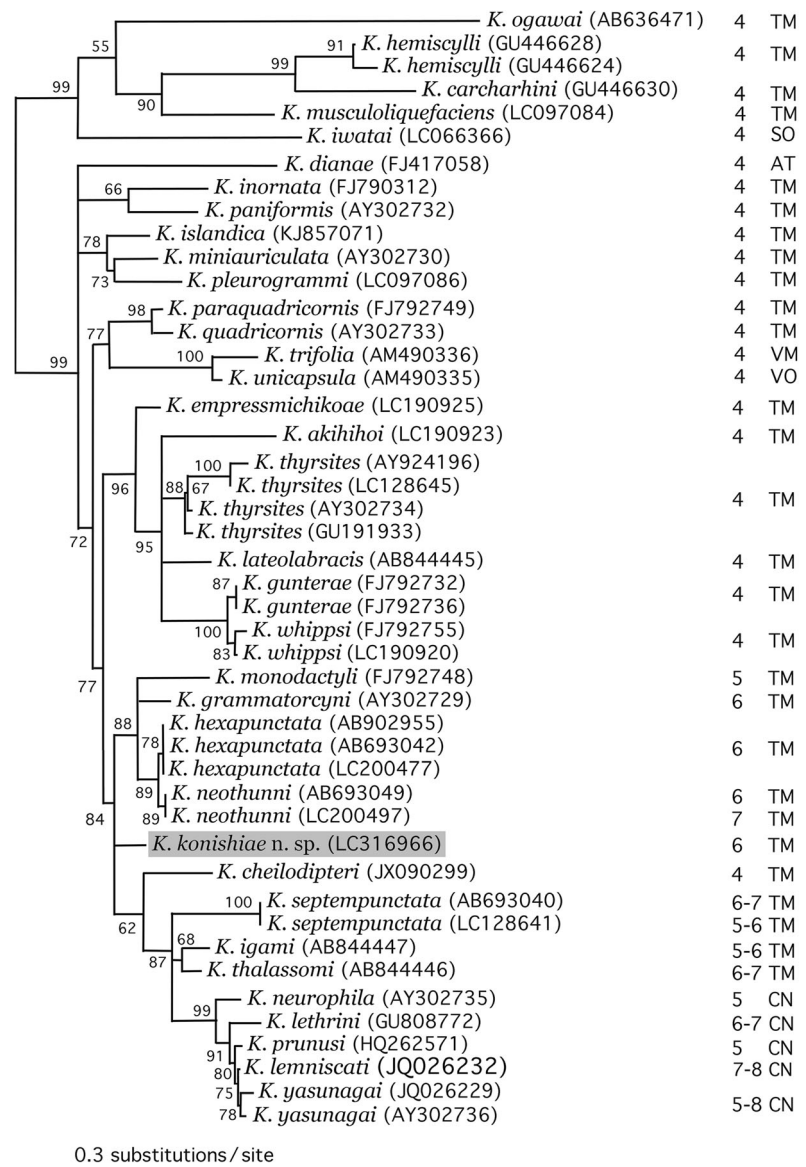
Species	<i>Kudoa konishiae</i> n. sp.	<i>Kudoa konishiae</i> n. sp.	<i>Kudoa scomberomori</i>	<i>Kudoa grammatorcyni</i>	<i>K. hexapunctata</i>	<i>K. hexapunctata</i>
Geographical origin	Sea of Japan off Yamaguchi, Japan	Northwestern Pacific Ocean off Kagoshima, Japan	Great Barrier Reef, Queensland, Australia	Great Barrier Reef, Queensland, Australia	Western Pacific Ocean off Kochi, Japan	Western Pacific Ocean off Japan
Number of SV/PC per spore ^a	6	6	6	6	6	6
Host	<i>Scomberomorus niphonius</i>	<i>Scomberomorus niphonius</i>	<i>Scomberomorus commerson</i>	<i>Grammatocyclus bicarinatus</i>	<i>Thunnus orientalis</i>	<i>Thunnus orientalis</i>
Reference	Present study	Present study	Adlard et al. 2005	Adlard et al. 2005	Yokoyama et al. 2014	Li et al. 2013
Number of examined spores	20	20	30	30	20	15
Spore width	8.1–9.7 (9.0)	8.1–9.6 (8.9)	6.8–8.2 (7.6)	8.0–9.0 (8.6)	8.9–11.4 (10.3)	9.5–11.4 (10.6)
Spore thickness	7.2–8.8 (8.2)	7.1–8.8 (8.1)	6.2–7.6 (6.8)	7.6–8.7 (8.1)	7.9–10.2 (9.0)	8.9–10.9 (9.7)
Sutural spore thickness	6.7–7.5 (7.1)	6.0–7.5 (6.7)	5.3–6.3 (5.9)	7.2–8.2 (7.7)	6.9–8.8 (7.7)	7.3–8.6 (8.1)
Spore length	7.1–7.9 (7.5)	7.1–8.8 (8.1)	5.0–6.2 (5.4)	6.3–6.7 (6.5)	6.7–7.9 (7.3)	7.3–7.7 (7.6)
Polar capsule length	2.7–4.2 (3.6)	3.2–4.7 (3.8)	3.0–3.6 (3.2)	3.6–3.8 (3.7)	2.7–4.0 (3.3)	3.6–4.1 (3.8)
Polar capsule width	1.2–1.8 (1.5)	1.3–2.5 (1.7)	1.3–1.5 (1.4)	1.7–1.8 (1.7)	1.5–2.3 (1.8)	1.8–2.3 (2.0)
Variation in size of polar capsules	Not evident	Not evident	Not evident	Not evident	Not evident	Not evident
Species	<i>K. hexapunctata</i>	<i>K. neothunni</i>	<i>K. neothunni</i>	<i>K. neothunni</i>	<i>K. septempunctata</i>	<i>K. septempunctata</i>
Geographical origin	East China Sea off Nagasaki, Japan	Western Pacific Ocean off Philippines	Western Pacific Ocean off Vanuatu	East China Sea off Nagasaki, Japan	Aquacultural facility in Korea	Sea of Japan off Tottori, Japan
Number of SV/PC per spore ^a	5–6 (4.96)	6	6	6–7 (9.91)	6–7 (26.74)	5–6 (28.72)
Host	<i>Thunnus tonggol</i>	<i>Thunnus albacares</i>	<i>Thunnus albacares</i>	<i>Thunnus tonggol</i>	<i>Paralichthys olivaceus</i>	<i>Thunnus modestus</i>
Reference	Kasai et al. 2017	Arai and Matsumoto 1953	Yokoyama et al. 2014	Kasai et al. 2017	Matsukane et al. 2010	Kasai et al. 2016b
Number of examined spores	20	20	20	20	10	20
Spore width	10.4–12.4 (11.0)	9.1–13.0 (11.0)	10.7–12.9 (11.7)	11.0–13.0 (12.1)	11.1–13.1 (11.8)	11.4–14.0 (12.5)
Spore thickness	8.8–10.9 (9.9)	— ^b	8.6–10.5 (9.7)	9.2–12.0 (10.7)	8.9–10.0 (9.4)	9.2–11.6 (10.5)
Sutural spore width	7.7–8.9 (8.4)	5.9–8.7 (7.1)	6.8–7.9 (7.4)	— ^b	— ^b	— ^b
Spore length	7.0–8.3 (7.6)	5.3–7.3 (6.2)	6.3–7.5 (7.0)	7.5–8.9 (8.1)	7.9–8.9 (8.5)	6.8–8.3 (7.5)
Polar capsule length	3.5–4.4 (4.0)	2.0–3.1 (2.5)	2.4–4.5 (3.5)	3.9–5.7 (4.8)	3.7–5.3 (4.6)	3.7–5.3 (4.6)
Polar capsule width	1.5–2.1 (1.8)	1.3–2.1 (1.6)	1.8–2.2 (2.0)	1.6–2.4 (2.0)	2.2–2.8 (2.4)	1.7–2.4 (1.9)
Variation in size of polar capsules	Not evident	Not evident	Not evident	Not evident	Evident	Evident

All measurements are in μm and expressed as range with mean in parentheses

^a Range of numbers of SV/PC per spore, followed by frequency of spores with each SV/PC number in parentheses

^b No available data

Fig. 4 ML phylogenetic tree based on the 28S rDNA sequence of representative *Kudoa* spp. forming pseudocysts. On the right side of the tree, the number of spore valves and polar capsules per myxospore, and parasite localization are shown as in Fig. 3. Additional abbreviations of tissue preference are VO, visceral organs; and SO, systemic organs. Similarly as in Fig. 3, gray bars on the rightmost edge indicate two different clades of *Kudoa* spp. with five or more shell valves/polar capsules

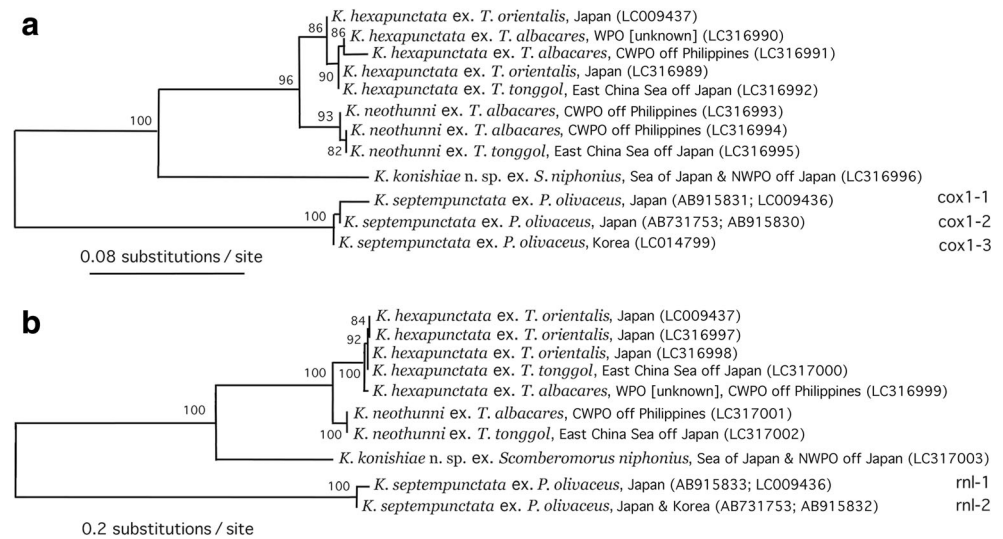


nucleotide sequences of *K. hexapunctata* (six isolates) were newly obtained. No intraspecific variation was found throughout the length of the 1368-bp *rns-rnl* nucleotide sequences of *K. neothunni*, whereas intraspecific variation of the same mitochondrial DNA region of *K. hexapunctata* was demonstrated at nine nucleotide sites; however, the nucleotide sequence identity between different isolates was never lower than 99.49%. Interspecific variation between *K. neothunni* and *K. hexapunctata* was found at 52 nucleotide sites (including two indels), resulting in approximately 95% identities. Both *K. neothunni* and *K. hexapunctata* showed approximately 80% nucleotide sequence identities with *K. konishiae* n. sp. The phylogenetic relationships of these three species along with *K. septempunctata* based on the partial *rnl* nucleotide sequences are shown in Fig. 5b.

Discussion

When Whipps et al. (2004) redefined the genus *Kudoa* Meglitsch, 1947, as myxosporeans having four or more SV/PC, there were only six *Kudoa* spp. with more than four SV/PC: *K. neurophila*, *K. cutanea*, *K. muscularis*, *K. shulmani*, *K. neothunni*, and *K. yasunagai*. Currently, such *Kudoa* spp. have increased in number to 18 (Sato and Kasai 2016), and many but not all of them have been genetically characterized based on the rDNA nucleotide sequences (Grossel et al. 2003, 2005; Whipps et al. 2003a, 2004; Adlard et al. 2005; Gunter et al. 2006; Burger et al. 2007; Burger and Adlard 2010b, 2011; Matsukane et al. 2010; Meng et al. 2011; Miller and Adlard 2012; Li et al. 2013; Yokoyama et al. 2014; Shirakashi et al. 2014; Kasai et al. 2017b). In Figs. 3 and 4 showing the

Fig. 5 ML phylogenetic trees based on the partial mitochondrial gene sequences (**a**, *cox-1* and **b**, *rnl*) of representative *Kudoa* spp. with six or more spore valves/polar capsules per myxospore. Parasite names are followed by host fish species, isolation localities, and DDBJ/EMBL/GenBank accession numbers. To the far right, the haplotype names of mitochondrial genes of *K. septempunctata*, defined by Takeuchi et al. (2016), are shown for reference



phylogenetic trees based on either the 18S or 28S rDNA nucleotide sequence, *Kudoa* spp. with more than four SV/PC were exclusively confined to two clades: the first clade containing muscle-preferring *Kudoa* spp. with mainly six SV/PC (exceptionally five or 13 SV/PC), such as *K. permulticapsula*, *K. monodactyli*, *K. grammatorcyni*, *K. scomberomori*, *K. neothunni*, *K. hexapunctata*, and the present new species *K. konishiae* n. sp.; the other clade containing *Kudoa* spp. with fluctuating numbers of SV/PC (between five and nine) even within a species, localized in the central nervous system or trunk muscles, such as *K. neurophila*, *K. lethrini*, *K. yasanagai*, *K. chaetodoni*, *K. lemniscati*, *K. prumusi*, *K. septempunctata*, *K. thalassomi*, and *K. igami*. Host fish for the former clade belonged exclusively to Scombrinae (Perciformes: Scombridae), whereas those for the latter clade were varied.

Recently, mitochondrial DNA genes (*cox-1* and *rnl*) of myxosporeans were analyzed for the first time to biogeographically characterize *K. septempunctata* isolated from olive flounders (*Paralichthys olivaceus*), farmed in Japan and Korea, or fished from the natural waters around Japan (Takeuchi et al. 2016). Takeuchi et al. (2016) differentiated three genotypes based on single nucleotide polymorphisms at seven *cox-1* and two *rnl* nucleotide sites, i.e., ST1 genotype (cox1-1 and rnl-1), ST2 genotype (cox1-2 and rnl-2), and ST3 genotype (cox1-3 and rnl-2), and suggested an almost exclusive prevalence of ST1 and ST2 genotypes in Japan and ST3 genotype in Korea. This molecular strategy to identify different parasite populations was applied to the ABU and KGS isolates of *K. konishiae* n. sp. from Japanese Spanish mackerels fished in the Sea of Japan and northwestern Pacific Ocean, respectively, since *S. niphonius* is thought to be divided into two ecological stocks, East China Sea (China) stock and Seto Inland Sea (Japan) stock (Shui et al. 2009), and it is possible that the two aforementioned isolates might originate from different spawning places of the host fish. The

analyses were conducted together with multiple isolates of *K. hexapunctata* and *K. neothunni* reported in our previous work (Kasai et al. 2017b), which show close morphological and phylogenetic resemblances to the new species.

As shown in Fig. 5, both the *cox-1* and *rnl* nucleotide sequences of mitochondrial DNA clearly separated the three different species with six SV/PC in stellate spores. Furthermore, unique *K. neothunni* spores with seven SV/PC recently isolated from the longtail tuna in the East China Sea (Kasai et al. 2017b) were demonstrated to be conspecific with the species with six SV/PC prevalent in the yellowfin tuna in the northwestern Pacific Ocean around the Philippines. This was ascertained from the finding of no or only a few nucleotide differences in the 18S, 5.8S, and 28S rDNA but substantial nucleotide differences in the internal transcribed regions (ITS1 and ITS2) of the rDNA (Kasai et al. 2017b). As far as examined here, no genetically distinct populations of any of these *Kudoa* species (*K. hexapunctata*, *K. neothunni*, or *K. konishiae* n. sp.) were noted by host fish or locality (sea area). Incorporation of the nucleotide sequencing of mitochondrial genes for closely related kudoid organisms could provide further insights into their phylogenetic or biogeographical relatedness after speciation and geographical dispersal of different species or intraspecific unique phenotype(s).

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