1 Phylogenetic study of the genus *Kudoa* (Myxozoa: Multivalvulida) with a description of

2 *Kudoa rayformis* sp. nov. from the trunk muscle of Pacific sierra *Scomberomorus sierra*

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20 **Abstract**

21 *Kudoa rayformis* n. sp. (Myxozoa; Multivalvulida) was observed in the trunk muscle
22 of Pacific sierra *Scomberomorus sierra* caught off the coast of Tonosi, Panama. The
23 species formed pseudocysts in myofibers and infection was subclinical. The myxospores
24 possessed four polar capsules and spore valves, one of which had a distinct filamentous
25 extension. This unique morphological characteristic of the myxospore validated this as a
26 new species of *Kudoa*. Genetically, *K. rayformis* n. sp. is closest to *K. inornata*, with 98%
27 and 91% similarity in 18S and 28S rDNA, respectively, but its spore shape was clearly
28 distinct. The 18S rDNA and concatenated sequences from *K. rayformis* were used in
29 molecular phylogenetic analyses of kudoids to examine the congruence of phylogeny
30 with infection site tropism, spore morphology and cyst/pseudocyst formation. The
31 results demonstrated that the phenotypic traits were correlated with the phylogeny of
32 Kudoidae, and that the biological features of *K. rayformis* originated from the ancient
33 Kudoidae as exhibited by the non-specific infection site tropism and the ability to infect
34 muscle and form pseudocysts.

35

36 *Keywords:* *Kudoa rayformis*; spore morphology; infection site tropism; cyst;
37 pseudocyst; Bayesian tip-association significance testing

38 1. Introduction

39 *Kudoa* (Meglitsch, 1947) the sole genus in the family Kudoidae, comprises
40 approximately 100 nominal species, making it one of the major genera in Myxozoa (Eiras
41 et al., 2014b). The myxospores of kudoid myxozoans possess four or more valves and
42 polar capsules (to date, a maximum of 13 have been observed) and this distinguishing
43 characteristic separate *Kudoa* from other myxozoans (Whipps et al., 2004). *Kudoa* are
44 predominantly found in marine fish, mostly teleosts, but also found in some
45 elasmobranchs (Gleeson et al., 2010). In general, they display high infection site tropism
46 (Burger et al., 2007). To date, the highest number of *Kudoa* species is found from the
47 skeletal muscle, followed by the central nervous system and heart and other organs such
48 as intestines, ovaries, and gills, can also be target sites for some species (Eiras et al.,
49 2014b; Sato, 2011). Within their preferred organs, *Kudoa* either form cysts or are found
50 as pseudocysts (only in skeletal muscle), depending on the species (Moran et al., 1999).

51 Some *Kudoa* species are considered important pathogens. Unsightly macroscopic
52 cysts in fish musculature (e.g., *Kudoa amamiensis* (Egusa et Nakajima, 1980) in
53 yellowtail *Seriola quinqueradiata*) or post-mortem myoliquefaction in commercially
54 important fish (e.g., *Kudoa thyrsites* (Gilchrist, 1924; Syn. *Chloromyxum thyrsites*) in
55 Atlantic salmon *Salmo salar*) cause economic losses in fisheries and aquaculture

industries (Kent et al., 2001; Lom and Dyková, 2006; Moran et al., 1999; Yokoyama, 2003). In addition, there is growing concern about the potential impact of *Kudoa* on public health (Kawai et al., 2012) and it has led to the importance of identifying *Kudoa* infections in fish for human consumption.

Recent increased attention has resulted in the discovery of new *Kudoa* species and a genetic approach is indispensable for the identification and description of *Kudoa*. Information from large (28S) and small subunit (18S) ribosomal DNA (rDNA) is commonly used for the identification and separation of *Kudoa* species (Burger et al., 2007; Whipps et al., 2004). However, even 28S rDNA, which has a greater resolving power than 18S rDNA, is limited in its usefulness for separating closely related *Kudoa* species (Burger and Adlard, 2010b; Meng et al., 2011). As a result, a growing amount of sequence data is accumulating in databases. However, few phylogenetic studies have assessed the evolution of *Kudoa* spp. and the statistical relationships between their morphological and bionomical characters (Takeuchi et al., 2015).

In the present study, we conducted phylogenetic analyses of *Kudoa* spp. with their sequences available on GenBank aiming to determine the relationship between their phylogenetic position and biological characteristics such as infection site tropism, spore morphotype, and cyst/pseudocyst formation. The analyses include a novel *Kudoa* species

found from the trunk muscle of Pacific sierra *Scomberomorus sierra* captured off the Pacific coast of Panama and we also provide the description of this new *Kudoa*.

2. Materials and methods

2.1. Fish sample

Pacific sierra were caught off the coast of Tonosi, Los Santos Province, Panama (7.36°N, 80.37°W) by lure fishing on December 17, 2014. Fish were kept at 4 °C for a day before checking *Kudoa* infection. Thin slices of muscle tissue were flattened between two glass plates and checked for the presence of pseudocysts under a dissecting scope with transmitted light. When infection was confirmed, the muscle tissues were kept frozen at –20 °C for subsequent morphological and molecular analyses.

2.2. Morphological examination

Pseudocysts were collected from semi-thawed muscle samples using a fine forceps. Wet-mounted preparations of squashed pseudocysts were observed under a light microscope and photographed at 1,000× magnification. Apical and lateral measurements of myxospores were taken from 20 spores each, originated from multiple pseudocysts, using the ImageJ image processing program (available at <http://rsb.info.nih.gov/ij/>). For

spore measurements, we followed the basic guidelines of Adlard et al. (2005) as modified by Yokoyama and Itoh (2005) due to the unequal shape of the spore valves.

2.3. Molecular analysis

DNA was extracted from pseudocysts using QIAamp DNA Mini Kit (QIAGEN Inc., Germany) following the manufacturer's instructions. Portions of 18S and 28S rDNA were amplified by PCR using a combination of primers that we (Kudo-ShinF, Kudo-ShinR, Kudo-ShinRS, Kudo 28S-ShinF, and Kud2400fR) and other groups (18e, ERIB 10, Kt28S1F, NLF1050, NLR1270, Kud2400f, Kud2400fR, 3R, and NLR3284) have designed (Barta et al., 1997; Bartosova et al., 2009; Grabner et al., 2012; Hillis and Dixon, 1991; Van der Auwera et al., 1994; Whipps et al., 2004) (Table 1). Both PCRs were conducted with the following cycling program: initial denaturation of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s or 90 s, and a final extension at 72 °C for 7 min. PCR products were treated with QIAquick Gel Extraction Kit (QIAGEN) to remove excess primers and dNTPs and directly sequenced with BigDye™ Terminator v3.1 in an ABI 3730xl Sequencer.

2.4. Database construction

A synoptic database of *Kudoa* was constructed based on Eiras et al. (2014b) and Sato (2011) with six additional species described since (including the one we describe in the present study). The reported geographical localities of *Kudoa* spp. were plotted in graphs to see their geographical distribution around the globe.

2.5. Phylogenetic analyses

Multiple alignments of each gene sequence were made by Clustal X 2.0 (Larkin et al., 2007) with the homologous sequences of other *Kudoa* species available on the GenBank database. The 18S rDNA sequences and combined matrices constructed by concatenation of the pre-aligned 18S rDNA and 28S rDNA sequences were analyzed separately, and *Enteromyxum leei* (Diamant, Lom, et Dykova, 1994) was used as an outgroup for both data sets. Bayesian inference (BI) was used to reconstruct the phylogenetic tree from datasets containing 56 sequences of the 18S rDNA and 45 sequences of the concatenated sequences from the myxosporean. For BI analysis, nucleotide substitution models were selected using the Bayesian information criterion (BIC) and Akaike information criterion (AIC) as implemented in jModeltest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012) and TVM + I + G and GTR + I + G were chosen as the best-fit nucleotide substitution models for the 18S and concatenated data sets, respectively. The

metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm implemented in MrBayes 3.2.4 (Ronquist et al., 2012) was run for a sufficient number of generations until the average standard deviation of the split frequencies was <0.01 . The sampling frequency was set at every 100 generations for 1,000,000 samples. The first 100,000 samples from each run were discarded as burn-in, and the remaining were analyzed using the “sumt” command in MrBayes. Gaps were treated as missing data. A consensus tree was created using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). The association between the phylogeny and biological traits was tested using a Bayesian MCMC approach implemented in Bayesian tip-association significance testing (BaTS) v0.1.1 (Parker et al., 2008). This program analyzes the posterior set of 9,000 trees generated by MrBayes 3.2.4, accounting for phylogenetic error (Ronquist et al., 2012). The presence of phylogenetic trait (morphological difference, cyst/pseudocyst formation and infection site tropism) correlations was assessed using the parsimony score (PS), association index (AI), and maximum monophyletic clade (MC) size, and the complete list of sequences is given in supplementary data. The null hypothesis of random phylogenetic trait associations was rejected at a significance level of 0.05 (P-value <0.05).

3. Results

3.1. *Kudoa* sp. in Pacific sierra

Long filamentous pseudocysts (2–3 mm long) were detected in the trunk muscle of both Pacific sierra examined (infection rate 2/2) (Fig. 1). The infection appeared to be minor as only a few pseudocysts could be found in a flattened muscle tissue of approximately 3 × 3 cm. The defrosted muscle tissues were not liquefied, but appeared to be softer than normal. All the examined pseudocysts contained myxospores with the same morphological characteristics. The myxospores possessed four polar capsules and spore valves, one of which had a distinct “tail-like” appendage (Fig. 2). Such a unique morphological characteristic of the myxospores provides compelling evidence that this is a new species of *Kudoa*.

3.2. Description

3.2.1. *Kudoa rayformis* n. sp. (Myxozoa: Multivalvulida)

Spores in apical view with bilateral symmetry in sagittal plane only, with four unequal spore valves. Peripheral edge of one valve rounded, those of two others pointed, and final one tapered with filamentous extension. Polar capsule within one valve with extension slightly larger than the other three. Number of coils of polar filament indiscernible. Spores pouch shaped in side view with prominent apical valve projections on apex.

164 Average $\pm$ standard deviation (range) of maximum thickness 8.2 ± 0.6 (7.5–9.2) μm ,
165 minimum thickness 6.7 ± 0.4 (6.1–7.5) μm , width 5.0 ± 0.3 (4.6–5.7) μm , length 5.4 ± 0.2
166 (5.0–5.7) μm . Length of large and small polar capsules in apical view 1.9 ± 0.3 (1.5–2.6)
167 μm and 1.7 ± 0.2 (1.2–2.0) μm , respectively, and width of large and small polar capsules
168 in apical view 1.9 ± 0.2 (1.5–2.5) μm and 1.8 ± 0.3 (1.4–2.5) μm , respectively. Length and
169 width of small polar capsules in lateral view 2.3 ± 0.2 (1.9–2.6) μm and 1.7 ± 0.2
170 (1.4–2.0) μm , respectively (Fig. 3).

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172 3.2.2. Taxonomic summary

173 Host: *Scomberomorus sierra*, Pacific sierra (Perciformes, Scombridae)

174 Locality: Coast of Pedasi, Los Santos Province, Panama

175 Site of infection: Trunk muscle

176 Type material: Syntype specimens (Diff-Quik stained smear of spores), accession
177 number, MPM Coll. No.21010, Meguro Parasitological Museum, Tokyo, Japan.

178 Etymology: The species name is derived from the ray-like form of the spores.

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180 Remarks

There are seven described *Kudoa* species possessing four unequal spore valves and polar capsules that infect the trunk muscle of marine fish: namely *K. cheilodipteri* (Heiniger, Cribb et Adlard, 2013), *K. gunterae* (Burger et Adlard, 2010), *K. lateolabracis* (Yokoyama, Whipps, Kent, Mizuno et Kawakami, 2004), *K. megacapsula* (Yokoyama et Itoh, 2005), *K. minithyrsites* (Whipps, Adlard, Bryant, Lester, Findlay et Kent, 2003), *K. thyrsites*, and *K. whippsi* (Burger et Adlard, 2010). *K. rayformis* n. sp. possesses unique morphological characteristics distinct from those species: a filamentous extension from one spore valve and apical valve projections on the apex. In addition, all the other seven species are stellate or cruciform (*K. megacapsula*) in apical view, whereas *K. rayformis* n. sp. is neither. Filamentous extensions at the peripheral edge of spore valves were also reported from *K. caudate* (Kovaleva et Gayevskaya, 1983), but this species is clearly different from *K. rayformis* n. sp. by having an extension from each of four equal valves and by not having an apical projection. The myxospores of *K. rayformis* n. sp. are smaller than the above listed species, except for those of *K. minithyrsites*, which are nearly equivalent (Table 2).

To date, four *Kudoa* species have been described from the fish hosts belonging to the genus *Scomberomorus*, namely *K. crumena* (Iversen et Van Meter, 1967) from *Scomberomorus maculatus*, *K. scomberomori* (Adlard, Bryant, Whipps et Kent, 2005)

and *K. permulticapsula* (Whipps, Adlard, Bryant et Kent, 2003) from *Scomberomorus commerson*, and *Kudoa* sp. (Eiras, Lima, Cruz et Saraiva, 2014) from *Scomberomorus brasiliensis*. *K. crumena* has quadrate shape spore with four equal polar capsules, form cyst in muscle and considerably larger than *K. rayformis* n. sp (Iversen and Van Meter, 1967). *K. scomberomori* and *K. permulticapsulata* are stellate with six or thirteen polar capsules, respectively (Adlard et al., 2005; Whipps et al., 2003a). These species are clearly different from *K. rayformis* n. sp. in morphology. *Kudoa* sp. isolated from *S. brasiliensis* shares some similarities with *K. rayformis* n. sp. It was found from similar geographical region Brazil, possesses four polar capsules, forms pseudocysts in trunk muscle and are similar in size (width 5.5 μm , thickness 4.3 μm , length 4.2 μm ; Eiras et al., 2014a). The microphotograph of this *Kudoa* sp. resembles with *K. rayformis* n. sp. However, the former species was described as having equal polar capsules and there was no description about a filamentous extension and apical valve projections as seen in *K. rayformis* n. sp. Unfortunately, no genetic information is available for this species.

Genetically, *K. rayformis* n. sp. is most close to *K. inornata* (Dykova, de Buron, Fiala et Roumillat, 2009), with 91% (1518/1659) and 98% (1703/1731) similarity in 28S (accession number FJ790312) and 18S rDNA (accession number FJ790311), respectively. *K. inornata* is also a small species (thickness, 5.9–6.1 μm) with four spore valves and

found as pseudocysts in the trunk muscle of fish, but its spores are quadrate without any extensions or apical projections (Dykova et al., 2009).

3.3. Molecular and phylogenetic analyses

Partial sequences of the 18S rDNA (1,731 bp) and 28S rDNA (3,266 bp) were obtained from *K. rayformis* n. sp. and deposited in GenBank (accession numbers KR140014 and KR140015, respectively), of which 1,567 bp of the 18S rDNA and 2,418 bp of the concatenated sequence of *K. rayformis* sp. n. were used for phylogenetic analyses. Although the tree topologies inferred by BI analysis were slightly different between the 18S and the concatenated sequence data sets in the position of several species, such as *K. islandica* (Kristmundsson et Freeman, 2014), *K. hemiscylli* (Gleeson, Bennett et Adlard, 2010), *K. carcharhini* (Gleeson, Bennett et Adlard, 2010), *K. trifolia* (Holzer, Blasco-Costa, Sarabeev, Ovcharenko et Balbuena, 2006), *K. unicapsula* (Yurakhno, Ovcharenko, Holzer, Sarabeev et Balbuena, 2007), and *K. ogawai* (Yokoyama, Yanagida et Shirakashi, 2012). *K. rayformis* was constantly clustered with *K. inornata*, *K. paniformis* (Kabata et Whitaker, 1981), *K. diana* (Dykova, Avila et Fiala, 2002), and *K. miniauriculata* (Whitaker, Kent et Sakanari, 1996) (Fig. 5 and Fig. 6). Based on the BaTS result, cyst/pseudocyst formation and spore morphotype of the Kudoidae showed a

significant relationship within both phylogenetic trees, whereas the infection site tropism, such as muscle, intestine, and heart, had significant differences in phylogeny constructed by concatenated sequences (Tables 3–5).

3.4. Database analyses

Since the description of *Kudoa quadratum* Thélohan, 1894 (Syn. *Chloromyxum quadratum*) by Thélohan in 1894, the number of *Kudoa* species gradually increased until the 1970s. In early years, species infecting trunk muscle dominated, probably because they were easier to find. The species discovery rate increased after the 1980's, with more species found from organs other than musculature (Eiras et al., 2014b; Sato, 2011). Today, *Kudoa* spp. infecting trunk muscle comprise over 60% (63/103) of known species, followed by species infecting the central nervous system (9%, 9/103) and heart (6%, 6/103) (Sato, 2011; Eiras et al., 2014b; with six additional species, such as *K. islandica*, *K. hexapunctata* (Yokoyama, Suzuki et Shirakashi, 2014), *K. quraishii* (Mansour, Harrath, Abd-Elkader, Alwasel, Abdel-Baki et Al Omar, 2014), *K. saudiensis* (Mansour, Harrath, Abdel-Baki, Al-Quraishy et Al Omar, 2015), *K. orbicularis* (Azevedo, Rocha, Matos, Oliveira, Matos, Al-Quraishy et Casal, 2015) and *K. rayformis*). Although *Kudoa* are cosmopolitan parasites and reported worldwide, remarkably more number of species

have been found from the Pacific Ocean, especially around Japan and Australia, reflecting these countries' higher research interests (Fig. 4). In most regions, the number of species infecting trunk muscle exceed those infecting other organs. However, this is not the case for western Asia and Africa.

4. Discussion

Kudoa rayformis n. sp. described in the present study has unique morphological characteristics. The combination of unequal polar capsules with a filamentous extension at one end, apical projections, and minute spore size is distinct from any other *Kudoa* spp. described to date, except for *Kudoa* sp. in *S. brasiliensis* from Brazil which lacks detail description and molecular information (Table 2). Such morphological characteristics and the results of molecular analyses support our proposal that this is a new species of kudoid myxozoan.

The traditional approach to classifying myxosporeans uses phenotypic differences, such as spore morphology and infection site tropism (Burger et al., 2007; Whipps et al., 2004). Whipps et al. (2004) suggested original paradigm for the quest for correlative and uniting characters in Kudoidae and Burger et al., (2007) compared host specificity, geography and tissue tropism to a genetic phylogeny. We also investigated the correlation

271 between biological features and phylogeny through 18S rRNA and concatenated
272 sequences including *K. rayformis* n. sp. Although our statistical analysis did not show a
273 correlation between the infection site tropism (except for the brain and ovary) and
274 concatenated sequence phylogeny, the BaTS result suggested that cyst/pseudocyst
275 formation, infection site tropism, and spore morphotype are important factors for
276 identifying and classifying Kudoidae (Tables 3–5). Previous study (Burger et al., 2007)
277 suggested the possibility that the infection site tropism of some Kudoids correlates with
278 genetic relatedness and we explained the correlation by utilizing concept of specific
279 infection-site tropism (SIT) and non-specific infection site tropism (NSIT). The
280 Kudoidae were classified into two groups according to the infection-site tropism. The
281 first group showed SIT for specific organs, such as trunk muscle or the heart. The second
282 group showed NSIT and infects various organs including trunk muscle, intestines,
283 ovaries, the brain, and the esophagus. A relationship between the biological traits and
284 phylogenetic clusters was also observed in another myxosporean group, Myxobolidae
285 (Shin et al., 2014). In Myxobolidae, the existence of SIT/NSIT groups were observed
286 only for the species infecting cyprinid hosts. However, in Kudoidae, this grouping was
287 not restricted to any particular host group. *K. rayformis* n. sp. was clustered with two

other *Kudoa* spp. (*K. inornata* and *K. paniformis*), and this clade was placed in the NSIT group.

The present study explains the correlation between spore morphotypes and SIT/NSIT grouping although previous studies suggested the spore valve number did not correlate with genetic relatedness within the Kudoidae (Burger et al., 2007; Whipps et al., 2004).

The myxospores of *Kudoa* spp. in the SIT group typically have a quadrate shape (Fig. 5). However, even within the SIT group, those infecting the heart possess narrower polar capsules than those infecting trunk muscle (Blaylock et al., 2004; Mansour et al., 2014).

A similar pattern was also observed in the SIT *Myxobolus* spp. from cyprinids (Shin et al., 2014). It may be that the changes in polar capsule shape occurred within the SIT group species during the adaptation to other infection sites. Interestingly, the NSIT group seemed to have more diverse spore morphotypes, such as a stellate shape with one larger shell valve or multi-shell valve and quadrate equal valves. In addition, some species in NSIT group with four spore valves possess posterior projection (*K. quadricornis* (Whipps, Adlard, Bryant et Kent, 2003) and *K. paraquadricornis* (Burger et Adlard, 2010)) or filamentous extension (*K. rayformis* n. sp.). Such morphological plasticity of Kudoidae may be a result of adaptation to a specific mode of transmission or life cycle (Kodadkova et al., 2015; Liu et al., 2010). For example, the posterior projection

306 appendages enlarge the spore surface and may induce better floatation, thus facilitating
307 dispersion in the water. The caudal filament of *K. rayformis* n. sp. may also help
308 transmission to the invertebrate host, although the mode of transmission is still unknown
309 in any *Kudoa* spp. The *Kudoa* spp. with five or more shell valves and polar capsules were
310 clustered together, and this was supported strongly in the concatenated phylogenetic tree
311 (Table 4 and Fig. 6). The phylogenetic analyses also revealed that the spore morphotypes
312 reflect the genetic relationships among *Kudoa* spp. However, more information and
313 studies are required to understand and discuss why some *Kudoa* spp. have evolved to
314 possess more shell valves and polar capsules.

315 Infections of *Kudoa* spp. are associated with either cyst or pseudocyst formation.
316 Moran et al. (1999) discussed the confusion in terminology of ‘cyst’ and ‘pseudocyst’.
317 According to them, the term “pseudocyst” should only be used for species that localize
318 within the host’s muscle fiber and lack a host cell envelope. We classified the *Kudoa* spp.
319 according to this standard and demonstrated the relationship between cyst/pseudocyst
320 formation and phylogeny by a test of statistical significance. Based on the result, we
321 found the *Kudoa* spp. forming pseudocysts were mainly classified in the NSIT group
322 (Table 5 and Fig 6). The *Kudoa* spp. known to cause postmortem myoliquefaction,
323 namely *K. lateolabracis*, *K. thyrsites*, *K. megacapsula*, *K. neothunni* (Arai et Matsumoto,

1953; Syn. *Hexacapsula neothuni*), *K. islandica*, *K. rosenbuschi* (Gelormini, 1944; Syn. *Chloromyxum rosenbuschi*), and *K. paniformis* form pseudocysts in trunk muscle and are also classified in the NSIT group (Abollo et al., 2005; Funk et al., 2008; Kristmundsson and Freeman, 2014; Yokoyama et al., 2004; Yokoyama et al., 2014; Yokoyama and Itoh, 2005). However, it is difficult to assert an absolute correlation between pseudocyst formation and postmortem myoliquefaction because there are *Kudoa* spp. that do not induce myoliquefaction even though they form a pseudocyst, such as *K. hexapunctata* and *K. septempunctata* (Matsukane, Sato, Tanaka, Kamata et Sugita-Konishi, 2010) (Matsukane et al., 2010; Yokoyama et al., 2014). Nevertheless, it is speculated that the alterations in infection site tropism, cyst/pseudocyst formation, and spore morphology appeared during the course of adaptation between the parasites and hosts, and the NSIT group appeared to have more variations in adaption than the SIT group. Based on this study, we speculate that the ancestor Kudoidae had quadrate spores, infected trunk muscle, and formed cysts. In addition, the biological characteristics of *K. rayformis* originated from an ancient NSIT Kudoidae.

The recent increase in the rate of descriptions of *Kudoa* spp. and the low number of species described from certain regions suggest that there are numerous *Kudoa* species yet to be discovered. Many undescribed *Kudoa* spp. may have a significant impact not only

on fisheries industries but also on human health and there have been relatively few *Kudoa* species described from South America, Africa, and Europe. With the aid of advanced technologies for detection and the accumulating knowledge on these microscopic parasites, finding new *Kudoa* species worldwide will provide important information about the general biology of this unique group of parasite and also their effects on fisheries and human health. In addition, the phylogenetic analysis methods used in this study can be applied to reveal the relationship between phenotypic traits and the phylogeny of other myxosporean species in order to provide important information on the genetic relationships between infection site tropism, the formation of cysts and pseudocysts, and spore morphology.

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Figure1

Figure2

Figure3

Figure4

Figure5

SIT

NSIT

Figure 6

Table 1

Oligonucleotide primers used for molecular analysis in this study.

Target	Name	Sequence (5'-3')	Position	Reference
18S rRNA	18e	CTGGTTGATCCTGCCAGT	3	Hillis and Dixon, 1991
	ERIB10	CTTCCGCAGGTTACCTA	1733	Barta et al., 1997
	Kudo-ShinF	AGGAAGGCAGCAGGCG	350	This study
	Kudo-ShinR	CACCAACTAAGAACGGCCAT	1100	This study
	Kudo-ShinRS	ATGGCCGTTCTTAGTTGGTG	1100	This study
28S rRNA	Kt28S1F	CAAGACTACCTGCTGAAC	15	Whipps et al., 2004
	NLF 1050	AATCGAACCATCTAGTAGCTGG	1050	Bartosova et al., 2009
	NLR 1270	TTCATCCCGCATCGCCAGTTC	1270	Bartosova et al., 2009
	Kudo 28S-ShinF	CTACTGCAGTGTCCGCATTA	1400	This study
	Kud2400f	CTTTACGAGTGGCAAGCATC	2400	Grabner et al., 2012
	Kud2400fR	GATGCTTGCCACTCGTAAAG	2400	This study
	3R	GAGCACTGGGCAGAAATC	2490	Whipps et al., 2004
	NLR 3284	TTCTGACTTAGAGGCGTTCAG	3550	Van der Auwera et al., 1994

Table 2

Spore measurements (in μm : mean $\pm$ standard deviation, when available, and range in parentheses) of *Kudoa rayformis* n. sp. and other *Kudoa* spp. with four unequal polar capsules reported from the trunk muscle of marine fishes.

<i>Kudoa</i> species (reference)	Reported host	Spore				Small polar capsule				Large polar capsule	
		T-max	T-min	Width	Length	Apical view		Lateral view		Apical view	
						Length	Width	Length	Width	Length	Width
<i>K. rayformis</i> n. sp. (Present study)	<i>Scomberomorus sierra</i>	8.2 $\pm$ 0.6 (7.5-9.2)	6.7 $\pm$ 0.4 (6.1-7.5)	5.0 $\pm$ 0.3 (4.6-5.7)	5.4 $\pm$ 0.2 (5.0-5.7)	1.7 $\pm$ 0.2 (1.2-2.0)	1.8 $\pm$ 0.3 (1.4-2.5)	2.3 $\pm$ 0.2 (1.9-2.6)	1.7 $\pm$ 0.2 (1.4-2.0)	1.9 $\pm$ 0.3 (1.5-2.6)	1.9 $\pm$ 0.2 (1.5-2.5)
<i>K. cheilodipteri</i> (Heiniger et al., 2013)	<i>Cheilodipterus quinquelineatus</i> <i>Ostorhynchus cyanosoma</i> <i>O. aureus</i>	10.1 $\pm$ 0.9 (8.3-11.7)		6.8 $\pm$ 0.3 (5.9-7.4)	6.1 $\pm$ 0.5 (4.9-7)		2.2 $\pm$ 0.2 (1.7-2.6)			4.5 $\pm$ 0.5 (3.5-5.4)	3.1 $\pm$ 0.3 (2.2-3.6)
<i>K. gunterae</i> (Burger and Adlard, 2010a)	Various Pomacentridae <i>Apogon properuptus</i>	10 $\pm$ 0.37 (9.3-10.5)		5.62 $\pm$ 0.15 (5.4-5.9)	5.42 $\pm$ 0.19 (5.2-6.0)		1.18 $\pm$ 0.03 (1.1-1.3)	2.44 $\pm$ 0.22 (2.1-3.0)		2.59 $\pm$ 0.13 (2.3-2.9)	2.4 $\pm$ 0.12 (2.2-2.7)
<i>K. lateolabracis</i> (Yokoyama et al., 2004)	<i>Lateolabrax</i> sp. <i>Paralichthys olivaceus</i>	11.5 (9.6- 12.9)			6.4 (5.4-6.9)	3.6 (3.0-4.0)	1.9 (0.5-2.0)			5.2 (4.0-5.9)	2.8 (2.5- 3.5)
<i>K. megacapsula</i> (Yokoyama and Itoh, 2005)	<i>Sphyræna pinguis</i> <i>Seriola quinqueradiata</i>	40.0 (30.3- 44.7)	30.9 (25.6- 34.9)	12.0 (11.0- 12.9)	13.4 (9.3- 15.4)	2.8 (1.5-4.4)	1.2 (1.0-1.5)			12.7 (11.7- 14.2)	5.8 (4.9-6.8)
<i>K. minithyrsites</i> (Wipps et al., 2003b)	<i>Pempheris ypsilychnus</i>	8.70 (7.79- 9.91)		6.36 (5.84- 6.75)	4.69 (4.31- 5.36)	2.15 (1.94- 2.34)	1.25 (1.05- 1.49)			3.63 (3.20- 3.83)	1.99 (1.33- 2.31)
<i>K. thyrsites</i> (Yokoyama and Itoh, 2005)	Various marine fish	12.6 (11.7- 14.6)			6.8 (5.6-7.3)	2.0 (1.5-2.7)	1.3 (1.0-1.7)			4.6 (3.2-6.8)	2.6 (2.2- 3.2)
<i>K. whippsi</i> (Burger and Adlard, 2010a)	Various marine fish	10.55 $\pm$ 0.44 (9.5-11.4)		5.77 $\pm$ 0.22 (5.3-6.2)	5.67 $\pm$ 0.23 (5.3-6.2)		1.63 $\pm$ 0.12 (1.4-2.0)	3.18 $\pm$ 0.25 (2.8-3.7)		3.35 $\pm$ 0.29 (2.8-4.0)	2.75 $\pm$ 0.1 7 (2.4-3.1)

Significant associations between 18S rDNA/concatenated sequence phylogeny and infection sites identified by BaTS testing.

Statistic	Observed mean	Lower 95% CI	Upper 95% CI	Null mean	Lower 95% CI	Upper 95% CI	Significance
AI	0.073/0.296	0.038/0.273	0.145/0.338	3.347/2.683	2.629/1.928	4.08/3.31	0.000/0.000
PS	8.003/8.000	8.000/8.0	8.000/8.000	19.455/15.453	18.000/14.000	20.00/16.000	0.000/0.000
MC(Muscle)	12.224/7.000	7.000/7.0	13.000/7.00	4.365/4.120	2.763/2.127	6.735/8.0	0.003/0.073
MC (Intestine)	2.000/2.000	2.000/2.0	2.000/2.000	1.083/1.139	1.000/1.000	1.923/2.0	0.040/0.103
MC (Ovary)	3.000/2.000	3.000/2.0	3.000/2.000	1.029/1.017	1.000/1.000	1.012/1.0	0.001/0.015
MC (Brain)	5.997/6.000	6.000/6.0	6.000/6.000	1.169/1.210	1.000/1.000	2.000/2.0	0.001/0.001
MC (Heart)	4.000/1.000	4.000/1.0	4.000/1.000	1.073/1.000	1.000/1.000	1.923/1.0	0.001/1.000
MC (Esophagus)	1.000/1.000	1.000/1.0	1.000/1.000	1.000/1.000	1.000/1.000	1.000/1.0	1.000/1.000
MC (Systemic infection)	1.000/1.000	1.000/1.0	1.000/1.000	1.000/1.000	1.000/1.000	1.000/1.0	1.000/1.000

Significant associations between 18S rDNA/concatenated sequence phylogeny and spore morphotypes identified by BaTS testing.

Table 5.

Significant associations between 18S rDNA/concatenated sequence phylogeny and cyst-pseudocyst formation identified by BaTS testing.

Statistic	Observed mean	Lower 95% CI	Upper 95% CI	Null mean	Lower 95% CI	Upper 95% CI	Significance
AI	0.066 /0.295	0.036/0.27 2	0.092/0.3 37	3.039/2.5 14	2.099/1.70 0	4.001/3.3 24	0.000/0.000
PS	7.978/6.59 6	7.000/6.00 0	9.000/7.0 00	19.558/15 .870	16.291/12. 992	22.718/18 .913	0.000/0.000
MC (Cyst)	11.000/8.0 01	11.000/8.0 00	11.000/8. 000	3.168/3.0 23	2.002/2.00 0	5.111/5.0 00	0.001/0.011
MC (Pseudocyst)	12.203/5.0 01	7.000/5.00 0	9.000/5.0 00	3.037/2.7 07	2.001/2.00 0	4.937/4.0 00	0.001/0.035
MC (Nocyst)	1.000/1.00 0	1.000/1.00 0	1.000/1.0 00	1.000/1.0 00	1.000/1.00 0	1.000/1.0 00	1.000/1.000