ORIGINAL PAPER

Characterization of the ribosomal RNA gene of *Kudoa neothunni* (Myxosporea: Multivalvulida) in tunas (*Thunnus* spp.) and *Kudoa scomberi* n. sp. in a chub mackerel (*Scomber japonicus*)

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Abstract *Kudoa neothunni* is the first described *Kudoa* species having six shell valves and polar capsules, previously assigned to the genus *Hexacapsula* Arai and Matsumoto, 1953. Since its genetic analyses remain to be conducted, the present study characterizes the ribosomal RNA gene (rDNA) using two isolates from a yellowfin tuna (*Thunnus albacares*) with post-harvest myoliquefaction and a northern bluefin tuna (*Thunnus thynnus*) without tissue degradation. Spores of the two isolates localized in the myofiber of trunk muscles, forming pseudocysts, and showed typical morphology of *K. neothunni* with six equal-sized shell valves radially arranged in apical view: spores ($n=15$) measuring 9.5–11.4 μm in width, 7.3–8.6 μm in suture width, 8.9–10.9 μm in thickness, and 7.3–7.7 μm in length; and polar capsules measuring 3.6–4.1 μm by 1.8–2.3 μm. In lateral view, the spores were pyramidal in shape without apical protrusions. Their 18S and 5.8S rDNA sequences were essentially identical, but variations in the ITS1 (62.4 % similarity across 757-bp length), ITS2 (66.9 % similarity across 599-bp length), and 28S (99.0 % similarity across 2,245-bp length) rDNA regions existed between the two isolates. On phylogenetic trees based on the 18S or 28S rDNA sequence, *K. neothunni* formed a clade with *Kudoa* spp. with more than four shell valves and polar capsules, particularly *K. grammatorcyni* and *K. scomberomori*. Semiquadrate spores of a kudoid species with four shell valves and polar capsules were detected from minute cysts (0.30–0.75 mm by 0.20–0.40 mm) embedded in the trunk muscle of a chub mackerel (*Scomber japonicus*) fished in the Sea of Japan. Morphologically, it resembled *K. caudata* described from a chub mackerel fished in the southeastern Pacific Ocean off Peru; however, it lacked filamentous projections on the shell valves of spores. Additionally, it morphologically resembled *K. thunni* described from a yellowfin tuna also fished in the Pacific Ocean; spores ($n=30$) measuring 8.2–10.5 μm in width, 7.0–8.8 μm in thickness, and 6.1–6.8 μm in length; and polar capsule measuring 2.5–3.4 μm by 1.3–2.0 μm. The similarities of the 18S and 28S rDNA sequences between these two species were 98.5 % and 96.3 %, respectively. Simultaneously, the dimensions of cysts in the trunk muscle formed by *K. thunni* are clearly larger than those of the present species from a chub mackerel: 1.3–2.0 mm by 1.1–1.4 mm ($n=14$) vs. 0.30–0.75 mm by 0.20–0.40 mm ($n=7$), respectively. Thus, *Kudoa scomberi* n. sp. is proposed for this multivalvulid species found in the chub mackerel.

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Introduction

Kudoa neothunni Arai and Matsumoto, 1953 (Myxosporea: Multivalvulida) was first described from a yellowfin tuna

(*Thunnus albacares*; syn. *Neothunnus macropterus*) with post-mortem myoliquefaction hauled from the Banda Sea and transported to the Tokyo Fish Market (Arai and Matsumoto 1953). The spores were packed in pseudocysts in the myofiber of trunk muscles, having six shell valves and polar capsules (SV/PC) arranged symmetrically. Due to this novel character and the archaic definition of the genus *Kudoa* at that time (Lom and Noble 1984), the new genus *Hexacapsula* was proposed based on this species (Arai and Matsumoto 1953). Similarly, the genera *Pentacapsula* (with five SV/PC) and *Septemcapsula* (with seven SV/PC) were added to the family Kudoidae in the order Multivalvulida (Naidenova and Zaika 1970; Hsieh and Chen 1984). When Whipps et al. (2003) described a new myxosporean species having 13 SV/PC, they placed it in the genus *Kudoa*, i.e., *K. permulticapsula*, because its 18S ribosomal RNA gene (rDNA) was closest to *K. thyrsites* and *K. quadricornis*, not forming a distinct clade from *Kudoa* spp. Subsequently, Whipps et al. (2004) demonstrated that the 18S rDNA of members classified in the genera *Pentacapsula*, *Hexacapsula* (with six SV/PC), and *Septemcapsula* rooted within a clade of *Kudoa* spp. with *Unicapsula* (Trilosporidae) as an outlier to these genera, and thus, proposed that all marine myxosporean parasites having four or more SV/PC should be classified in the genus *Kudoa*.

As myxosporean spores are small and often possess only a few morphological features useful for differentiation, reliable species identification requires not only thorough morphological observations but also molecular analyses of rDNA sequences and other biological characterizations such as tissue tropism, host specificity, pathogenicity, and geographical distribution. To differentiate *K. alliaria* from *K. rosenbuschi* exhibiting a similar spore morphology and almost identical 18S rDNA sequences (99.8 % similarity across 1,680-bp length), Whipps and Diggles (2006) described that unlike the latter species, the former species was not associated with tissue degradation. Furthermore, Burger et al. (2007) found identical 18S rDNA sequences in two *Kudoa* isolates with distinct morphologies and clearly disparate host origins; *K. yasunagai* with seven polar capsules from *Paralichthys olivaceus* in Japan (Yokoyama et al. 2004) and *Kudoa* sp. with five and six SV/PC from sand whitings (*Sillago ciliata*) in Australia. They suggested that the 18S rDNA sequence was incapable of distinguishing some combinations of *Kudoa* spp. Later, however, Burger and Adlard (2010b) concluded that the molecular results indicated conspecificity rather than 18S rDNA analysis reaching its level of resolution.

Of 80 or more nominal *Kudoa* spp., approximately half have been genetically characterized using mainly 18S rDNA sequences (Burger and Adlard 2011; Sato 2011). Although genetically characterized *Kudoa* spp. face new taxonomic challenges as mentioned above, the species differentiation of *Kudoa* spp. known only by morphology is far more difficult, particularly when morphologically similar spores are detected

from hosts other than the type host or fishes listed in the original description, or in geographically distant areas from the original record (Moran et al. 1999; Kent et al. 2001; Yokoyama 2003; Diamant et al. 2005; Whipps and Diggles 2006; Whipps and Kent 2006; Burger and Adlard 2010a, 2011).

In this study, we compared the rDNA sequences of two isolates of *K. neothunni* with/without post-harvest myoliquefaction in order to clarify the taxonomic relationship of these two isolates. Furthermore, a kudoid species having semiquadrate spores with four SV/PC from minute cysts in the trunk muscle of a chub mackerel (*S. japonicus*) fished in the Sea of Japan was examined morphologically and genetically, and compared with known species including *K. caudata* Kovaleva and Gaevskaya, 1983 described from the same host species but in the southeastern Pacific Ocean off Peru (Kovaleva and Gaevskaya 1983).

Materials and methods

Fish samples and parasitological examination

A frozen block of the trunk muscle of a northern bluefin tuna (*T. thynnus*) containing kudoid spores was provided by a Japanese-style restaurant located in Kanagawa Prefecture, Japan during the summer of 2010. The fish was fished from an unknown site in the Pacific Ocean. This fish did not show post-harvest myoliquefaction.

Thin slices of the frozen trunk muscle were placed in physiological saline and minced with fine forceps to release the parasite. The released spores were observed using a microscope equipped with differential interference contrast imaging, photographed at a magnification of ×400, and transformed into photographs with Adobe® Photoshop® ver. 11.0 (Adobe Systems, San Jose, California, 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. Following removal of a portion of the spores for DNA extraction, the parasite was fixed in 10 % neutral-buffered formalin solution and used for scanning electron microscopy as previously described (Matsukane et al. 2010). Briefly, the preserved sample was washed three times in 0.2 M Na_2HPO_4-NaH_2PO_4-buffered solution, pH 7.8 (PB) and immersed in 2.5 % glutaraldehyde in PB overnight. After washing three times in PB, the specimen was post-fixed in 1 % (*w/v*) osmium tetroxide in PB for 1 h. Following three washes in PB, the sample was placed in a 1.5-ml tube then dehydrated through a graded ethanol series, immersed in warmed *t*-butyl-alcohol, and cooled in a refrigerator at 4 °C for 2 h. The sample was then freeze-dried in a freeze-drying device (model JFD-300; JEOL, Akishima, Tokyo, Japan), mounted on stubs, and

sputter-coated with gold-palladium at 200 Å in an ion sputtering device (model JFC-1500; JEOL). Specimens were examined using a scanning electron microscope (model JSM-6100; JEOL) at an accelerating voltage of 15 kV.

Another *K. neothunni* isolate was obtained from a yellowfin tuna (*T. albacares*), 36 kg in body weight, which was fished in the southwestern Pacific Ocean near Mindanao Island, Philippines (approximate sea position at 7–8N, 131E) during 15–20 October 2011 and landed at Naha, Okinawa Prefecture, Japan on 5 November 2011. This fish showed post-harvest myoliquefaction. The kudoid isolate from this fish was examined under a light microscopy as above.

A fresh fillet of a chub mackerel (*S. japonicus*) with several macroscopic but minute white spots was obtained from a local fish market near Yamaguchi University on 11 February 2011. This fish was fished in the Sea of Japan off Nagasaki Prefecture, Japan, and was examined parasitologically as described above.

Specimens collected in the present work were deposited in the National Museum of Nature and Science, Tokyo, Japan under specimen numbers NSMT-Pr320–Pr330.

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

Parasite DNA was extracted from kudoid spore-rich samples 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 (listed in Table 1). The PCR cycling protocol was 3 min at 94 °C, then 40 cycles of 45 sec at 94 °C, 1 min at 64 °C or 62 °C (see Table 1), and 1 min at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR products were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany), and sequenced directly using the primers listed in Table 1. When direct sequencing was not satisfactory, the purified PCR products were cloned into a 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 NucleoSpin® Plasmid kit (MACHEREY-NAGEL GmbH, Düren, Germany) and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

Phylogenetic analysis

For phylogenetic analysis, the newly obtained 18S and 28S rDNA sequences of *Kudoa* spp. in the present study (DDBJ/EMBL/GenBank accession nos. AB693040–

AB693049) and *Kudoa*-related 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 sequences were excluded from subsequent analyses (Smythe et al. 2006); 1,341 characters, of which 341 were variable, remained for subsequent analysis for 18S rDNA, and 506 characters, of which 286 were variable, remained for subsequent analysis for 28S rDNA. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the "phylogeny.fr" website (http://www.phylogeny.fr/). The probability of inferred branch was assessed by the approximate likelihood-ratio test (aLRT), an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006). *Unicapsula* sp. (Trilosporidae, Multivalvulida; DDBJ/EMBL/GenBank accession nos. AY302725 and AY302727) was used as an outgroup for the construction of ML phylogenetic trees.

Results

Morphological observations

The trunk muscle of the northern bluefin tuna showed a normal gross appearance, whereas that of the yellowfin tuna exhibited striking post-harvest myoliquefaction. One gram of muscle removed from the northern bluefin tuna contained 7×10^7 spores (Fig. 1), with six equal-sized shell valves radially arranged in apical view (Fig. 2). In lateral view, the spores were pyramidal in shape without apical protrusions. This finding was confirmed by scanning electron microscopy (Fig. 3). Spores collected from jellied meat and intact meat of the yellowfin tuna showed the same morphology. All the isolates exhibited a morphology typical of *K. neothunni*: 9.5–11.4 (mean [*n*=15], 10.6) μm in width; 7.3–8.6 (8.1) μm in suture width; 8.9–10.9 (9.7) μm in thickness; and 7.3–7.7 (7.6) μm in length. The polar capsules were equal in dimensions: 3.6–4.1 (3.8) μm in length and 1.8–2.3 (2.0) μm in width (Table 2). Coils in the polar capsules were not visible under a light microscope.

Although our specimens of *K. neothunni* had larger polar capsules in comparison to the original description by Arai and Matsumoto (1953), this species was differentiated from *K. grammatorcyni* by having larger spores, a more pointed shell valve edge in apical view, and a different lateral view (pyramidal versus ovoid; Table 2). *K. neothunni* and *K. scomberomori* had an obvious resemblance in spore morphology, but the former species had larger spores and polar capsules as well as a different lateral view (Table 2). *K.*

Table 1 Primers used to amplify and sequence seven overlapping segments of 18S and 28S rDNA of *Kudoa* spp.

Segment no.	Primer name[a]		Sequence	Annealing temperature	Position of 5′-end[b]
	for amplifying	for sequencing			
1	F: NSF4/18		5′-CTGGTTGATCCTGCCAGT-3′	64 °C	4
	R: KUDOA-SSU926R		5′-AAACACTCTTGGCGAATGCT-3′		933
		R: NSR581/18	5′-TCTCAGGCTCCCTCTCCGG-3′		382
2	F: NSF573/19		5′-CGCGGTAATTCCAGCTCCA-3′	64 °C	555
	R: S.r.18S-SSU18R		5′-TGATCCTTCYGCAGGTTCAC-3′		1,740
		F: NSF1419/20	5′-ATAACAGGTCTGTGATGCCC-3′		1,380
3	F: NSF1419/20		5′-ATAACAGGTCTGTGATGCCC-3′	62 °C	1,380
	R: Kudoa-28S-113R		5′-TCCGGGAAGATTAAACGATG-3′		3,263
		F: NC13(ITS2)/F	5′-ATCGATGAAGAACGCAGC-3′		2,482
		R: NC13(ITS1)/R	5′-GCTGCGTTCTTCATCGAT-3′		2,499
4	F: NSF1419/20		(see above)	62 °C	1,380
	R: Kudoa-28S-275R		5′-AGGTTGCAGTCCCAAACAAC-3′		3,429
		F: NC13(ITS2)/F	(see above)		2,482
		R: NC13(ITS1)/R	(see above)		2,499
5	F: KUDOA-Kt28S1F		5′-CAAGACTACCCTGCTGAAC-3′	62 °C	3,150
	R: KUDOA-28S-795R		5′-CTGTSTTTCAAGACGKGTCG-3′		3,927
6	F: KUDOA-Kt28S1F		(see above)	64 °C	3,150
	R: NLR2362/20		5′-ACATTCAGAGCACTGGGCAG-3′		5,641
		F: Kudoa 28S-260F	5′-TTGGGATTGCAACCTAAAGC-3′		3,415
		F: NLF1999/19	5′-CCGCAKCAGGTCTCCAAG-3′		5,191
		R: 28S-1284R	5′-GTTGATTCGGCAGGTGAGTT-3′		4,566
		R: 28S-2132R	5′-AGAGGCTGTTCACCTTGGAG-3′		5,221
7	F: NLF3090/24		5′-AGGGAACGTGAGCTGGGTTTAGAC-3′	62 °C	-194
	R: Mbseq2r		5′-CCTTGGTGAGCCACTACCTC-3′		330

[a] Primers used for amplifying and sequencing a certain segment are shown as "for amplifying," and those only for sequencing as "for sequencing"

[b] The relative position of the 5′-end of each primer in *Kudoa neothunni* rDNA sequence (DDBJ/EMBL/GenBank accession no. AB693042)

F forward, *R* reverse

thalassomi was differentiated from *K. neothunni* by having spores with lateral projections, whereas *K. septempunctata* was differentiated by its spore shape and demonstrating variations in polar capsule size (Table 2).
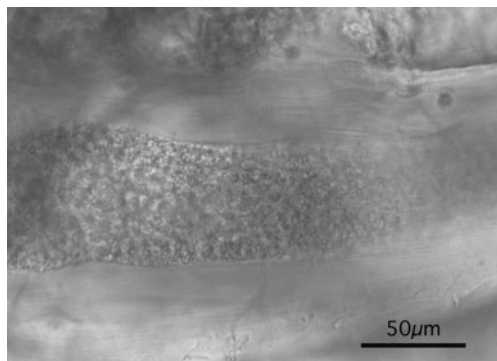

Fig. 1 Partial view of a pseudocyst of *Kudoa neothunni* in a myofiber of a northern bluefin tuna under a light microscope. Note the intact striated muscle myofibrils in the parasitized myofiber

In the chub mackerel fillet, fished in the Sea of Japan, there were 20 white parasitic cysts delineated by a thin layer of fibrous tissue. The range of cyst sizes (mean±SD; $n=7$) was 0.30–0.75 (0.49±0.15)mm by 0.20–0.40 (0.26±0.07)mm. Spores were semiquadrate with four equal-sized shell valves in apical view (Figs. 4 and 5). In lateral view, the spores were ovoid in shape and apical projections on shell valves were not evident. Polar capsules were drop-like or ovoid. The morphometries of the spores were compared with other *Kudoa* spp. having similar morphology (Table 3).

Molecular phylogenetic analysis

The rDNA sequence of *K. neothunni* collected from the northern bluefin tuna was 6,226 bp in length: partial external transcribed spacer (ETS), 170 bp; 18S, 1,741 bp; internal transcribed spacer (ITS) 1 region, 705 bp; 5.8S, 158 bp; ITS2 region, 529 bp; and partial 28S, 2,923 bp (DDBJ/EMBL/GenBank accession no. AB693042). The rDNA sequence of *K. neothunni* collected

Fig. 2 Photographs of fresh spores of *Kudoa neothunni* from a northern bluefin tuna under a light microscope. *Upper photographs* are apical views of spores; *lower photographs* are lateral views of spores
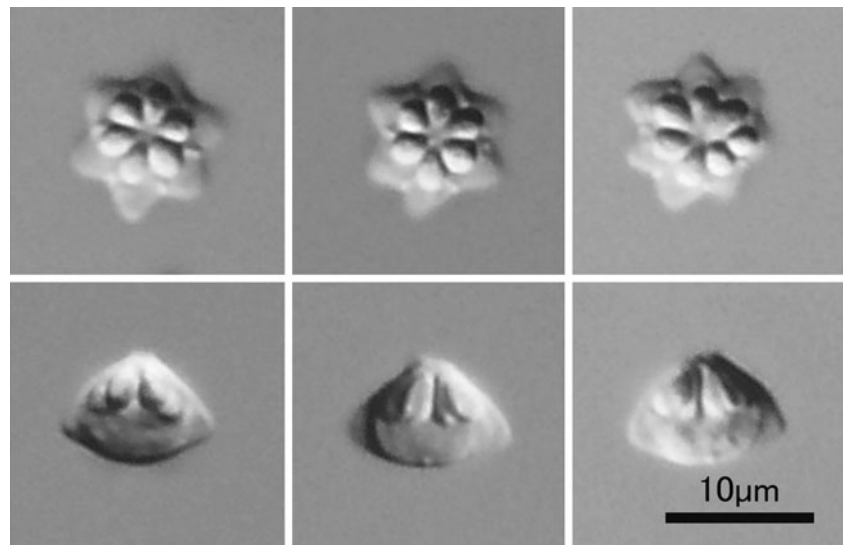
from the yellowfin tuna was 5,609 bp in length: partial 18S, 1,720 bp; ITS1 region, 705 bp; 5.8S, 158 bp; ITS2 region, 588 bp; and partial 28S, 2,438 bp (DDBJ/EMBL/GenBank accession no. AB693049). The 18S and 5.8S rDNA sequences of these two isolates were essentially identical (99.9 % similarity across 1,720-bp length and 100 % similarity across 158-bp length, respectively). However, variations in the ITS1 (62.4 % similarity across 757-bp length), ITS2 (66.9 % similarity across 599-bp length), and 28S (99.0 % similarity across 2,245-bp length) rDNA sequences were observed between the two isolates.

In the ML phylogenetic trees based on the 18S and 28S rDNA sequences, *K. neothunni* positioned closest to *K. grammatorcyni*, forming a clade with *Kudoa* spp. with five or more SV/PC and tissue tropism to trunk muscles except for *K. septempunctata* and *K. thalassomi* (Figs. 6 and 7). The latter two *Kudoa* spp. formed a clade with kudoids with five or more SV/PC and characteristic tissue tropism to the brain. The similarities of the 18S and 28S rDNA sequences between *K. neothunni* and *K. grammatorcyni* were 99.4 % (10 base

differences across 1,680-bp length) and 93.4 % (48 base differences across 726-bp length), respectively. The similarity of the 18S rDNA sequence between *K. neothunni* and *K. scomberomori* was 99.6 % (6 base differences across 1,680-bp length). Comparison of the 28S rDNA sequences was not possible due to a lack of deposited 28S rDNA sequences of *K. scomberomori* in the DDBJ/EMBL/GenBank databases.

The kudoid species isolated from the chub mackerel formed a clade with *K. thunni* and "*K. crumena*" in both ML phylogenetic trees (Figs. 6 and 7). The similarities of the 18S and 28S rDNA sequences between this kudoid isolate and *K. thunni* were 98.5 % (27 base differences across 1,765-bp length) and 96.3 % (30 base differences across 804-bp length), respectively. Similarly, those between the present isolate and "*K. crumena*" were 98.8 % (20 base differences across 1,631-bp length) and 96.6 % (28 base differences across 815-bp length), respectively. The similarities of the 18S and 28S rDNA sequences between the present isolate and *K. trachuri* were 96.2 % (across 1,780-bp length) and 90.4 % (across 771-bp length), respectively. This morphologically and genetically characterized kudoid is described as a new species.

Description

Kudoa scomberi n. sp. (Myxosporea: Multivalvulida) (Figs. 4 and 5; Table 3).

Plasmodia were delineated by a thin layer of fibrous tissue in the trunk muscle, seen as minute white cysts measuring 0.30–0.75 (average 0.49)mm by 0.20–0.40 (average 0.26)mm (*n*=7).

Spores semiquadrate in shape in apical view, with four shell valves and polar capsules of equal sizes. Shell valves without ornamentation, at least by light microscopy assessment. Polar capsules drop-like, located at the anterior portion of shell valves. In apical view, polar capsules accounted for approximately 50 % or less of the total radius of shell valves.
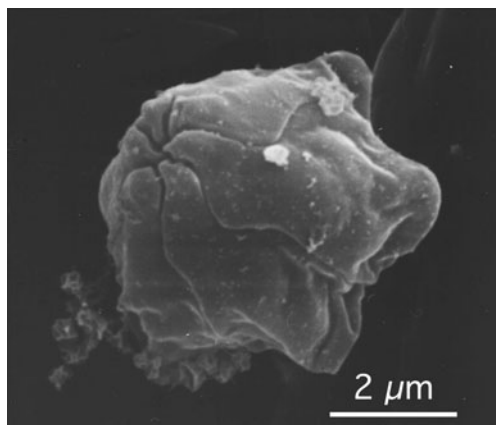
Fig. 3 Scanning electron microscopy of a *Kudoa neothunni* spore in oblique view

Table 2 Measurements of *Kudoa* spp. having six shell valves/polar capsules and characteristic tissue tropism to somatic muscles[a]

Species	K. neothunni	K. neothunni	K. grammatorcyni	K. scomberomori	K. thalassomi	K. septempunctata
Number of shell valves/polar capsules	6	6	6	6	6/7[b]	6/7[c]
Representative host	*Thunnus thynnus*	*Thunnus albacares*	*Grammatorcynus bicarinatus*	*Scomberomorus commerson*	*Thalassoma lunare*	*Paralichthys olivaceus*
Post-harvest myoliquefaction	No	Yes	No	No	No	No
Reference	The present study	Arai and Matsumoto (1953)	Adlard et al. (2005)	Adlard et al. (2005)	Adlard et al. (2005)	Matsukane et al. (2010)
Number of examined spores	15	–[d]	30	30	30	10
Width	9.5–11.4 (10.6)	9.1–13.0 (11.0)	8.0–8.9 (8.6)	6.8–8.2 (7.6)	9.5–11.8 (10.7)	11.1–13.1 (11.8)
Thickness	8.9–10.9 (9.7)	–	7.6–8.7 (8.1)	6.2–7.6 (6.8)	8.6–10.8 (9.4)	8.9–10.0 (9.4)
Suture width	7.3–8.6 (8.1)	5.9–8.7 (7.1)	7.2–8.2 (7.8)	5.3–6.3 (5.9)	6.8–8.8 (8.0)	–
Length	7.3–7.7 (7.6)	5.3–7.3 (6.2)	6.3–6.7 (6.5)	5.0–6.2 (5.4)	6.2–7.1 (6.7)	7.9–8.9 (8.5)
Polar capsule length	3.6–4.1 (3.8)	2.0–3.1 (2.5)	3.6–3.8 (3.7)	3.0–3.6 (3.2)	4.7–5.0 (4.9)	3.7–5.3 (4.6)
Polar capsule width	1.8–2.3 (2.0)	1.3–2.1 (1.6)	1.6–1.8 (1.7)	1.3–1.5 (1.4)	2.0–2.2 (2.1)	2.2–2.8 (2.4)
Apical view of spores	Radially symmetrical	Radially symmetrical	Radially symmetrical	Radially symmetrical	Radially symmetrical	Irregularly radial
Lateral view of spores	Pyramidal	Pyramidal	Ovoid	Ovoid	Pyramidal	Pouch-shape
Ornamentation of valve	None	None	None	None	Lateral projections	None
Variation in size of polar capsules	Not evident	Not evident	Not evident	Not evident	Not evident	Evident

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

[b] Spores with six shell valves/polar capsules are dominant. See Burger and Adlard (2011)

[c] Ratio of spores with six or seven shell valves/polar capsules was variable by host individuals, e.g., 26:74, 33:67, 60:40, and 66:34 (Matsukane et al. 2010; Sato, unpublished data)

[d] Data not available

In lateral view, spores ovoid with somewhat flattened bottom. Coils of polar filament were not visible in wet preparations. Spores ($n=30$; average and range in parentheses; measurements in μm): width 9.2 (8.2–10.5), thickness 8.1 (7.0–8.8), length 6.4 (6.1–6.8), polar capsule length 2.9 (2.5–3.4), and polar capsule width 1.6 (1.3–2.0).

Partial nucleotide sequences of the 18S, ITS1, and 5.8S rDNA (2,232 bp in length) and partial 5.8S, ITS2, and 28S rDNA (1,052 bp in length) have been deposited in DDBJ/EMBL/GenBank (accession nos. AB693044 and AB693045).

Taxonomic summary

Type host: *S. japonicus* Houttuyn, 1782, chub mackerel (Actinopterygii: Perciformes: Scombridae).

Fig. 4 Photographs of fresh spores of *Kudoa scomberi* n. sp. from a chub mackerel fished in the Sea of Japan
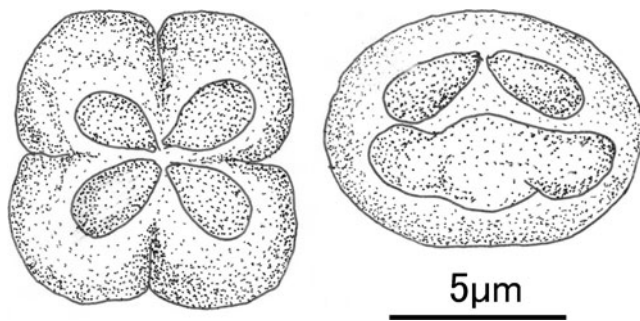
Fig. 5 Stylized diagrams of *Kudoa scomberi* n. sp. in apical (*left*) and lateral (*right*) views

Site of infection: Cysts in somatic muscles.
Type locality: Sea of Japan, off Nagasaki, Nagasaki Prefecture, Japan.
Specimen deposited: Hapantotype NSMT-Pr323, National Museum of Nature and Science, Tokyo, Japan.
Etymology: The specific name comes from its host, *S. japonicus*.
Prevalence: Unknown.

Remarks

K. caudata has been reported from the same host species (*S. japonicus*), but fished in the southeastern Pacific Ocean off Peru (Kovaleva and Gaevskaya 1983). Although the general appearances of *K. caudata* and *K. scomberi* n. sp. spores resemble one another, *K. caudata* is differentiated from the new species by possession of a hair-like filamentous projection on the lateral surface of each shell valve. Similar fine but shorter projections on the lateral surface of each shell valve have been documented in *K. aequidens* from *Aequidens plagiozonatus* in the Amazonian estuarine region and *K. camarguensis* from *Pomatoschistus minutus* in the Mediterranean Sea (Pampoulie et al. 1999; Casal et al. 2008). Since its original description, *K. caudata* has not been recorded again. The rDNA sequence of this kudoid is currently unavailable, so its genetic relationship with the new species is unknown. *K. scomberi* n. sp. formed a clade with *Kudoa* spp. with semiquadrate spores such as *K. thunni*, "*K. crumena*," and *K. trachuri* in both of the phylogenetic trees based on 18S and 28S rDNA sequences. In addition to a substantially lower similarity of the 18S and 28S rDNA sequences (98.8 % or lower, and 96.6 % or lower, respectively), the new species is morphologically differentiated from *K. crumena* on the basis of a different lateral view of spores (ovoid versus pouch-shaped with truncated apex), smaller spore length, and smaller polar capsule size. Furthermore, it is morphologically differentiated from *K. trachuri* by a larger spore size (Table 3). The morphological differentiation of *K. scomberi* n. sp. from *K. thunni* based solely on light microscopy assessment is difficult. In addition to a lower similarity of the 18S and

28S rDNA sequences (98.5 % and 96.3 %, respectively), the dimensions of cysts in the trunk muscle formed by *K. thunni* are clearly larger than those of the new species (Matsukane et al. 2011): 1.5 (1.3–2.0) mm by 1.2 (1.1–1.4) mm ($n=14$) vs. 0.49 (0.30–0.75) mm by 0.26 (0.20–0.40) mm ($n=7$), respectively.

As reported previously (Matsukane et al. 2011), *K. crumena* has been recorded from Spanish mackerel (*Scomberomorus maculatus*) in southern Florida, USA (Iversen and Van Meter 1967), but also from yellowfin tuna (*T. albacares*) collected in North Carolina, USA (Moran et al. 1999). The 18S and 28S rDNA sequences deposited in DDBJ/EMBL/GenBank for *K. crumena* (accession nos. AF378347 and FJ417057, respectively) were based on an isolate from *T. albacares* in North Carolina, USA, and another isolate from southern bluefin tuna (*T. maccoyii*) in Tasmania, Australia, respectively, and not from the type host for *K. crumena*, *Scomberomorus maculatus* (Kent et al. 2001; Bartosová et al. 2009). The closeness of the relationships of the 18S and 28S rDNA sequences of *K. thunni* with the deposited sequences of "*K. crumena*" from *Thunnus* spp. requires further confirmation in order to establish whether *K. crumena* described by Iversen and Van Meter (1967) and "*K. crumena*" from *Thunnus* spp. are indeed identical.

Discussion

K. neothunni has been recorded not only from the jellied meat of yellowfin tuna but also from the jellied meat of bigeye tuna (*T. obesus*) fished in the Pacific Ocean near Taiwan and northern bluefin tuna in the Pacific Ocean near northern Japan (Arai and Matsumoto 1953; Konagaya 1982, 1984). In the present study, we have characterized morphologically and genetically two *K. neothunni* isolates from different tuna species (*T. albacares* and *T. thynnus*) with/without post-harvest myoliquefaction. In addition to morphological similarity, the 18S rDNA sequences of these two isolates were essentially identical; only two base substitutions were detected across the 1,720-bp length of the 18S rDNA sequence. There are only two base substitutions across the comparable 1,679-bp length of 18S rDNA sequence of *K. alliaria* infecting Argentinian hoki (*Macruronus magellanicus*) without myoliquefaction and *K. rosenbuschi* recovered from Argentinean hake (*Merluccius hubbsi*) with post-harvest myoliquefaction. Furthermore, these two species show an identical morphology and geographical distribution (Abollo et al. 2005; Whipps and Diggles 2006). This illustrates the use of a single pathophysiological character (post-mortem tissue degradation) as a major criterion for species discrimination. When we apply this taxonomic code, it appears likely that the two *K. neothunni* isolates found in tunas with/without post-harvest myoliquefaction are distinct species. In this regard, however, we should take into account the fact the degree of post-

Table 3 Measurements of *Kudoa* spp. having quadrate or semiquadrate spores in apical view and forming macroscopic cysts in the trunk muscle[a]

Species	Kudoa scomberi n. sp.	Kudoa caudata	Kudoa thunni	Kudoa crumena	Kudoa trachuri	Kudoa trachuri
Host	Scomber japonicus	Scomber japonicus	Thunnus alalunga	Scomberomorus maculatus	Trachurus japonicus	Trachurus japonicus
Location in the host	Elliptical cysts between myofibers	–[b]	Ovoid cysts between myofibers	Ellipsoidal cysts between myofibers	Elliptical cysts between myofibers	Elliptical cysts between myofibers
Locality	Sea of Japan off Nagasaki	Southeastern Pacific Ocean off Peru	Pacific Ocean	Miami, Florida	Sea of Japan off Nagasaki	Sea of Japan off Nagasaki
Date of collection	11 Feb 2011	25 Jan 1980	3 Oct 2008	–	11 Sep 2008	4 Dec 2008
References	The present study	Kovaleva & Gaevskaya (1983)	Matsukane et al. (2011)	Iversen & Van Meter (1967)	Matsukane et al. (2011)	Matsukane et al. (2011)
Number of examined spores	30	–	20	10	20	20
Width	8.2–10.5 (9.2)	8.0–8.6	9.2–9.9 (9.5)	9.3–10.4 (9.9)	7.0–8.5 (7.9)	5.9–6.8 (6.3)
Thickness	7.0–8.8 (8.1)	6.7	7.7–9.0 (8.3)	8.2–9.7 (9.0)	5.3–6.2 (5.8)	4.5–5.6 (5.1)
Suture thickness	6.1–8.0 (7.1)	–	–	–	–	–
Length	6.1–6.8 (6.4)	5.3–6.7	6.4–6.6 (6.5)	6.8–8.2 (7.5)	5.5–6.9 (6.1)	6.7–7.5 (7.0)
Polar capsule length	2.5–3.4 (2.9)	2.0–2.7	2.2–2.9 (2.5)	3.2–4.6 (4.0)	2.6–3.5 (2.9)	2.2–2.9 (2.7)
Polar capsule width	1.3–2.0 (1.6)	1.6–2.0	1.9–2.2 (2.1)	2.1–2.9 (2.5)	1.6–2.2 (2.0)	1.7–2.2 (2.0)
Apical view of spore	Semiquadrate	Semiquadrate	Semiquadrate	Semiquadrate	Semiquadrate	Semiquadrate
Lateral view of spore	Ovoid	Almost ovoid	Ovoid	Pouch-shaped with truncated apex	Ovoid	Ovoid
Ornamentation of shell valve	None	Filamentous projection	None	None	None	None
Polar capsule	Drop-like	Drop-like	Drop-like	Elongated, drop-like	Drop-like	Drop-like

[a] All measurements are in μm and expressed as range and mean in parentheses

[b] Data not available

harvest myoliquefaction, i.e. enzymatic digestion of the host muscle, can be affected by several factors such as time of evaluation post-mortem, temperature during storage, intensity of the infection, developmental stage of plasmodia, host response

to infected myofibers, muscle pH associated with the inherent flesh quality, etc. (Konagaya 1984; Dawson-Coates et al. 2003; Funk et al. 2007, 2008; Zhou and Li-Chan 2009). Even if a close association between particular *Kudoa* spp. and post-harvest

Fig. 6 ML phylogenetic tree based on the 18S rDNA sequence. On the *right side*, the number of shell valves and polar capsules per spore, and the location in the host are shown for each species. *B* brain, *CT* connective tissue, *GO* general body, *OV* ovary, *PC* pericardium, *SM* smooth muscle, *TM* trunk muscle
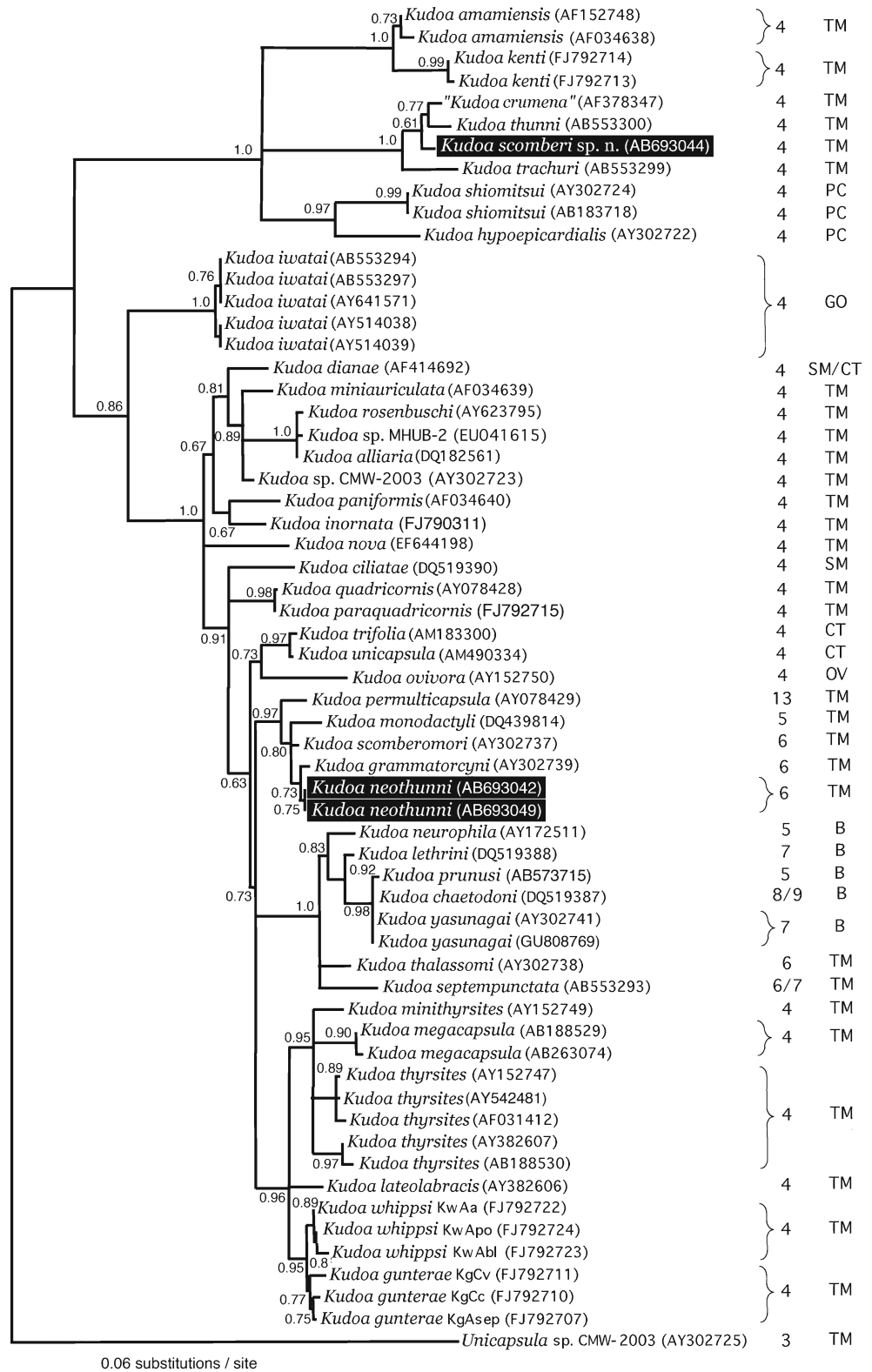
myoliquefaction is regarded as a general tendency, the condition of fish meats at a particular time point could be highly variable, and for an individual case of infection it is inadvisable to use this character as a major criterion for species differentiation.

In contrast to their 18S rDNA sequence, the two *K. neothunni* isolates collected in this study had significant differences in the nucleotide sequences of their ITS1 (only 62.4 % similarity across 757-bp length), ITS2 (only 66.9 % similarity across 599-bp length), and 28S (22 base changes across 2,245-bp length) rDNA regions. Collection of more isolates from different origins and genetic characterization of their ITS region and/or 28S rDNA may disclose cryptic species or different lineages of *K. neothunni*, and could contribute to understanding the significance(s) of such ITS variation in the species. When conducting such research, kudoid specimens should be collected from infected fish with post-harvest myoliquefaction and from infected fish with an intact appearance, although it is easier to collect

samples from the former. In a work on cosmopolitan *K. thyrsites*, assumed to be a species complex by many researchers, the 18S rDNA sequences obtained from hosts in different regions (seas around Canada, USA, Chile, UK, South Africa, Australia, and Japan) showed little sequence divergence (0.2–1.0 %). Greater variation in the 28S rDNA sequences (3.0–9.6 % variation across 700-bp length) and ITS1 regions (maximum difference of 65.6 % for 393–493-bp length) was found (Whipps and Kent 2006). Greater base variation in the 28S rDNA sequence than in the 18S rDNA sequence has been shown for several *Kudoa* spp. (Burger and Adlard 2011). Genetic comparison of 18 *K. thalassomi* isolates from 18 fish species of six different families on the Great Barrier Reef, Queensland, Australia revealed a minimum 99.7 % identity for 18S rDNA (range of 0–10 nucleotide differences across 1,539-bp length) and 98.5 % identity for 28S rDNA (range of 0–10 nucleotide differences across 814-bp length) regardless of host origin (Burger and

Fig. 7 ML phylogenetic tree based on the 28S rDNA sequence. On the *right side*, the number of shell valves and polar capsules per spore, and the location in the host are shown for each species. *B* brain, *CT* connective tissue, *GO* general body, *SM* smooth muscle, *TM* trunk muscle
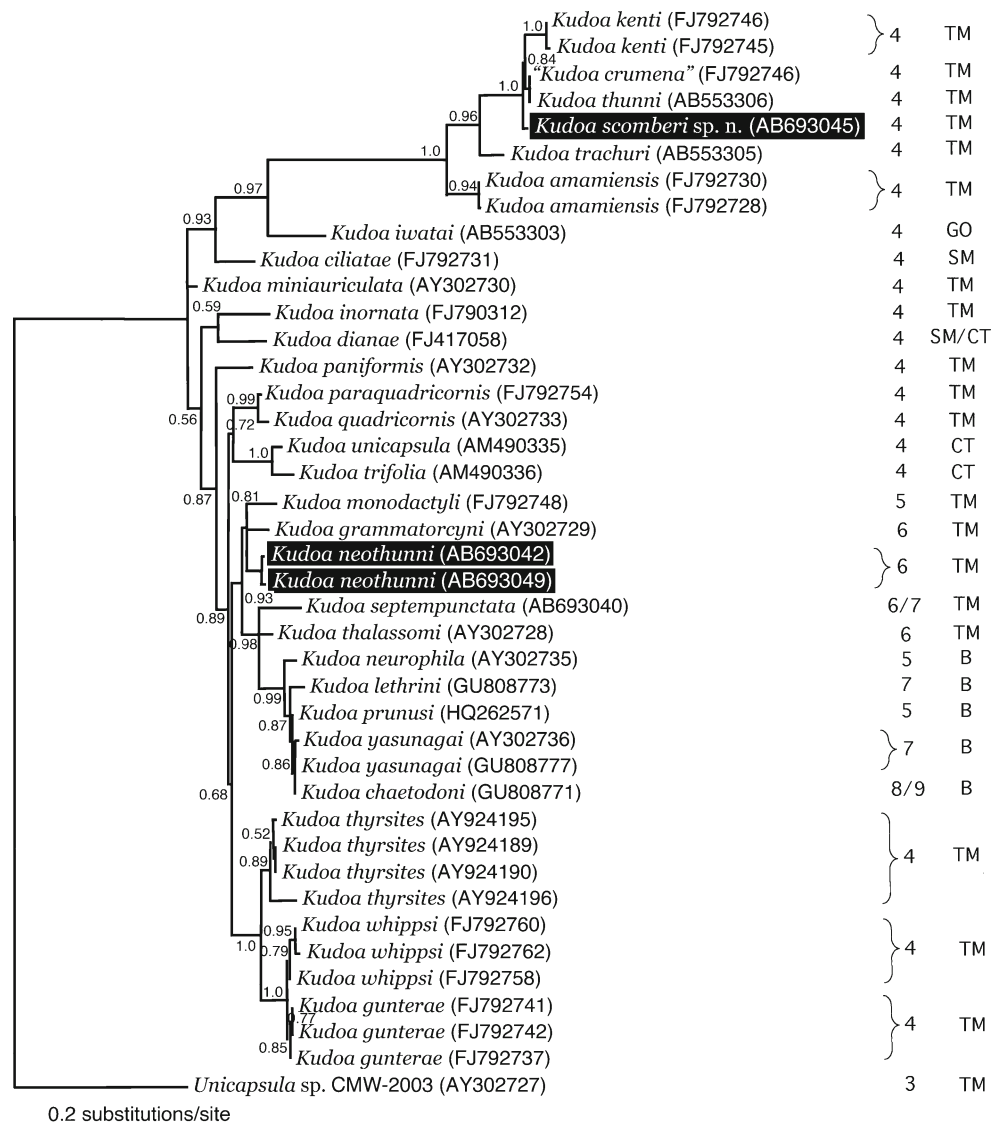
Adlard 2011). The possible usefulness of molecular markers other than the 18S rDNA sequence awaits evaluation for *Kudoa* spp. including *K. neothunni*.

Recently, Burger and Adlard (2010b) identified a *Kudoa* isolate having five SV/PC in the brain of sand whitings around Australia as *K. yasunagai*—originally described as having seven SV/PC in the brain of olive flounders (*P. olivaceus*) and bluefin tunas—based on 100 % identity of the 18S rDNA. This morphological variation of a single species appears to be distinct from the previously reported plasticity of numbers of SV/PC in a single plasmodium of *K. chaetodoni* (Burger et al. 2007), *K. crumena* (Iversen and Van Meter 1967), *K. iwatai* (Egusa and Shiomitsu 1983; Diamant et al. 2005), *K. lethrini* (Burger et al. 2007), *K. monodactyli* (Gunter et al. 2006), *K. neurophila* (Grossel et al. 2003), *K. permulticapsula* (Whipps et al. 2003), *K. septempunctata* (Matsukane et al. 2010), *K. thalassomi* (Burger and Adlard 2011), and *K. yasunagai* (Hsieh and Chen 1984). The same researchers (Burger et al. 2007), however, differentiated brain-parasitizing *K. chaetodoni* from *K. yasunagai* based on notable differences in spore sizes and the number of polar capsules (8/9), although these two species have 0.19 % difference (3 bases) in the 18S rDNA and 0.6–1.3 % difference (4–8 bases) in the partial 28S rDNA. Similarly, Burger and Adlard (2010a) found dimensional differences, but not in shape, between spores of *K. paraquadricornis* and *K. quadricornis*, although these two species have only one nucleotide difference in the 18S rDNA of 1,683-bp length (99.94 % identity) and 27 base differences in the 28S rDNA of 715-bp length (96.22 % identity). Likewise, *K. prunusi*, recorded from the brain of aquacultured northern bluefin tunas, shows a close resemblance in morphology with *K. yasunagai* ex *S. ciliata*, i.e., having five SV/PC, and has a 0.3 % (5 bases) difference in the 18S rDNA and a 1.7 % (11 bases) difference in the 28S rDNA from those of *K. yasunagai* (Meng et al. 2011). As mentioned above, collection of more *Kudoa* isolates from different origins and geographical areas and subsequent genetic characterization of them together with intensive morphological observation may help us to determine whether the observed divergence is ascribed to intraspecific variation or critical difference separating species of the myxosporean parasites.

Although a close relationship of *K. scomberi* n. sp. to *K. thunni* appears apparent in both ML phylogenetic trees based on the 18S and 28S rDNA sequences (Figs. 6 and 7), this is ascribed not to conspecificity of these two species, but to extensive trimming of certain variable areas containing gaps for construction of the molecular phylogenetic tree. Since kudoid species with quadrate or semiquadrate spores with four SV/PC resembling each other are abundant, it is critical to perform genetic analyses not only for 18S rDNA but also for 28S rDNA for their species identification.

Recently, a subtle but significant public health impact of *K. septempunctata* has been indicated by Japanese researchers (Konishi 2011; Ohnishi 2011; Grabner et al. 2012; Harada et al. 2012; Kawai et al. 2012). Indeed, two to three dozen outbreaks of symptomatic patients per year have been recorded in the last few years. This food poisoning, with a moderate prognosis, is manifested by vomiting and diarrhea within 12 hours after consumption of fresh flounder slices in the raw state as sashimi or sushi. The Ministry of Health, Labour and Welfare of Japan has issued an official notice to regard *K. septempunctata* as a potential cause of food poisoning following consumption of fresh fish foods. Since this food poisoning is dose- and spore viability-dependent, it is advisable to avoid consumption of fresh fish with kudoid infection in the raw state. With kudoid species that form pseudocysts in the trunk muscle, like *K. septempunctata* in the flounder and *K. neothunni* in the tuna, it is difficult to perceive their infection with the naked eye, even heavy ones as shown in Fig. 1, resulting in the oral intake of kudoid spores in bulk. In Spain, Martínez de Velasco et al. (2008) noticed a potential public health impact of *Kudoa* spp. as a food-borne allergen for humans with allergic gastrointestinal symptoms. Feasible and reliable techniques to identify *Kudoa* spp. defined by taxonomical experts would facilitate public health workers to approach and control the cause of diseases, and specify the fish species that put our health at risk.

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