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FISH PARASITOLOGY - ORIGINAL PAPER

# New host records of three *Kudoa* spp. (*K. yasunagai*, *K. thalassomi*, and *K. igami*) with notable variation in the number of shell valves and polar capsules in spores

Haruya Sakai[1] · Takao Kawai[2] · Jinyong Zhang[1,3] · Hiroshi Sato[1]

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## Abstract

To date, 26 *Kudoa* spp. (Myxozoa: Myxosporea: Multivalvulida) have been recorded in edible marine fishes in Japan. In the future, it is likely that even more marine fish multivalvulid myxosporeans will be characterized morphologically and genetically, which will aid the precise understanding of their biodiversity and biology. We examined 60 individuals of six fish species collected from the Philippine Sea off Kochi or from the border between the Philippine Sea and East China Sea around Miyako Island, Okinawa, i.e., the southern part of Japan. Newly collected parasite species included *Kudoa yasunagai* from the brain of Japanese meagre (*Argyrosomus japonicus*) and Japanese parrotfish (*Calotomus japonicus*), *Kudoa miyakoensis* n. sp. and *Kudoa thalassomi* from the brain and trunk muscle, respectively, of bluespine unicornfish (*Naso unicornis*), and *Kudoa igami* from the trunk muscle of Carolines parrotfish (*Calotomus carolinus*), African coris (*Coris gaimard*), and Pastel ringwrasse (*Hologymnosus doliatus*). With the exception of Japanese parrotfish for *K. yasunagai*, all these fish are new host records for each kudoid species. Notable variation in the number of shell valves (SV) and polar capsules (PC) was observed for all four kudoid species. In particular, spores with seven or eight SV/PC were prominent in *K. igami* isolates, despite the original Japanese parrotfish-derived description characterizing it as having spores with six, or less commonly five, SV/PC. However, molecular genetic characterization based on the ribosomal RNA gene (rDNA) and mitochondrial DNA (cytochrome *c* oxidase subunit 1 and ribosomal RNA small and large subunits) found no significant differences in the nucleotide sequences of isolates with different phenotypical features as far as examined in the present study. A newly erected species, *K. miyakoensis* n. sp., was determined to be phylogenetically closest to brain-parasitizing species, such as *K. chaetodoni*, *K. lemniscati*, and *K. yasunagai* based on rDNA nucleotide sequences, but differed from them morphologically.

**Keywords** *Kudoa yasunagai* · *Kudoa miyakoensis* n. sp. · *Kudoa thalassomi* · *Kudoa igami* · Morphological variation · Mitochondrial DNA

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✉ Hiroshi Sato
sato7dp4@yamaguchi-u.ac.jp

[1] Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

[2] Osaka Institute of Public Health, 1-3-69 Nakamachi, Higashinari-ku, Osaka 537-0023, Japan

[3] Fish Diseases Laboratory, State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

## Introduction

*Kudoa* Meglitsch, 1947, is the largest genus in the order Multivalvulida (Cnidaria: Myxozoa: Myxosporea), with currently more than 100 nominal species (Moran et al. 1999; Lom and Dyková 2006; Eiras et al. 2014; Sato and Kasai 2016). The genus is defined as myxosporeans with four or more shell valves and polar capsules (SV/PC) in equal numbers (Whipps et al. 2003, 2004; Lom and Dyková 2006; Fiala et al. 2015). Over the last two decades, new geographical and host records as well as new species descriptions of *Kudoa* spp. have rapidly accrued due to the introduction of molecular genetic technologies which allow the differentiation of morphologically simple myxospores. Furthermore, myxospores often show

phenotypic plasticity, e.g., different numbers of SV/PC in a single species and different morphometrics (Burger et al. 2008; Burger and Adlard 2010, 2011; Matsukane et al. 2010, 2011; Kasai et al. 2016). To ensure accurate species identification, the accumulation of nucleotide sequence data of the ribosomal RNA gene (rDNA) and mitochondrial DNA (mtDNA) (currently focused on cytochrome *c* oxidase subunit 1 (*cox-1*) and ribosomal RNA small and large subunits (*rns* and *rnl*)) (Takeuchi et al. 2016; Sakai et al. 2018) is of critical importance.

Up to 2018, 26 *Kudoa* spp. and two *Unicapsula* spp. have been recorded from edible marine fishes in the natural seawater around Japan or commercial marine fish consumed daily by Japanese people after importation from foreign countries, such as Southeast Asian countries, USA, or Russia (Sato and Kasai 2016; Sakai et al. 2018). A precise and confident identification of kudoid or other myxosporean species is of critical importance for the municipal public health laboratory when identification of a possible causative agent of "*Kudoa* food poisoning" is immediately sought after an outbreak of clinical cases following consumption of raw fish (Sugita-Konishi et al. 2014). Daily surveys for myxosporean infections in marine fishes not only contribute to the aforementioned practical task but also help us to understand the real biodiversity and ecological situations of multivalvulid species.

In the present study, we characterized morphologically and genetically three known *Kudoa* spp., but with notable phenotypical variation in the number of SV/PC. To clarify the phylogenetic relationships between different isolates of a single species, their rDNA and mtDNA nucleotide sequences were compared. With the exception of Japanese parrotfish for *K. yasunagai*, all host records of *Kudoa* spp. in the present study were new for each species. In addition, a new kudoid species with brain tropism, *Kudoa miyakoensis* n. sp., was differentiated from an array of brain-parasitizing kudoids based on morphological and molecular genetic analyses of their myxospores.

## Materials and methods

### Fish samples examined

Whole fish bodies of 60 individuals of six fish species were purchased from local fish markets in Kochi or a fisherman on Miyako Island, Okinawa, southern Japan, during the period August 17, 2016 to June 23, 2017 (Table 1). These fishes included two Japanese meagre (*Argyrosomus japonicaus*), three Japanese parrotfish (*Calotomus japonicus*), 38 bluespine unicornfish (*Naso unicornis*), three Carolines parrotfish (*Calotomus carolinus*), 10 African coris (*Coris gaimard*), and four Pastel ringwrasse (*Hologymnosus doliatus*), caught in the Philippine Sea, off Kochi, Japan, or at the border between the Philippine Sea and East China Sea, off Miyako Island, Okinawa, Japan. A package of several fillets of longtail tuna (*Thunnus tonggol*) from the East China Sea, off Nagasaki, Japan, was purchased in a fish market near Yamaguchi University.

### Parasitological and morphological examination

Following transportation of fresh samples on ice (Japanese meagre and Japanese parrotfish) or frozen samples (bluespine unicornfish, Carolines parrotfish, African coris, and Pastel ringwrasse), fishes were thawed if necessary, cut open and their gills, viscera, and brain removed and examined under a dissection microscope. Fish meat fillets were examined on the day of arrival or frozen until examination. To check for the presence of myxosporean cysts or pseudocysts, thin slices of muscle fillet were pressed between two glass plates and examined under a dissection microscope. The commercial package's fish fillets were examined in a similar manner.

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 brain or myofiber was executed with fine forceps. Myxospores were observed using a light microscope, photographed at a magnification of ×800, then transformed into digital images with Adobe® Photoshop ver. 11.0 (Adobe Systems, San Jose, California, USA). Measurements were conducted on digital 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 or 70% ethanol solution. Specimens collected in the present work were deposited in the Meguro Parasitological Museum, Tokyo, Japan, under collection nos. 21379 and 21380 (*K. yasunagai* from Japanese meagre and Japanese parrotfish, respectively), 21428 (*K. thalassomi* from bluespine unicornfish), 21429 (*K. miyakoensis* n. sp. from bluespine unicornfish), 21430 (*K. hexapunctata* from longtail tuna), 21431 (*K. igami* from Carolines parrotfish), 21432 (*K. igami* from African coris), and 21433 (*K. igami* from Pastel ringwrasse).

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

Parasite DNA was extracted from a kudoid plasmodium 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 25-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

**Table 1** Fish samples examined in the present study

| Fish species | Number of samples examined | Collection date | Locality | Standard body length[a] (cm) | Body weight[a] (g) | Parasite species[b] | Location of parasite | Parasitic form and intensity (number of plasmodia/fish) |
|---|---|---|---|---|---|---|---|---|
| Japanese meagre (*Argyrosomus japonicus*) | 2 | Aug. 17, 2016 | Philippine Sea, off Kochi, Japan | 85 | 6600 | *K. yasunagai* (1/2) | Brain | Pseudocyst (1) |
| Japanese parrotfish (*Calotomus japonicus*) | 3 | Oct. 12, 2016 | Philippine Sea, off Kochi, Japan | 30 | 784–890 | *K. yasunagai* (1/3) | Brain | Pseudocyst (1) |
| Bluespine unicornfish (*Naso unicornis*) | 38 | Jun.23, 2017 | Border between Philippine Sea and East China Sea, off Miyako Is., Okinawa, Japan | 18.6–32.3 (25.5) | 261–971 (516) | *K. miyakoensis* n. sp. (2/38) | Brain | Pseudocyst (7 or 5) |
| | | | | | | *K. thalassomi* (2/38) | Trunk muscle | Pseudocyst (48 / 42 g muscle or 44 / 59 g muscle) |
| Carolines parrotfish (*Calotomus carolinus*) | 3 | | | 21.0–23.9 (21.9) | 276–418 (333) | *K. igami* (3/3) | Trunk muscle | Pseudocyst (175 / 4.8 g muscle – 308 / 1.7 g muscle) |
| African coris (*Coris gaimard*) | 10 | | | 19.5–27.0 (22.4) | 146–468 (240) | *K. igami* (3/10) | Trunk muscle | Pseudocyst (13 / 27.3 g muscle – 68 / 15.4 g muscle) |
| Pastel ringwrasse ( *Hologymnosus doliatus*) | 4 | | | 21.9–28.5 (26.1) | 115–351 (245) | *K. igami* (3/4) | Trunk muscle | Pseudocyst (9 / 8.9 g muscle – 28 / 7.6 g muscle) |
| Longtail tuna (*Thunnus tonggol*) | 1 package | Aug. 26, 2017 | East China Sea, off Nagasaki, Japan | – | – | *K. hexapunctata* (1/1) | Trunk muscle | Pseudocyst (63 / 9.8 g muscle) |

[a] Range and average in parentheses

[b] Number of positive fish/number of examined fish in parentheses

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 cells (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 mtDNA, i.e., *cox-1* and *rns-rnl*, according to our previous study (Sakai et al. 2018).

The nucleotide sequences obtained in the present study are available from the DDBJ/EMBL/GenBank databases under the accession nos. LC316967, LC316968, LC381986–LC382002 (rDNA), LC382003–LC382010 (*cox-1*), and LC382036–LC382041 (*rns-rnl*).

### Phylogenetic analysis

For phylogenetic analysis, the newly obtained rDNA nucleotide sequences of *Kudoa* spp. in the present study and related kudoid 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 or ambiguous base in any sequence, except for one base site mentioned below, were excluded from subsequent analyses; 1414 characters, of which 83 were variable, and 624 characters, of which 170 were variable, remained for subsequent analyses for the 18S and 28S rDNA, respectively. An ambiguous base "K" (Keto; T or G) at the 640th site of an 18S rDNA nucleotide sequence of *K. hexapunctata* (DDBJ/EMBL/GenBank accession no. LC200469) was replaced by "T"—which was the common nucleotide at this position in all the other *K. hexapunctata* isolates—because the nucleotide at the 640th base site is important to characterize the 18S rDNA sequence of *K. chaetodoni* and consequently could not be excluded from the phylogenetic analysis. Replacement of the ambiguous base "K" at the 640th site of the same 18S rDNA nucleotide sequence of *K. hexapunctata* (LC200469) by "G" showed no changes in topological relationships of brain-infecting *Kudoa* spp. in the phylogenetic trees. Maximum likelihood (ML) analysis was performed with the program PhyML as described previously (Matsukane et al. 2010; Li et al. 2013).

Similarly, partial mtDNA sequences of *cox-1* and *rns-rnl* were aligned; 437 characters, of which 168 were variable, and 1007 characters, of which 384 were variable, remained for subsequent analyses for *cox-1* and *rnl*, respectively. ML phylogenetic trees were constructed with multiple sequences of *K. septempunctata*, *K. hexapunctata*, *K. neothunni*, and *K. konishiae*, which have been characterized in previous reports (Takeuchi et al. 2016; Sakai et al. 2018).

## Results

### Incidence of kudoid infection

Myxosporean plasmodia, one and four in number, were detected on the brain surface of one Japanese meagre and one Japanese parrotfish, respectively (Table 1). Myxospores found in the plasmodium isolated from the Japanese meagre were not fully developed, whereas plasmodium myxospores from the Japanese parrotfish were well developed and oval in shape, measuring 0.35–0.97 (0.54) mm by 0.31–0.83 (0.45) mm ($n = 4$). A single plasmodium from both fish hosts contained kudoid spores with predominantly six SV/PC but also five or seven SV/PC (Supplemental Fig. 1).

Seven and five plasmodia were found on the brain surface of two individuals (nos. 27 and 37) of 38 bluespine unicornfish samples examined. Spherical plasmodia, measuring 0.44–1.14 (0.79) mm in diameter ($n = 7$) or 0.43–0.85 (0.69) mm ($n = 5$), contained kudoid spores with seven or eight SV/PC (Fig. 1). In the myofibers of trunk muscle of two bluespine unicornfish individuals (nos. 34 and 38), kudoid plasmodia forming pseudocysts were abundantly detected; 48 plasmodia per 42-g muscle and 44 plasmodia per 59-g muscle. Elongated pseudocysts tapered at both sides, measuring 1.11–4.79 (2.44) mm in length by 0.06–0.27 (0.16) mm in width ($n = 13$), contained kudoid spores with six or seven SV/PC (Supplemental Fig. 2).

Similarly, pseudocysts containing spores with seven to nine SV/PC (Supplemental Fig. 3) occurred frequently in the myofibers of trunk muscle of all three individuals of Carolines parrotfish, three of 10 individuals of African coris, and three of four individuals of Pastel ringwrasse. Kudoid infection in Carolines parrotfish was extremely high, with grossly visible elongated pseudocysts occupying almost the full length of myofibers (Fig. 2). Spore formation in the plasmodium was active, hence both ends of pseudocysts were blunt. Kudoid plasmodia also grew well in African coris and Pastel ringwrasse: 2.02–4.82 (3.63) mm in length by 0.11–0.28 (0.20) mm in width ($n = 20$) in Carolines parrotfish; 1.47–3.57 (2.30) mm in length by 0.19–0.26 (0.22) mm in width ($n = 5$) in African coris; and 3.15–4.86 (3.64) mm in length by 0.23–0.29 (0.26) mm in width ($n = 5$) in Pastel ringwrasse.

Numerous plasmodia of *K. hexapunctata* with six SV/PC were found in the muscle fillet of longtail tuna as pseudocysts, with an intensity of 63 plasmodia per 9.8-g muscle. Other

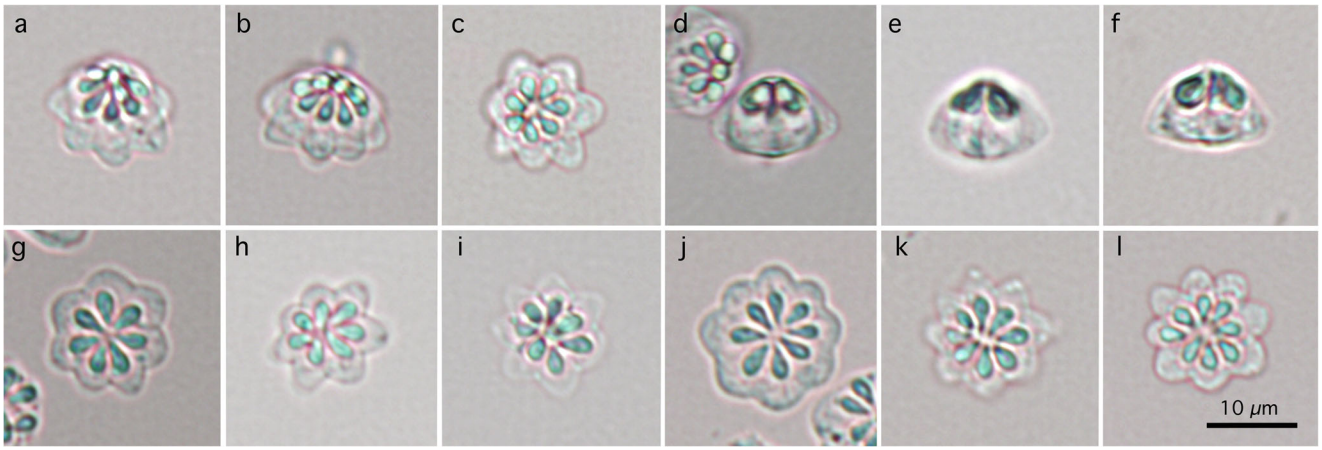


**Fig. 1** Fresh spores of *Kudoa miyakoensis* n. sp. from the brain of *Naso unicornis*. Spores are shown in apical (**a**–**c**, **g**–**l**) and lateral (**d**–**f**) views, and spores shown in (**g**–**l**) are fully extended ones to clearly display the number and shape of their PC. All photographs are at the same magnification, with the scale shown on the lower furthest right photograph (**l**)

characters were similar to a previous report of *K. hexapunctata* parasitism in a longtail tuna (Kasai et al. 2017b).

## Morphological and molecular genetic analyses of isolated kudoids

Kudoid species isolated from the brain or muscles of perciform fish classified in the families Sciaenidae, Scaridae, Acanthuridae, and Labridae were characterized as having myxospores with five or more SV/PC (Fig. 3). Furthermore, the number of SV/PC in spores growing in a single plasmodium was not fixed and varied among plasmodia and consequently fish individuals.

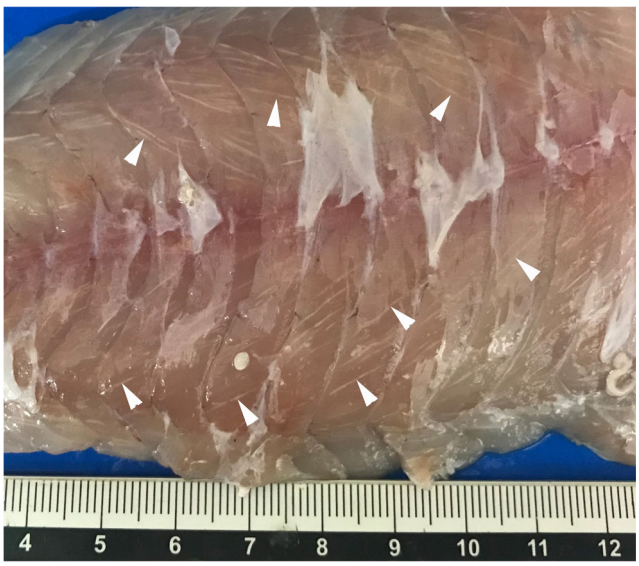


**Fig. 2** Gross lesions of kudoid infection in the trunk muscle of *Calotomus carolinus*. White stripes in the muscle are the plasmodia of *Kudoa igami* growing in the myofiber, and some of them are indicated by arrowheads. Minimum unit of the scale shown below the fish sample is 1 mm

The kudoid spores in plasmodia growing on the brain surface of Japanese meagre and Japanese parrotfish (Supplemental Fig. 1) had predominantly six, or six and seven, SV/PC. The ratio of spores with different numbers of SV/PC in a single plasmodium is shown in Table 2. The morphology of these two *K. yasunagai* isolates corresponded well to descriptions in previous studies. Long nucleotide sequences of the rDNA of the two isolates from the two aforementioned fish species were successfully obtained. They consisted of partial 18S rDNA (1719 bp), internal transcribed spacer 1 (ITS1; 365 bp or 377 bp), 5.8S rDNA (158 bp), internal transcribed spacer 2 (ITS2; 461 bp or 465 bp), and partial 28S rDNA (2408 bp or 2843 bp). The nucleotide sequences of the 18S and 5.8S rDNA were absolutely identical, while those of the 28S rDNA showed just a few substitutions (99.88% identity [2405/2408]). The ITS1 nucleotide sequences showed a sequential 12-bp long insertion/deletion (indel) over a length of 377 bp, while the ITS2 nucleotide sequences showed three sites of indels with one to four nucleotides and 3.04% (14/460) nucleotide substitutions.

The kudoid spores in plasmodia growing on the brain surface of bluespine unicornfish had seven or eight SV/PC (Fig. 1 and Table 3). Spore dimensions of the present isolate were evidently bigger than those of *K. yasunagai* except for a few isolates from *Liza vaigiensis* and *Lutjanus ehrenbergii* in the Ningaloo Reef, Western Australia, and smaller than those of *K. chaetodoni* and *K. lemniscati*, all of which show brain tropism (Tables 2 and 3). Concurrently, the PC dimensions of the present isolate were distinctly smaller than those recorded in *K. chaetodoni* and *K. lemniscati* spores collected from the Coral Sea of Western Australia. Two long nucleotide sequences of the rDNA of the bluespine unicornfish isolate were successfully obtained. They consisted of partial 18S rDNA (1719 bp), ITS1 (306 bp or 312 bp), 5.8S rDNA (158 bp), ITS2 (460 bp or 465 bp), and partial 28S rDNA (2843 bp). These two sequences showed intra-individual nucleotide variations only in the ITS1 and ITS2 regions; specifically, two sites of indel with 16 bp and

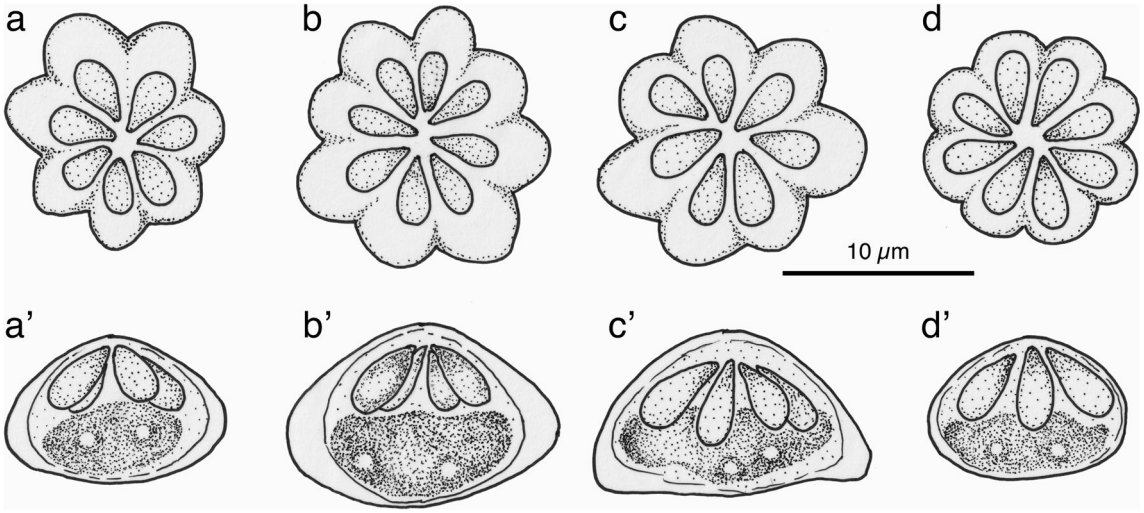


**Fig. 3** Stylized diagrams of the four *Kudoa* spp. recorded in the present study. *Kudoa yasunagai* (**a** and **a′**), *Kudoa miyakoensis* n. sp. (**b** and **b′**), *Kudoa thalassomi* (**c** and **c′**), and *Kudoa igami* (**d** and **d′**) in apical (upper) and lateral (lower) views

three nucleotide substitutions in the former region, and one site of indel with 5 bp in the latter region. As far as our comparison showed, the nucleotide sequences of the ITS regions of *K. yasunagai* and the present isolate were rather different (approximately 60–70% identities), and other regions of the rDNA, i.e., 18S, 5.8S, and 28S rDNA, were highly similar among *Kudoa* spp. with brain tropism. A detailed comparison of the nucleotide sequences is shown later, and the isolate from the brain of bluespine unicornfish is considered as a new species, *K. miyakoensis* n. sp.

In the trunk muscle of two out of 38 bluespine unicornfish samples, plasmodia containing kudoid spores with six or seven SV/PC were frequently detected (Supplemental Fig. 3 and Table 4). Partial 18S rDNA sequences (1697 bp), identical between the two isolates from the two different fish samples in the present study, showed high nucleotide identities with deposited sequences in the DDBJ/EMBL/GenBank databases of *K. thalassomi* ranging from 99.65 (1426/1431) to 100% (1431/1431 or 1565/1565). The 18S rDNA nucleotide sequences showing 100% identity with the present isolate included *K. thalassomi* from *Calotomus japonicuas* fished in the Philippine Sea, off Wakayama Prefecture, Japan, and *Thalassoma lutescens*, *Thalassoma lunare*, and *Chrysiptera cyanea* fished in the Coral Sea of Australia (DDBJ/EMBL/GenBank accession nos. AB844443, HM022111, HM022116, and AY302738). Partial 28S rDNA sequences (2893 bp), identical between the two isolates from the two different fish samples in the present study, showed the highest identity (99.72% [724/726]) with that of *K. thalassomi* from *C. japonicus* in Japan. Between different *K. thalassomi* isolates, identities of the 28S rDNA nucleotide sequences ranged between 98.54 (675/685) and 99.85% (684/685).

In the trunk muscle of Carolines parrotfish, African coris, and Pastel ringwrasse, plasmodia containing kudoid spores with seven to nine SV/PC were frequently found. Partial 18S rDNA sequences (1697 bp), identical among the three isolates from the three different fish hosts in the present study, showed 100% nucleotide identity (over 1565-bp or 1610-bp lengths) with *K. igami* from *C. japonicus* and *Paralichthys olivaceus* fished or aquacultured in the Philippine Sea, off Wakayama Prefecture, Japan (accession nos. AB844444 and KX163080). Partial 28S rDNA sequences (2055 bp), identical among the three isolates from the three different fish hosts examined in the present study, showed 100 (724/724) or 99.95% (2009/2010) nucleotide identities with *K. igami* from *C. japonicus* and *P. olivaceus* in Japan (accession nos. AB844447 and KX163081). Although the original description by Shirakashi et al. (2014) characterizes *K. igami* as having five or six SV/PC in spores with their morphometrics shown in Table 5, it is valid to identify the present isolate as *K. igami*.

## Phylogeny of isolated kudoids based on their rDNA and mtDNA

As mentioned above, the spores of all four kudoid species collected in the present study (*K. yasunagai*, *K. miyakoensis* n. sp., *K. thalassomi*, and *K. igami*) showed notable variation in the number of SV/PC. Phylogenetic trees constructed based on the 18S and 28S rDNA of *Kudoa* spp. characterized by spores with more than four SV/PC were constructed (Supplemental Figs. 4 and 5, respectively). The topology of these kudoid species with more than four SV/PC was essentially identical in the two phylogenetic trees based on different rDNA portions, or those including a variety of kudoids with four or more SV/PC (see Figs. 3 and 4 of Sakai et al. 2018). The phylogenetic relationships of four kudoid species with brain tropism, i.e., *K. yasunagai*, *K. chaetodoni*, *K. lemniscati*, and *K. miyakoensis* n. sp., were extremely close due to only a few nucleotide substitutions

(Table 6). These four species showed just one or two nucleotide substitutions over a minimum length of 1432 bp of the 18S rDNA sequences and four to 10 nucleotide substitutions over a minimum length of 637 bp (GU808769) of the 28S rDNA sequences. At least two *K. yasunagai* isolates from *L. vaigiensis* and *L. ehrenbergii* in the Ningaloo Reef, Western Australia showed highly divergent 28S rDNA nucleotide sequences from other isolates (accession nos. JQ026230 and JQ026229; five and 10 nucleotide substitutions compared with *K. yasunagai* isolates from Japan), as shown in Table 6, and their morphometrics were moderately bigger than those of other *K. yasunagai* isolates (Table 2). Apart from these isolates, the four species analyzed here had unique rDNA and appreciable morphological differences.

To supplement the assessment of phylogenetic relationships between different isolates based on the rDNA, nucleotide sequencing of the mtDNA, i.e., *cox-1* and *rns-rnl*, was conducted using primers described in Takeuchi et al. (2016) and Sakai et al. (2018). As their usability had been demonstrated for *K. hexapunctata*, *K. neothunni*, and *K. konishiae*, all having spores with six or seven SV/PC, in a recent report from our laboratory (Sakai et al. 2018), the *cox-1* and *rns-rnl* nucleotide sequences of the newly collected *K. hexapunctata* isolate from a longtail tuna were successfully amplified and sequenced. The same primers for *cox-1* and *rns-rnl* amplified targeted sequences of four and two kudoid species collected in the present study, respectively. Phylogenetic trees based on the *cox-1* and *rns-rnl* nucleotide sequences (Fig. 4) were coincident with the basic topological relationships demonstrated by the rDNA phylogenetic trees. However, they separated over a longer distance between different species due to a higher frequency of nucleotide substitution in the mtDNA compared with the rDNA.

## Description

### *Kudoa miyakoensis* n. sp. (Myxosporea: Multivalvulida) (Figs. 1 and 3, and Table 3)

Small spherical plasmodia, 0.44–1.14 (0.79) mm ($n = 7$) or 0.43–0.85 (0.69) mm ($n = 5$) in diameter, attached to the brain surface. Polysporic and synchronized spore development. Myxospores stellate with seven or eight equal SV/PC in apical view, without SV ornamentation and apical projection. Myxospores with different numbers of SV/PC occur in the same plasmodium, and different plasmodia exhibit variable ratios of spores with seven or eight SV/PC. Round peripheral expansion of SV with slightly visible suture lines between them in apical view. In lateral view, myxospores rounded pyramidal in shape, PC teardrop-like, occupying apical 1/3 of myxospores. Spore bottom convexed and maximum lateral width of spores at 1/5 to 1/4 of spore length upper from bottom. Coils of polar filament not seen in wet preparations. Spores having dimensions of ($n = 20$): width 12.8–15.6 (14.5); thickness 12.3–15.1 (13.6); sutural thickness 10.0–12.8 (11.6); length 8.2–11.3 (9.8); PC length 2.8–4.4 (3.5); and PC width 1.2–1.8 (1.5).

Nucleotide sequences of the 18S to 28S rDNA (5486 and 5497 bp in length), including partial 18S rDNA (1719 bp), 5.8S rDNA (158 bp), and partial 28S rDNA (2843 bp), were obtained for spores in the same plasmodium (DDBJ/EMBL/GenBank accession nos. LC381986 and LC381987). Minor intra-individual nucleotide changes were found in the ITS regions. Partial nucleotide sequences of the mitochondrial gene *cox-1* (437 bp) were similarly obtained (DDBJ/EMBL/GenBank accession no. LC382004).

### Taxonomic summary

Host: *Naso unicornis* (Forsskål, 1775), bluespine unicornfish (Actinopterygii: Perciformes: Acanthuridae).

Locality: Border between Philippine Sea and East China Sea around Miyako Island, Okinawa, Japan.

Site of infection: Plasmodia in the subarachnoid space, attached to the brain surface.

Materials deposited: Hapantotype no. 21429, Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: Two of 38 fish (5.3%) with five or seven plasmodia per host.

Etymology: The species is named after the locality of host fish collection.

### Remarks

Dimensions of myxospores with seven or eight SV/PC of the present new species are evidently bigger than those of *K. yasunagai*, *K. lethrini*, *K. neurophila*, and *K. prunusi* and smaller than those of *K. chaetodoni* and *K. lemniscati* (Tables 2 and 3). Dimensions of PC of *K. miyakoensis* n. sp. are comparable to those of *K. yasunagai* and *K. lethrini*, but bigger than those of *K. neurophila* and *K. prunusi*. Due to distinctly smaller PC dimensions of *K. miyakoensis* n. sp. compared with those of *K. chaetodoni* and *K. lemniscati*, posterior ends of PC do not extend over 50% of spore length in the new species, whereas those of *K. chaetodoni* and *K. lemniscati* extend more than 50% in lateral view. Morphological uniqueness of each *Kudoa* sp. with brain tropism mentioned above is reflected in their 18S and 28S rDNA sequences, with minor but critical nucleotide differences in either rDNA region allowing differentiation of these seven kudoid species (Table 6).

## Discussion

The number of SV/PC per myxospore of kudoids has attracted intense interest from myxosporean researchers as it was thought to be the most critical character for the systematics of myxosporeans (Lom and Dyková 2006). In the past, this

**Table 2** Morphological characteristics of *Kudoa* spp. with brain tropism and smaller spore dimensions[a]

| Species | *K. yasunagai* | *K. yasunagai* | *K. yasunagai* | *K. yasunagai*[b] | *K. yasunagai*[b] | *K. lethrini* | *K. neurophila* | *K. prunusi* |
|---|---|---|---|---|---|---|---|---|
| Host | *Argryosomus japonicas* | *Calotomus japonicas* | *Scolopsis monogramma* | *Liza vaigiensis* | *Lutjanus ehrenbergii* | *Gymnocranius audleyi* | *Seriola lalandi* | *Thunnus orientalis* |
| Locality | Northwestern Pacific Ocean off Kochi, Japan | Northwestern Pacific Ocean off Kochi, Japan | Great Barrier Reef, Queensland, Australia | Ningaloo Reef, Western Australia | Ningaloo Reef, Western Australia | Great Barrier Reef, Queensland, Australia | Great Barrier Reef, Queensland, Australia | Aquacultured in Wakayama Prefecture, Japan (Philippine |
| Reference | Present study | Present study | Burger and Adlard (2010) | Miller and Adlard (2012) | Miller and Adlard (2012) | Burger et al. (2007) | Burger and Adlard (2010) | Meng et al. (2011) |
| Number of examined spores | 20 | 20 | ca. 30 | ca. 30 | ca. 30 | 65 | ca. 30 | 20 |
| Spore width | 10.5–12.3 (11.5) | 10.6–12.8 (11.6) | 10.7–12.3 (11.5) | 11.5–14.6 (13.2) | 11.1–14.5 (12.9) | 10.2–12.4 (11.4) | 8.6–10.3 (9.2) | 8.5–10.3 (9.6) |
| Spore thickness | 8.6–11.2 (10.3) | 8.7–11.6 (10.4) | 9.5–11.1 (10.3) | 11.0–14.0 (12.7) | 11.6–13.8 (12.8) | 9.5–11.9 (10.6) | 6.8–8.0 (7.3) | 6.7–8.6 (7.5) |
| Spore sutural thickness | 7.0–8.6 (8.0) | 7.2–9.0 (8.4) | 7.4–8.6 (8.0) | 7.4–11.0 (8.8) | 6.8–10.2 (9.0) | 9.2–11.5 (10.0) | 7.2–8.6 (7.8) | _[c] |
| Spore length | 6.6–8.4 (7.5) | 7.0–8.5 (7.7) | _[c] | _[c] | _[c] | 7.8–9.3 (8.4) | _[c] | 6.7–8.6 (7.5) |
| Polar capsule length | 3.6–4.8 (4.2) | 3.9–5.0 (4.3) | 2.3–2.7 (2.5)[d] | 2.8–3.9 (3.3)[d] | 2.7–3.5 (3.1)[d] | 2.7–4.4 (3.8) | 2.0–2.6 (2.3) | 2.8–4.1 (3.7) |
| Polar capsule width | 1.6–2.6 (2.1) | 1.8–2.7 (2.2) | 1.4–1.8 (1.6) | 1.7–2.3 (1.9) | 1.6–2.2 (1.9) | 1.7–2.2 (2.0) | 1.4–1.8 (1.6) | 1.7–2.2 (2.0) |
| Variation of SV/PC number[e] | Marginal 4.3% (5) : 95.7% (6) [n=69] | Evident 72% (6) : 26% (7) : 2% (8) [n=50 | Evident 6–7 (ratio not indicated) | Evident 7–8 (ratio not indicated) | Evident 6–7 (ratio not indicated) | Marginal 4.9% (6) : 95.1% (7) [n=?] | Evident 4–5 (ratio not indicated) | Evident 80% (5) : 20% (6) [n=?] |

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

[b] Taxonomic situation of these two "*Kudoa yasunagai*" isolates may be disputable regarding relatively larger spore dimensions and unique 28S rDNA nucleotide sequences. For details, see the text

[c] " –" indicates no available data

[d] Apical measurement, somewhat different from that for other isolates

[e] Ratio of spores with different number of SV/PC indicated in parentheses is shown

**Table 3** Morphological characteristics of *Kudoa* spp. with brain tropism and larger spore dimensions[a]

| Species | *K. miyakoensis* n. sp. | *K. miyakoensis* n. sp. | *K. chaetodoni* | *K. chaetodoni* | *K. lemniscati* |
|---|---|---|---|---|---|
| Host | *Naso unicornis* | *Naso unicornis* | *Chaetodon unimaculatus* | *Casio cuning* | *Lutjanus lemniscatus* |
| Locality | Border between Philippine Sea and East China Sea, off Miyako Is. | Border between Philippine Sea and East China Sea, off Miyako Is. | Great Barrier Reef, Queensland, Australia | Great Barrier Reef, Queensland, Australia | Ningaloo Reef, Western Australia |
| Reference | Present study | Present study | Burger et al. (2007) | Miller and Adlard (2012) | Miller and Adlard (2012) |
| Number of examined spores | 20 | 12 | ca. 30 | ca. 30 | ca. 30 |
| Spore width | 12.8–15.6 (14.5) | 12.2–14.6 (13.6) | 15.6–17.8 (16.8) | 11.7–17.0 (15.3) | 14.1–16.6 (15.7) |
| Spore thickness | 12.3–15.1 (13.6) | 11.2–13.7 (12.6) | 14.2–17.0 (15.9) | 11.5–17.1 (15.1) | 13.1–16.5 (15.3) |
| Spore sutural thickness | 10.0–12.8 (11.6) | 8.8–14.9 (10.8) | 11.2–14.2 (12.4) | 9.6–13.6 (11.8) | 7.4–12.0 (10.0) |
| Spore length | 8.2–11.3 (9.8) | 8.3–10.2 (9.3) | 7.8–10.9 (10.0) | –[b] | 9.1–9.5 (9.3) |
| Polar capsule length | 2.8–4.4 (3.5) | 2.3–4.5 (3.7) | 5.1–7.1 (6.3) | 3.2–4.9 (4.2)[c] | 4.3–5.0 (4.7)[c] |
| Polar capsule width | 1.2–1.8 (1.5) | 1.3–1.7 (1.5) | 2.4–3.2 (2.7) | 1.7–2.5 (2.2) | 1.8–2.6 (2.3) |
| Variation of SV/PC number[d] | Evident | Evident | Evident | Evident | Evident |
| | 37% (7):63% (8) [$n$ = 100] | 62% (7):38% (8) [$n$ = 100] | 1.3% (7):51.3% (8):47.4% (9) [$n$ =?] | 7–8 (ratio not indicated) | 7–8 (ratio not indicated) |

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

[b] "–" indicates no available data

[c] Apical measurement, somewhat different from that for other isolates

[d] Ratio of spores with different number of SV/PC indicated in parentheses is shown

**Table 4** Morphological characteristics of *Kudoa thalassomi* with different origins[a]

| Species | *Kudoa thalassomi* | *Kudoa thalassomi* | *Kudoa thalassomi* | *Kudoa thalassomi* | *Kudoa thalassomi* |
|---|---|---|---|---|---|
| Host | *Naso unicornis* | *Naso unicornis* | *Thalassoma lunare; Moon wrasse* | *Abudefduf bengalensis* | *Calotomus japonicus* |
| Locality | Border between Philippine Sea and East China SEa off Miyako Is., Okinawa, Japan | Border between Philippine Sea and East China SEa off Miyako Is., Okinawa, Japan | Great Barrier Reef, Queensland, Australia | Great Barrier Reef, Queensland, Australia | Philippine Sea, off Wakayama, Japan |
| Reference | Present study | Present study | Adlard et al. (2005) | Burger and Adlard (2011) | Shirakashi et al. (2014) |
| Number of examined spores | 20 | 20 | 30 | 30 | 20 |
| Spore width | 13.0–16.1 (14.1) | 13.0–15.5 (14.2) | 9.5–11.8 (10.7) | 12.4–14.9 (13.7) | 12.1–15.0 (13.7) |
| Spore thickness | —[b] | —[b] | 8.6–10.8 (9.4) | 10.4–12.6 (11.5) | 9.7–13.8 (11.5) |
| Spore sutural thickness | 6.8–10.9 (8.1) | 10.9–13.2 (12.0) | 6.8–8.8 (8.0) | 8.6–10.2 (9.1) | 8.5–10.0 (9.2) |
| Spore length | 6.8–10.9 (8.1) | 7.0–9.8 (8.0) | 6.2–7.1 (6.6) | 6.7–7.8 (7.3) | 6.6–8.9 (7.7) |
| Polar capsule length | 3.2–5.2 (4.3) | 3.3–4.7 (4.0) | 4.7–5.0 (4.9) | 3.9–4.6 (4.3) | 3.2–4.3 (3.9) |
| Polar capsule width | 1.8–2.3 (2.0) | 1.7–2.2 (1.9) | 2.0–2.2 (2.1) | 1.6–2.3 (2.0) | 1.5–2.1 (1.8) |
| Variation of SV/PC number[c] | Evident | No | No | Evident | Evident |
| | 44% (6):56% (7) [$n$ = 100] | 100% (7) [$n$ = 100] | 100% (6) [$n$ = ?] | 6–7 (ratio not indicated) | 29.8% (6):70.2% (7) [$n$ = 84] |

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

[b] "—" indicates no available data

[c] Ratio of spores with different number of SV/PC indicated in parentheses is shown

**Table 5** Morphological characteristics of *Kudoa igami* with different origins[a]

| Species | *Kudoa igami* | *Kudoa igami* | *Kudoa igami* | *Kudoa igami* |
|---|---|---|---|---|
| Host | *Calotomus carolinus* | *Coris gaimard* | *Hologymnosus doliatus* | *Calotomus japonicus* |
| Locality | Border between Philippine Sea and East China Sea off Miyako Is., Okinawa, Japan | Border between Philippine Sea and East China Sea off Miyako Is., Okinawa, Japan | Border between Philippine Sea and East China Sea off Miyako Is., Okinawa, Japan | Philippine Sea, off Wakayama, Japan |
| Reference | Present study | Present study | Present study | Shirakashi et al. (2014) |
| Number of examined spores | 20 | 20 | 20 | 20 |
| Spore width | 9.2–11.9 (10.9) | 10.5–12.1 (11.4) | 10.2–12.7 (11.6) | 9.2–10.8 (9.9) |
| Spore thickness | —[b] | —[b] | —[b] | 8.5–9.6 (9.1) |
| Spore sutural thickness | 8.3–10.8 (9.8) | 8.6–11.0 (10.3) | 9.2–11.3 (10.1) | 7.0–9.1 (7.9) |
| Spore length | 6.0–7.6 (6.6) | 5.6–8.2 (7.1) | 6.1–7.9 (6.8) | 6.2–7.3 (6.5) |
| Polar capsule length | 3.4–4.9 (4.4) | 3.9–5.4 (4.6) | 3.9–5.9 (5.0) | 2.8–4.0 (3.3) |
| Polar capsule width | 1.6–2.4 (1.9) | 1.7–2.2 (2.0) | 1.9–2.5 (2.2) | 1.4–2.1 (1.7) |
| Variation of SV/PC | Evident | Evident | Evident | Evident |
| | A: 8.1% (7):83.0% (8):8.90% (9) [$n = 700$] | A: 2.0% (7):70.0% (8): 28.0% (9) [$n = 100$] | A: 53.8% (7):45.2% (8):1.0% (9) [$n = 400$] | |
| | B: 30.2% (7):69.0% (8):0.8% (9) [$n = 400$] | B: 23.0% (7):69.2% (8):7.8% (9) [$n = 400$] | B: 17% (7):74.5% (8):8.5% (9) [$n = 400$] | 19.0% (5) : 81.0% (6) [n=100] |
| | C: 46.6% (7):49.4% (8):4.0% (9) [$n = 400$] | | C: 6.0% (7):81.2% (8):12.8% (9) [$n = 400$] | |

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

[b] " —" indicates no available data

[c] Ratio of spores with different number of SV/PC indicated in parentheses is shown. Counting of SV/PC number was conducted on 100 spores per plasmodium, thus "$n = 700$" means counting was conducted on seven plasmodia per host fish

Groups A, B, and C mean counting in different fish individuals

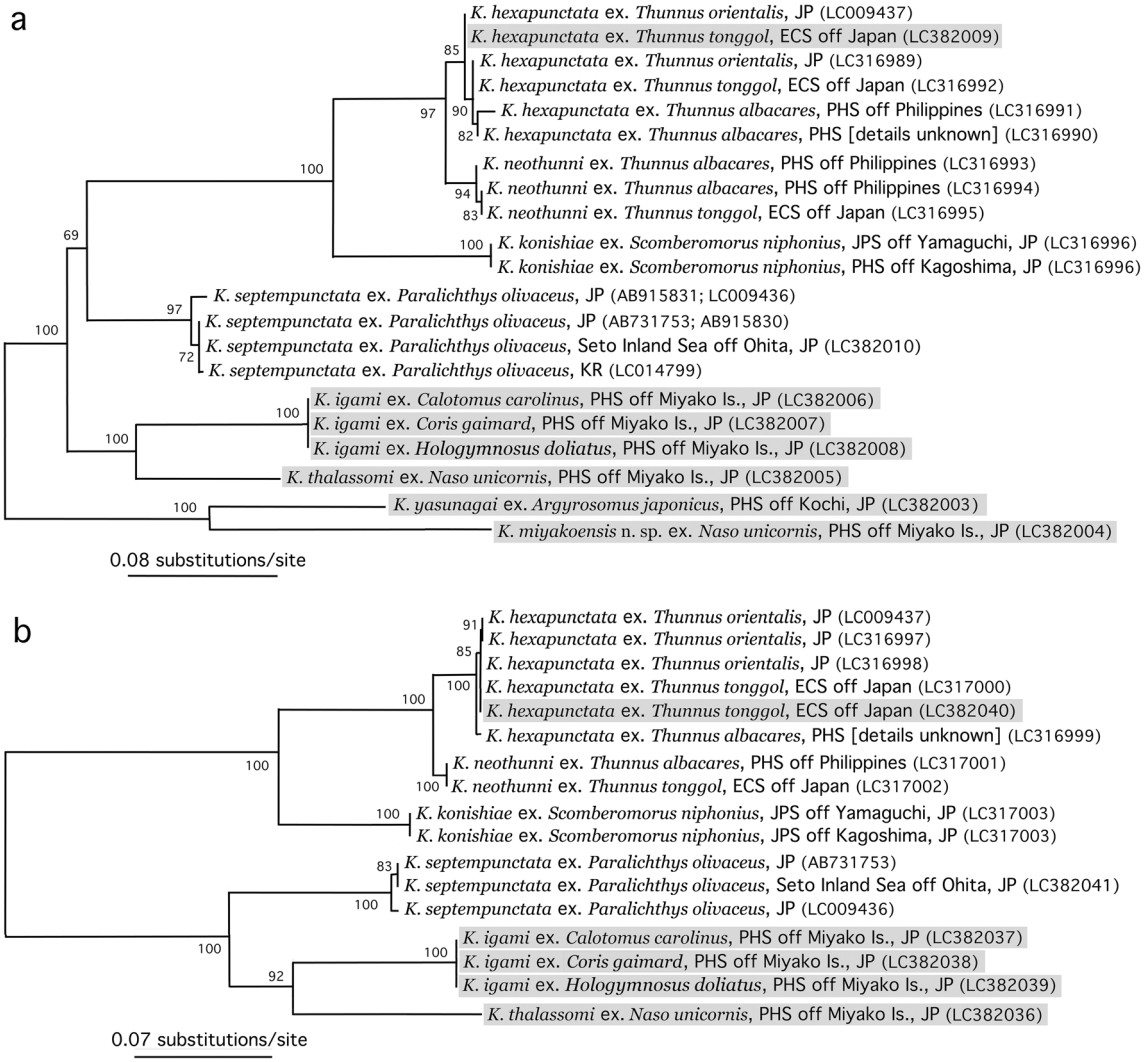


**Fig. 4** Unrooted ML phylogenetic trees based on partial mitochondrial gene sequences (**a**, *cox-1* and **b**, *rnl*) of representative *Kudoa* spp. with five or more SV/PC per myxospore. The species name of the isolates collected in the present study (with gray background) is followed by the name of the fish host, country or sea of collection, and DDBJ/EMBL/GenBank accession number. Abbreviations of country or sea names: AU, Australia; ECS, East China Sea; JP, Japan; JPS, Japan Sea; KR, Korea; and PHS, Philippine Sea

notion was embodied in the erection of *Hexacapsula* Arai et Matsumoto, 1953, *Pentacapsula* Naidenova et Zaika, 1970, and *Septemcapsula* Hsieh et Chen, 1984. When Whipps et al. (2003) described a new myxosporean species with 13 SV/PC, i.e., *K. permulticapsula*, they recognized that the number of SV/PC, particularly four or more, was not associated with the phylogenetic division of myxosporeans with different numbers of SV/PC, i.e., classical genera *Kudoa* (four SV/PC), *Pentacapsula* (five SV/PC), *Hexacapsula* (six SV/PC), and *Septemcapsula* (seven SV/PC). Consequently, Whipps et al. (2004) redefined the genus *Kudoa* as myxosporeans with four or more SV/PC based on phylogenetic analyses of representative species of the aforementioned genera utilizing the 18S rDNA.

Subsequently, Burger and Adlard (2010) demonstrated phenotypical plasticity of a single species by proving the conspecificity of kudoid isolates with different numbers of SV/PC and geographical distributions based on their identical nucleotide sequences of the 18S and 28S rDNA, i.e., *K. yasunagai* with five SV/PC from the brain of sand whiting (*Sillago ciliata*) around Australia and *K. yasunagai* with typically six or seven SV/PC from the same tissue of different fish hosts in the Far East. This situation is very similar to the demonstration of conspecificity of *K. neothunni* with seven SV/PC from the longtail tuna with its original description of six SV/PC in isolates from the yellowfin tuna (Kasai et al. 2017b) or *K. igami* with seven to nine SV/PC with its original description of five or six SV/PC in an isolate from the Japanese parrotfish (present study). Notable plasticity in the number of SV/PC of spores, even within the same plasmodium, has also been reported for *K. septempunctata* (Matsukane et al. 2010; Kasai et al. 2016), *K. thalassomi* (Burger and Adlard 2011), *K. neurophila*,

**Table 6** Nucleotide substitutions observed in partial 18S rDNA (minimum 1432-bp length) and 28S rDNA (minimum 637-bp length)

**A) 18S rDNA**

| Species | DDBJ/EMBL/GenBank accession no. | Host and Locality[a] | Position of nucleotide substitutions[b] 163 | 618 | 640 | 790 | 1641 |
|---|---|---|---|---|---|---|---|
| *Kudoa yasunagai* | | | | | | | |
| | LC316967 | *Argyrosomus japonicus* ; JP | T | C | T | T | G |
| | LC316968 | *Calotomus japonicus* ; JP | • | • | • | • | • |
| | AY302741 | *Paralichthys olivaceus* ; JP | • | • | • | • | • |
| | GU808769 | *Sillago ciliata* ; AU | • | • | • | • | |
| | JQ026226 | *Liza vaigiensis* ; AU | • | • | • | • | |
| | JQ026224 | *Lutjanus ehrenbergii* ; AU | • | • | • | • | |
| *Kudoa chaetodoni* | | | | | | | |
| | JQ026223 | *Lutjanus carponotatus* ; AU | C | • | A | – | – |
| | JQ026218 | *Caesio cuning* ; AU | C | • | A | • | |
| | DQ519387 | *Chaetodon unimaculatus* ; AU | C | • | A | • | |
| *Kudoa lemniscati* | | | | | | | |
| | JQ026222 | *Lutjanus lemniscatus* ; AU | C | T | • | • | • |
| *Kudoa miyakoensis* n. sp. | | | | | | | |
| | LC381986 | *Naso unicornis* ; JP | C | • | • | • | • |

**B) 28S rDNA**

| Species | DDBJ/EMBL/GenBank accession no. | Host and Locality[a] | Position of nucleotide substitutions[c] 133 | 136 | 181 | 447 | 460 | 463 | 468 | 471 | 472 | 479 | 481 | 482 | 518 | 532 | 535 | 558 | 580 | 611 | 667 | 736 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Kudoa yasunagai* | | | | | | | | | | | | | | | | | | | | | | |
| | LC316967 | *Argyrosomus japonicus* ; JP | C | T | A | C | C | C | G | C | A | A | T | G | T | G | C | T | T | G | C | T |
| | LC316968 | *Calotomus japonicus* ; JP | • | • | R | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| | AY302736 | *Paralichthys olivaceus* ; JP | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| | KX163085 | *Seriola lalandi* ; JP | • | • | G | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| | GU808775 | *Sillago ciliata* ; AU | • | • | G | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| | GU808777 | *Scolopsis monogramma* ; AU | • | • | G | • | • | • | • | T | • | • | • | • | • | • | T | • | • | • | T | • |
| | JQ026230 | *Liza vaigiensis* ; AU | T | • | G | • | G | • | • | T | • | • | • | • | • | • | T | • | • | A | • | |
| | JQ026229 | *Lutjanus ehrenbergii* ; AU | T | • | G | T | G | T | A | T | • | • | C | A | • | • | • | • | • | A | T | |
| *Kudoa chaetodoni* | | | | | | | | | | | | | | | | | | | | | | |
| | GU808771 | *Chaetodon unimaculatus* ; AU | • | C | G | • | • | • | • | T | • | • | • | • | C | T | T | • | A | • | T | • |
| | JQ026231 | *Caesio cuning* ; AU | • | C | G | • | • | • | • | T | • | • | • | • | C | T | T | • | A | • | T | |
| *Kudoa lemniscati* | | | | | | | | | | | | | | | | | | | | | | |
| | JQ026232 | *Lutjanus lemniscatus* ; AU | • | • | G | • | • | • | • | T | G | • | • | • | • | T | T | • | • | • | T | |
| *Kudoa miyakoensis* n. sp. | | | | | | | | | | | | | | | | | | | | | | |
| | LC381986 | *Naso unicornis* ; JP | • | C | G | • | • | • | • | T | G | G | • | • | • | T | G | C | A | • | T | C |

[a] Locality is expressed by country name (AU, Australia; JP, Japan)

[b] Nucleotide position relative to the 5′-terminus of the 18S rDNA of *Kudoa yasunagai* (LC316967) is shown. Dots denote an identical base to the uppermost nucleotide sequence, and blanks indicate no available data. "—" indicates nucleotide deletion at the base site. Nucleotide substitutions emphasized by gray background are important ones for specific differentiation

[c] Nucleotide position relative to the 5′-terminus of the 28S rDNA of *Kudoa yasunagai* (LC316967) is shown. For meanings of dots, blanks, "—," and gray background refer to the upper explanation

*K. lethrini*, and *K. lemniscati* (Burger and Adlard 2010), all of which form a well-supported clade with *K. yasunagai* and *K. igami* (Supplemental Figs. 4 and 5).

In addition to phenotypical plasticity, minor genetic differences in the rDNA sequences of kudoids with brain tropism make their specific differentiation challenging. As shown in Table 6, which excluded *K. neurophila*, *K. lethrini*, and *K. prunusi* due to multiple nucleotide substitutions (more than five and 10 nucleotide substitutions compared with the Japanese *K. yasunagai* 18S and 28S rDNA sequences, respectively), the comparison of four species, i.e., *K. yasunagai*, *K. chaetodoni*, *K. miyakoensis* n. sp., and *K. lemniscati*, revealed few (one or two sites) or several (four to 10 sites) nucleotide substitutions in the 18S and 28S rDNA sequences, respectively. The frequency of interspecific nucleotide substitutions in the 18S and 28S rDNA sequences of these four *Kudoa* spp. with brain tropism was less than that of intraspecific nucleotide substitutions of *K. thalassomi*, for which the maximum nucleotide substitutions in the 18S and 28S rDNA were five sites over a 1431-bp length and 10 sites over a 685-bp length, respectively. Apart from different predominant numbers of SV/PC per spore, the apical and lateral views of spores, shape and localization of PC, and morphometric values of spores and PC were also different among the aforementioned brain-infecting *Kudoa* spp. despite their highly similar rDNA nucleotide sequences. A similar taxonomic situation is seen between *K. hexapunctata* and *K. neothunni*, which display highly similar rDNA nucleotide sequences and spore morphologies (Li et al. 2013; Yokoyama et al. 2014; Kasai et al. 2017b). Taxonomic validity of these two species parasitizing muscles of *Thunnus* tunas was demonstrated further by the clustering of distant geographical isolates of each species into distinct sister clades in phylogenetic trees based on the *cox-1* and *rnl* mtDNA (Sakai et al. 2018). The maximum nucleotide identity of *cox-1* between *K. hexapunctata* and *K. neothunni* was 97.32% over a 437-bp length, although the amino acid sequences of these two species were absolutely identical. Between *K. yasunagai* and *K. miyakoensis* n. sp., however, the maximum nucleotide identity of *cox-1* was 83.75% (366/437) and the maximum amino acid identity was 89.04% (130/146), suggesting that the analysis of mtDNA genes provides a clearer specific differentiation of phylogenetically sister kudoids. Further assessment of the applicability of mtDNA nucleotide and amino acid sequences for the clear and reliable specific differentiation of *Kudoa* spp., particularly those with brain tropism, is vital and requires the addition of more species and isolates.

The striking biodiversity of kudoid myxosporeans has become increasingly apparent over the last few decades through the introduction of molecular genetic analyses, particularly nucleotide sequencing of the 18S and 28S rDNA, to their taxonomy. Furthermore, this molecular technology has affirmed the conspecificity of different morphotypes of myxospores, e.g., *K. yasunagai* with five or seven SV/PC, *K. chaetodoni* with eight or nine SV/PC, *K. thalassomi* with six or seven SV/PC, and *K. septempunctata* with six or seven SV/PC (Burger et al. 2007; Burger and Adlard 2010, 2011; Kasai et al. 2016), as well as *K. igami* with six or eight SV/PC as shown in the present study. Therefore, the addition of genetic analyses to the phenotypical characterization of kudoid myxospores is critically important to facilitate their unambiguous specific identification. Nevertheless, appreciable variation in the nucleotide sequences of the 18S and 28S rDNA—genes believed to be relatively consistent and not rapidly evolving in a species—has been reported as discussed previously (Kasai et al. 2017a). As having been demonstrated by Burger and Adlard (2011) or illustrated in Supplemental Fig. 5, nucleotide variations of the rDNA sequences are evident in *K. thalassomi* isolates of different host origins but collected in the same waters (Coral Sea) around Australia. It will not be clear whether a similar genetic complexity of a single species applies to more *Kudoa* spp. until the rDNA of multiple isolates is sequenced instead of only one isolate/a few isolates as is currently the case. We have shown here that certain kudoid myxosporeans with brain tropism display few nucleotide variations in their 18S and 28S rDNA sequences (Table 6), while exhibiting appreciably different phenotypical characters. To understand more fully the taxonomic significance of different degrees of nucleotide variation in the rDNA sequences of kudoids, further nucleotide sequencing of mtDNA genes may provide additional insight. Not only kudoids with more than four SV/PC (Sakai et al. 2018; present study) but also *Kudoa* spp. with four SV/PC, which make up approximately 85% of the genus, should have their mtDNA genes sequenced in the near future.

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