

Patterns of relatedness in the Kudoidae with descriptions of *Kudoa chaetodoni* n. sp. and *K. lethrini* n. sp. (Myxosporea: Multivalvulida)

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SUMMARY

Two morphologically novel *Kudoa* species are characterized from brain tissue of fish, *Kudoa chaetodoni* n. sp. from *Chaetodon unimaculatus* (Chaetodontidae) and *Kudoa lethrini* n. sp. from *Gymnocranius audleyi* and *Lethrinus harak* (Lethrinidae). Additionally we characterized a 5-spore valve (SV) *Kudoa* species from the brain of *Sillago ciliata* (Sillaginidae). Intriguingly, its 18S rDNA sequence was identical to that of the 7 SV *Kudoa yasunagai* extracted from the brain of a paralichthyid halibut in Japan. These 2 species may either prove to be con-specific, even though morphology and distribution differ, or demonstrate the limit of specific resolution in the small subunit rDNA gene region. Small subunit rDNA sequences from these new species were used in molecular phylogenetic analyses of kudoids to examine congruence of phylogeny with tissue tropism, geographical distribution, and host specificity. There was significant correlation between tissue tropism in the form of well-supported brain and heart-infecting clades. Host specificity and geographical distribution showed some correlations with genotype.

Key words: Myxosporea, Kudoidae, *Kudoa*, phylogeny, tissue tropism, geographical distribution, host specificity.

INTRODUCTION

The Kudoidae Meglitsch, 1960 is a family of myxosporean, spore-forming parasites that infect a large range of fishes. They are most widely known for their impacts on meat quality in aquaculture caused by post-mortem myoliquefaction. Myxosporeans are morphologically simple organisms that have few distinctive features on which to characterize species. Families within the Multivalvulida Shulman, 1959 were traditionally distinguished by the number of polar capsules and spore valves. However, Whipps *et al.* (2004) found that phylogenetic analysis of 18S and 28S rDNA of myxosporeans within the Multivalvulida showed that clades were not consistent in the number of spore valves. The analysis found that species of the families Pentacapsulidae Naidenova & Zaika, 1970, Hexacapsulidae Shulman, 1959 and Septemcapsulidae Hsieh & Chen, 1984 all fell within the Kudoidae. In order to maintain monophyly of *Kudoa* Meglitsch, 1947, they proposed that the related families found within the *Kudoa* clade should be synonymized with Kudoidae and that the diagnosis of the genus *Kudoa* should be

amended to accommodate myxosporeans possessing 4 or more polar capsules. Presently there are only 2 genetically monophyletic families in the Multivalvulida, Trilosporidae Shulman 1959 with 3 genera, *Trilospora* Noble, 1939, *Trilosporoides* Koie, 2005, and *Uncapsula* Davis, 1924, and the Kudoidae with a single genus, *Kudoa*.

As a result of these taxonomic changes, *Kudoa* contains approximately 69 species reported globally from a broad range of fish families and tissue sites. At present there is no real understanding of the mechanisms that have underpinned this distribution. The goal of this study is to explore factors that may correlate with patterns of genetic relatedness. In so doing we propose 2 new species of *Kudoa*, *K. chaetodoni* n. sp. and *K. lethrini* n. sp. and characterize a third, *Kudoa* sp. from *Sillago ciliata* (Fig. 1), all isolated from the brain of their hosts. At this stage, we are not formally proposing *Kudoa* sp. as new because the 18S rDNA sequence data from this species has 100% homology with another already characterized species, *Kudoa yasunagai* Hsieh & Chen, 1984, despite significant morphological differences.

Recognizing patterns of morphological and biological characters that match molecular relationships will increase our understanding of the biology of these organisms and make identification of species, and control strategies (if warranted), more practical

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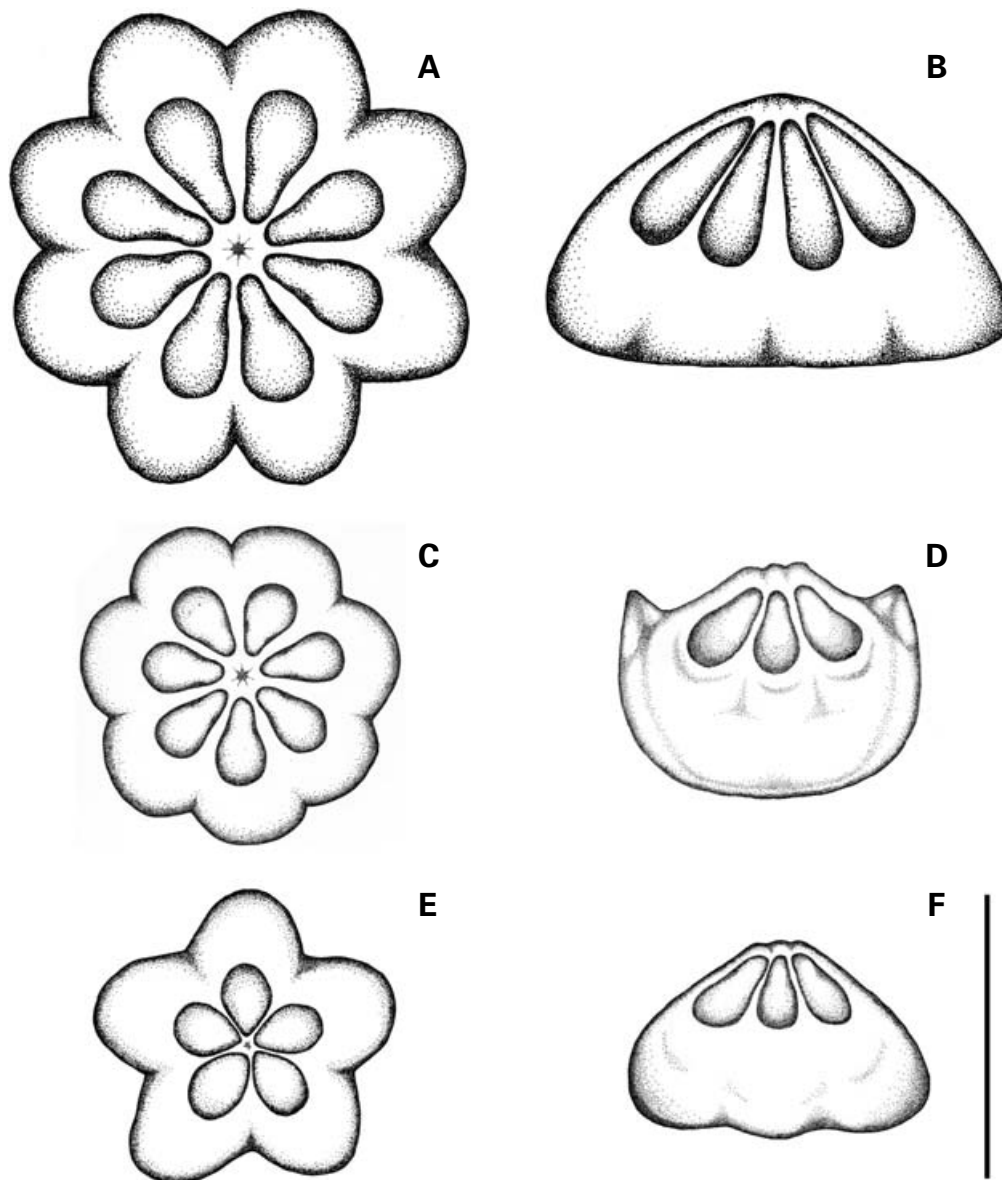


Fig. 1. Stylized diagrams of new species in apical and lateral view. (A and B) *Kudoa chaetodoni* n. sp.; (C and D) *Kudoa lethrini* n. sp.; (E and F) *Kudoa* sp. from *Sillago ciliata*. Scale bar = 10 μ m.

than relying solely on molecular data. Tissue tropism, geographical distribution, and host-specificity are evaluated in this study to determine whether a combination of these biological characteristics together with morphology can characterize the clades inferred from molecular phylogenetic analysis.

MATERIALS AND METHODS

Collection and dissection

Teleost fish were collected from 3 locations along the Queensland coastline of Australia: North Stradbroke Island, Heron Island, and Lizard Island. Fish were collected either by speargun, seine net, or by line fishing then examined using routine necropsy procedures for myxosporean parasites.

Fish were euthanized by immersion in clove oil or by pithing. To obtain brain tissue for examination, a cut was made along the dorsal surface of the mid-sagittal section starting from the premaxillary region to the end of the cranium. The cranium was prized open and the brain removed. A subsample (approximately 3 mm³) of brain tissue was placed on a glass microscope slide with a drop of saline, macerated with scalpel blades and covered with a glass cover-slip. Subsamples of gut wall cysts were also examined. When the tissue was found to contain myxosporean spores by observation using light microscopy at 400 \times magnification, remaining tissue was preserved by freezing (for morphological analysis), in 90% ethanol (for DNA extraction), and in 10% neutral buffered formalin (for histology). Where material was limited, priority went to frozen

samples from which both morphological information and DNA could be recovered.

Histology

Tissue samples preserved in 10% formalin were sectioned using standard histological methods. Alternating slides were stained either with haematoxylin and eosin (H & E) or Giemsa and eosin.

The slides were examined using compound light microscopy at 100 \times , 200 \times , and 400 \times magnification. Digital photographs of myxosporean spores were taken at all magnifications using a Panasonic Wv-CP610 CCD camera attached through a capture device to Snappy Video Snapshot (Play Inc.) image capture software.

Voucher specimens of *Kudoa ciliatae* (Giemsa and Eosin and H & E-stained histological sections) were deposited to the Queensland Museum, South Brisbane, Australia with Accession numbers G464887–G464894.

Morphological analysis of brain myxosporeans

Morphological measurements of spores from fresh and frozen material followed the guidelines proposed by Lom and Arthur (1989) for species descriptions of Myxosporidia. Additional measurements were required to fully characterize spores, including spores with an uneven number of spore valves (see Fig. 2). Where a significant number of spores showed plasticity in valve number, measurements were made for each morphotype present. Length (lateral) measurements were taken from all spores present since valve number could not be determined from this elevation. A minimum of 30 measurements of each character was taken from digital images of spores at 400 \times magnification calibrated against a stage micrometer slide. An additional 30 measurements were taken from formalin-preserved spores of the *Kudoa* sp. from *Sillago ciliata* and used for comparison with morphometrics provided by Hsieh and Chen (1984) for *Kudoa yasunagai*. The ratio of the different morphotypes in each sample was noted.

18S rDNA sequencing

DNA of *Kudoa* spp. was extracted from 5 fish host tissues: *Chaetodon unimaculatus* Bloch, 1787 brain, *Gymnocranius audleyi* Ogilby, 1916 brain, *Lethrinus harak* Forsskål, 1775 brain, *Sillago ciliata* Cuvier, 1829 and *S. maculata* Quoy & Caimard, 1824 brain, and *S. ciliata* gut wall cyst. The gut wall cyst contained spores identified as *Kudoa ciliatae* and was included for sequencing because the available 18S rDNA sequence on GenBank was much shorter (872 base pairs) than the 1580 average length of 18S rDNA from other myxosporean species and thus would not allow robust sequence analyses.

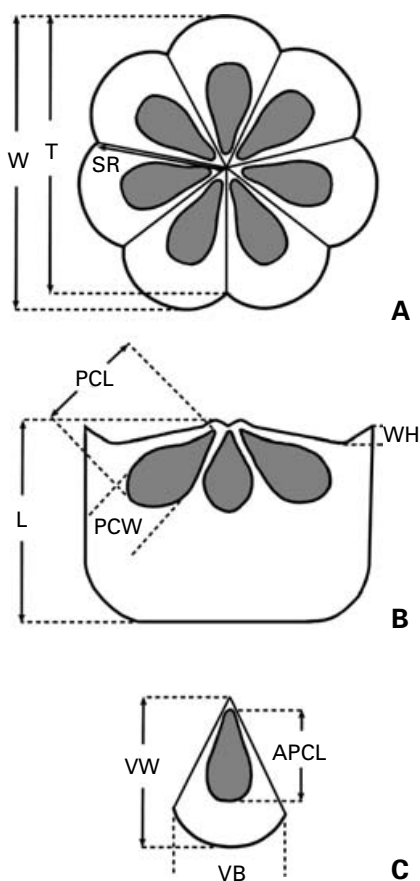


Fig. 2. Schematic diagram of spore measurements. (A) Apical view of spore with uneven number of valves: W, width; T, thickness; SR, suture radius. (B) Lateral view of spore with lateral apical extensions: L, length; PCL, polar capsule length; PCW, polar capsule width; WH, wing height. (C) Valve measurements: VW, valve length; VB, valve breadth; APCL, apical polar capsule length.

DNA was extracted from 2 mm³ sections of tissues using QIAgen DNeasy Kit (QIAGEN Inc., Valencia, California) as per manufacturer's instructions. Myxosporean 18S ribosomal DNA (rDNA) was amplified using PCR primers specific to 18S rDNA sequences conserved among members of the Order Multivalvulida and universal primers (Table 1). PCR fragments were amplified using primer combinations: 18e-Kud1r and Kud6F-18g. Reaction mixtures (25 μ l) were made up of 15.9 μ l of autoclaved Millipore water, 2.5 μ l of 10 \times Hotmaster Taq Buffer (Eppendorf, Hamburg, Germany), 2.0 μ l of 10 mM dNTPs, 1.25 μ l of 10 μ M forward primer, 1.25 μ l of 10 μ M reverse primer, 0.1 μ l of 5U Hotmaster Taq (Eppendorf) and 2 μ l of template DNA (undetermined concentration as contains both host and myxosporean DNA). PCR reactions were performed in a cp2-01 thermocycler (Corbett Research, Australia) with an initial denaturation at 94 $^{\circ}$ C for 2 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 20 sec, annealing at 50 $^{\circ}$ C for 20 sec and extension at 65 $^{\circ}$ C for 60 sec; with a final extension at 65 $^{\circ}$ C for 10 min.

Table 1. Primer sequences and positions used to amplify/sequence 18S ribosomal DNA

Primer	Source	Primer sequence	Position*
18e	Hillis and Dixon (1991)	CTG GTT GAT CCT GCC AGT	1
Mbseq3r	This study	CCT TGG TGA GCC ACT ACC TC	290
Kud6F	Whipps <i>et al.</i> (2003a)	TCA CTA TCG GAA TGA ACG	480
NgKud1r	Gunter <i>et al.</i> (2006)	GCG TTT TAC CGC AAC AAT TT	566
Mbseq2r	This study	CGC CTG CTT TGA GCA CTG TGC	719
Mbseq1ar	This study	CTT CGA ACC TCC AAC TTT CG	927
Mbseq1r	This study	CAA TCC TAT CAA TGT CTG GAC CTG	1154
Kud1r	Whipps <i>et al.</i> (2003a)	CAG ACC TGT TAT TGC CAC	1353
18g	Hillis and Dixon (1991)	GGT AGT AGC GAC GGG CGG CGT G	1585

* Relative to 18e.

Twenty μ l of each of the PCR products were isolated on a 1.4% agarose TA gel stained with 0.2 μ g/ml ethidium bromide run at 70 volts for 1 h.

Amplified PCR products were cut from the gel and purified using the Perfectprep[®] Gel Cleanup Kit (Eppendorf).

Sequencing reactions were performed in both directions following standard sequencing protocol for ABI BigDye[®] Terminator (Applied Biosystems) as described by Gunter *et al.* (2006).

Additional sequencing primers (see Table 1) were designed for this study based on conserved regions of already acquired sequence data using Primer3 (Rozen and Skalersky, 2000).

Phylogenetic analysis

DNA sequence information was edited using BioEdit (Hall, 1999) and aligned with other *Kudoa* 18S sequences available from Genbank (see Table 2) using ClustalW (Thompson *et al.* 1994). The alignment was then checked and adjusted by eye.

Unicapsula sp., a multivalvulidan in the family Trilosporidae was used as an outgroup as it represents the closest known relative external to the Kudoidae.

Neighbour joining, parsimony, and maximum likelihood analyses were conducted using PAUP* 4.0b10 (Swofford, 2002) and Bayesian analysis conducted using Mr Bayes 3.0B4 (Heulsenbeck and Ronquist, 2001). Neighbour-joining and parsimony analyses were given bootstrap confidence values based on 1000 replicates. Parsimony analysis employed a heuristic search with 50 repetitions of random sequence addition and tree bisection and reconnection branch swapping.

Optimum evolutionary models were used for maximum likelihood analyses as determined by Modeltest 3.7 (Posada and Crandall, 1998). Two models were utilized: GTR+I+G as determined by Akaike information criterion analyses of the sequence data and (TrN+I+G) selected by hierarchical likelihood ratio tests.

Bayesian analysis was conducted with 2 million generations of Markov chain Monte Carlo analysis, a set at 4 simultaneous chains with a burnin of 3000 trees and saving current trees to file every 100 generations.

RESULTS

Characterization of brain myxosporean species

Phylum Myxozoa

Class Myxosporea

Order Multivalvulida

Family Kudoidae Meglitsch, 1960

Genus *Kudoa* Meglitsch, 1947

Kudoa chaetodoni n. sp.

Type host: *Chaetodon unimaculatus* Bloch, 1787, Teardrop butterflyfish (Teleostei, Chaetodontidae) adult.

Location in the host: Brain. Pseudocysts sometimes visible as amorphous areas (up to 1 mm in diameter) that were paler than surrounding brain tissue.

Type locality: Turtle Beach (14° 39'S; 145° 27'E), Lizard Island, Great Barrier Reef, Queensland, Australia.

Type Material: Hapantotype G464863 (Giemsa and eosin-stained histological section) deposited in the Queensland Museum, South Brisbane, Australia.

Etymology: specific name refers to the genus of host fish in which it was found.

Prevalence: One of 7 (1 of 4, Lizard Island; none of 3 from North Wistari Reef, Capricorn-Bunker Group).

Description. *Spores*. (Figs 1A, B, 3A–D). Spores stellate in apical view, radiating from centre with 7–9 equal shell valves and polar capsules (in hapantotype, 8 SV spores slightly dominate 9 SV spores (ratio of 51.3:47.4) and 1.3% of spores have 7 SV), pyramidal in lateral view. Suture lines between valves not visible; shell distal margins tapering smoothly to point. Polar capsules pyriform, in anterior portion of shell valve and occupy approximately 62% of total length

Table 2. Species isolates used for 18S rDNA sequence analysis

Species	Host	Valve no.	Location	Accession no.	Reference
<i>Kudoa alliarina</i>	<i>Macruronus magellanicus</i>	4	Argentina	DQ182561	Whipps and Diggles (2006)
<i>Kudoa amamiensis</i> 1	<i>Pempheris ypsilychnus</i>	4	Australia	AY152748	Whipps <i>et al.</i> (2003b)
<i>Kudoa amamiensis</i> 2	<i>Seriola quinqueradiata</i>	4	Japan	AF034638	Hervio <i>et al.</i> (1997)
<i>Kudoa chaetodoni</i> n. sp.	<i>Chaetodon unimaculatus</i>	8/9	Australia	DQ519387	This study
<i>Kudoa ciliatae</i>	<i>Sillago ciliata</i>	4	Australia	DQ519390	Lom <i>et al.</i> (1992)
<i>Kudoa clupeiidae</i>	<i>Brevoortia tyrannus</i>	4	Virginia	AY197771	Webb <i>et al.</i> (2005)
<i>Kudoa crumena</i>	<i>Thunnus albacares</i>	4	North Carolina	AF378347	Kent <i>et al.</i> (2001)
<i>Kudoa funduli</i>	<i>Fundulus heteroclitus</i>	4	North America	AY312279	Akaishi <i>et al.</i> (2004)
<i>Kudoa grammatorcyni</i>	<i>Grammatorcynus bicarinatus</i>	6	Australia	AY302739	Whipps <i>et al.</i> (2004)
<i>Kudoa hypoepicardialis</i>	<i>Nomeus gronovii</i>	4	Gulf of Mexico	AY302722	Blaylock <i>et al.</i> (2004)
<i>Kudoa iwatai</i> J	<i>Pagrus major</i>	4	Japan	AY641571	Diamant <i>et al.</i> (2005)
<i>Kudoa iwatai</i> RS1	<i>Sparus aurata</i>	4	Israel	AY514038	Diamant <i>et al.</i> (2005)
<i>Kudoa iwatai</i> RS2	<i>Siganus rivulatus</i>	4	Israel	AY514039	Diamant <i>et al.</i> (2005)
<i>Kudoa lateolabraxis</i>	<i>Lateolabrax</i> sp.	4	Japan	AY382606	Yokoyama <i>et al.</i> (2004)
<i>Kudoa lethrini</i> n. sp.	<i>Gymnocranius audleyi</i>	7	Australia	DQ519388	This study
<i>Kudoa lutjanus</i>	<i>Lutjanus erythropterus</i>	4	Taiwan	AY644704	Wang <i>et al.</i> (2005)
<i>Kudoa megacapsula</i>	<i>Seriola quinqueradiata</i>	4	South Korea	AB263074	Yokoyama <i>et al.</i> direct submission
<i>Kudoa megacapsula</i>	<i>Sphyaena pinguis</i>	4	China	AB188529	Yokoyama and Itoh (2005)
<i>Kudoa miniauriculata</i>	<i>Sebastes paucispinis</i>	4	California	AF034639	Hervio <i>et al.</i> (1997)
<i>Kudoa minithyrsites</i>	<i>Pempheris ypsilychnus</i>	4	Australia	AY152749	Whipps <i>et al.</i> (2003b)
<i>Kudoa monodactyli</i>	<i>Monodactylus argenteus</i>	5	Australia	DQ439814	Gunter <i>et al.</i> (2006)
<i>Kudoa neurophila</i>	<i>Latris lineata</i>	5	Australia	AY172511	Whipps <i>et al.</i> (2004)
<i>Kudoa ovivora</i>	<i>Thalassoma bifasciatum</i>	4	Panama	AY152750	Whipps <i>et al.</i> (2003b)
<i>Kudoa paniformis</i>	<i>Merluccius productus</i>	4	British Columbia	AF034640	Hervio <i>et al.</i> (1997)
<i>Kudoa permulticapsula</i>	<i>Scomberomorus commerson</i>	13	Australia	AY078429	Whipps <i>et al.</i> (2003a)
<i>Kudoa quadricornis</i>	<i>Carangoides fulvoguttatus</i>	4	Australia	AY078428	Whipps <i>et al.</i> (2003a)
<i>Kudoa rosenbuschi</i>	<i>Merluccius hubbsi</i>	4	Argentina	AY623795	Abollo <i>et al.</i> (2005)
<i>Kudoa scomberomori</i>	<i>Scomberomorus commerson</i>	6	Australia	AY302737	Whipps <i>et al.</i> (2004)
<i>Kudoa shiomitsui</i> 1	<i>Takifugu rubripes</i>	4	Gulf of Mexico	AY302724	Blaylock <i>et al.</i> (2004)
<i>Kudoa shiomitsui</i> 2	<i>Paralichthys olivaceus</i>	4	Japan	AB183718	Direct submission
<i>Kudoa</i> sp.	<i>Sillago ciliata</i>	5	Australia		This study
<i>Kudoa thalassomi</i>	<i>Thalassoma lunare</i>	6	Australia	AY302738	Whipps <i>et al.</i> (2004)
<i>Kudoa thyrsites</i>	<i>Scomber scombrus</i>	4	England	AY542482	Whipps <i>et al.</i> (2004)
<i>Kudoa thyrsites</i>	<i>Sardinops ocellatus</i>	4	South Africa	AY542481	Whipps <i>et al.</i> (2004)
<i>Kudoa thyrsites</i>	<i>Paralichthys olivaceus</i>	4	Japan	AY382607	Yokoyama <i>et al.</i> (2004)
<i>Kudoa thyrsites</i>	<i>Coryphaena hippurus</i>	4	Australia	AY152747	Whipps <i>et al.</i> (2003b)
<i>Kudoa thyrsites</i>	<i>Thyrsites atun</i>	4	South Africa	AY078430	Whipps <i>et al.</i> (2003b)
<i>Kudoa thyrsites</i>	<i>Salmo salar</i>	4	British Columbia	AF031412	Hervio <i>et al.</i> (1997)
<i>Kudoa thyrsites</i>	<i>Aulorhynchus flavidus</i>	4	British Columbia	AF031413	Hervio <i>et al.</i> (1997)
<i>Kudoa trifolia</i>	<i>Liza aurata</i>	4	Spain	AM183300	Holzer (2006) Direct submission
<i>Kudoa yasunagai</i>	<i>Paralichthys olivaceus</i>	7	Japan	AY302741	Whipps <i>et al.</i> 2004
<i>Unicapsula</i> sp.	<i>Argyrosomus japonicus</i>	3	Australia	AY302725	Whipps <i>et al.</i> (2004)

of shell valve in apical view. Spore measurements are shown in Table 3.

Kudoa lethrini n. sp.

Type host: *Gymnocranius audleyi* Ogilby, 1916, Collared large-eye bream (Teleostei, Lethrinidae) adult.

Other host: *Lethrinus harak* Forsskål, 1775, Thumbprint emperor (Teleostei, Lethrinidae) adult.

Location in the host: Brain

Type locality: North Wistari Reef (23° 27'S; 151° 55'E), Capricorn-Bunker Group, Great Barrier Reef, Queensland, Australia.

Other localities: Lizard Island (14° 40' S; 145° 27' E), Great Barrier Reef, Queensland, Australia.

Type material: Hapantotype G464864 (Giemsa and eosin-stained histological section); parahapantotypes G464865–G464866 (Giemsa and eosin-stained histological sections) and G464867–464870 (H&E-stained histological sections) deposited in the Queensland Museum, South Brisbane, Australia.

Etymology: specific name refers to the family of host fish in which it was found.

Prevalence: *Gymnocranius audleyi* – 4 of 7 from North Wistari Reef, Capricorn-Bunker Group; *Lethrinus harak* – 3 of 6 from Lizard Island.

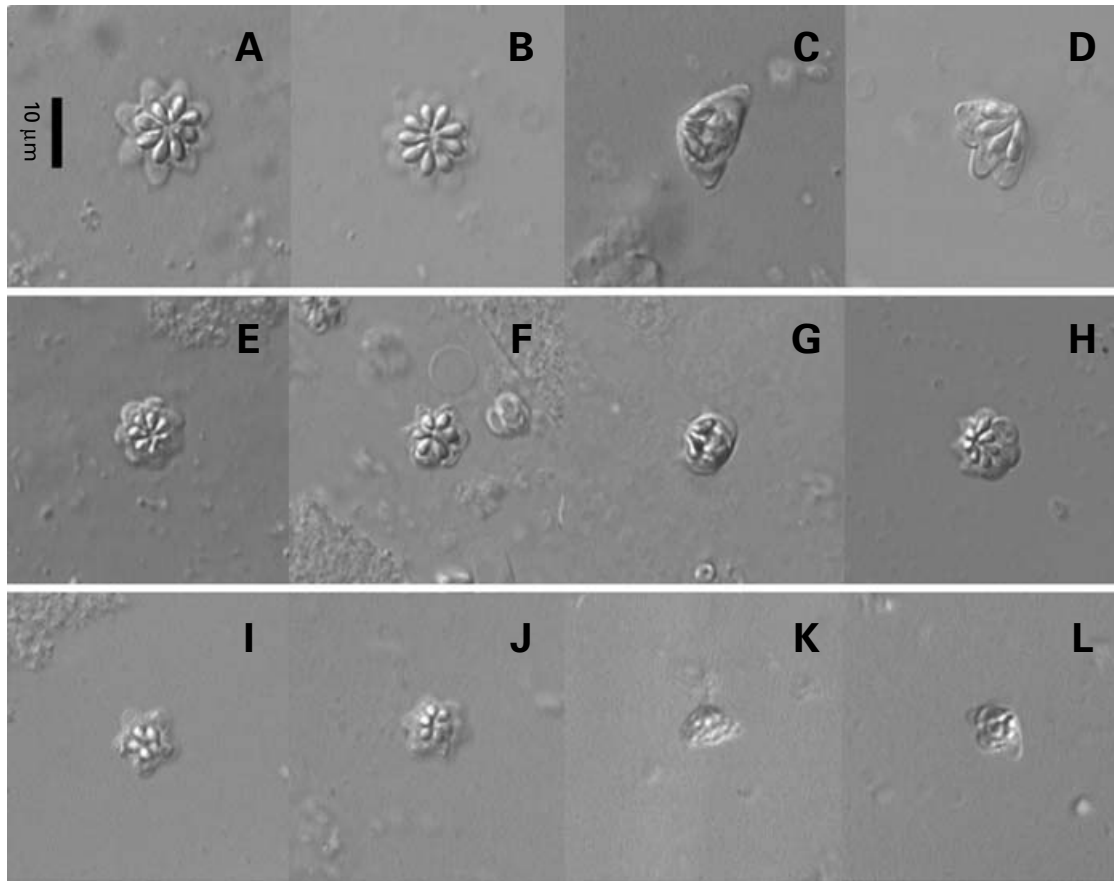


Fig. 3. Phase-contrast micrographs of fresh spore preparations. *Kudoa chaetodoni* n. sp. (A) 8-spore valve morphotype apical view. (B) 9-spore valve morphotype apical view. (C) Side view. (D) Oblique view. *Kudoa lethrini* n. sp. (E) 7-spore valve morphotype apical view. (F) 6-spore valve morphotype. (G) Side view. (H) Oblique view. *Kudoa* sp. I = 5-spore valve morphotype apical view. (J) 6-spore valve morphotype apical view, K = side view, L = oblique view.

Description. Spores. (Fig. 1C, D, 3E–H). Spores stellate in apical view, radiating from centre with 7 equal shell valves (95:1:4.9 ratio of spores with 7 shell valves to those with 6 shell valves), cup shape in lateral view. Suture lines between valves not visible; shell distal margins rounded at base and lateral apical extensions taper smoothly to point in flower petal-like form. Polar capsules pyriform, in anterior portion of shell valve and occupy approximately 55% of total length of shell valve in apical view. Spore measurements are shown in Table 3.

Kudoa sp.

Hosts: *Sillago ciliata* Cuvier, 1829, Sand whiting (Teleostei, Sillaginidae) adult and *S. maculata* Quoy & Gaimard, 1824, Winter whiting adult.

Location in the host: Brain.

Localities: One Mile (27° 29'S; 153° 24'E) and Amity Point (27° 23'S; 153° 26'E), North Stradbroke Island, Queensland, Australia.

Material: Vouchers G464871–G464876 (Giemsa and eosin stained histological sections) and G464877–G464882 (H&E-stained histological sections) deposited in the Queensland Museum, South Brisbane, Australia.

Prevalence: *Sillago ciliata* – 9 of 35 (8 of 19 from One Mile, 1 from 2 at Amity Point, North Stradbroke Island; none of 1 from Shark Bay, Heron Island; none of 12 from Casuarina Beach, Lizard Island); *Sillago maculata* – 2 of 9 (from One Mile).

Description. Spores. (Fig. 1E, F, 3I–L). Spores stellate in apical view, radiating from centre with 5 or 6 equal shell valves (in voucher specimen, 5 polar capsule spores dominate 6 polar capsule spores (ratio of 86.2:13.8 in *Sillago ciliata* and 67.3:32.7 in *Sillago maculata*)), pyramidal in lateral view. Suture lines between valves not visible; shell distal margins tapering smoothly to point. Polar capsules pyriform, in anterior portion of shell valve and occupy approximately 45% of total length of shell valve in apical view. Spore measurements are shown in Table 3.

Morphometric data of brain myxosporean species

The morphometric data from each of the myxosporean species from brain tissue is shown in Table 3. Measurements of *Kudoa chaetodoni* n. sp.

Table 3. Spore measurements in μm (with standard deviation) of myxosporeans isolated from brain tissue (SV = spore valve; PCL = polar capsule length; PCW = polar capsule width; n/a = measurement not appropriate for that morphotype. See Fig. 2 for measurement schematics.)

	<i>Kudoa chaetodoni</i> n. sp.		<i>Kudoa lethrini</i> n. sp.	<i>Kudoa</i> sp. ex <i>Sillago ciliata</i>	
	8 SV Spore	9 SV Spore	7 SV Spore	5 SV Spore	6 SV Spore
<i>Number of spores</i> (measured in apical view)	45	31	65	53	31
Width	16.82 ± 0.49	17.22 ± 0.71	11.41 ± 0.41	10.84 ± 0.46	11.55 ± 0.56
Range	(15.59–17.8)	(15.93–18.31)	(10.17–12.37)	(10.16–11.86)	(10.51–12.88)
Thickness	15.93 ± 0.57	15.58 ± 0.76	10.56 ± 0.47	9.96 ± 0.43	10.68 ± 0.57
Range	(14.24–16.95)	(14.07–16.95)	(9.49–11.86)	(9.15–10.85)	(9.83–12.2)
Suture radius	6.23 ± 0.24	6.81 ± 0.37	5.01 ± 0.23	4.45 ± 0.24	4.19 ± 0.20
Range	(5.59–7.12)	(6.1–7.63)	(4.58–5.76)	(3.9–5.08)	(3.82–4.56)
Valve width	8.47 ± 0.33	8.89 ± 0.43	5.87 ± 0.22	5.66 ± 0.29	5.72 ± 0.28
Range	(7.8–9.15)	(7.97–9.83)	(5.42–6.27)	(4.58–6.44)	(5.42–6.27)
Valve breadth	4.95 ± 0.29	4.86 ± 0.24	4.47 ± 0.22	5.02 ± 0.21	4.57 ± 0.2
Range	(4.41–5.76)	(4.41–5.42)	(4.07–5.08)	(4.41–5.42)	(4.41–5.08)
Apical PCL	5.27 ± 0.39	5.47 ± 0.43	3.21 ± 0.27	2.52 ± 0.24	2.6 ± 0.26
Range	(0.53–0.72)	(4.75–6.27)	(2.71–3.9)	(2.2–3.22)	(2.2–3.22)
Apical PCL:Valve width	0.62 ± 0.05	0.62 ± 0.04	0.55 ± 0.05	0.45 ± 0.04	0.46 ± 0.18
Range	(0.53–0.72)	(0.54–0.68)	(0.43–0.66)	(0.37–0.56)	(0.38–0.56)
<i>Number of spores</i> (measured in lateral view)		30	67		31
Length		9.97 ± 0.56	8.43 ± 0.35		7.31 ± 0.34
Range		(7.8–10.85)	(7.78–9.32)		(6.78–8.13)
PCL		6.28 ± 0.5	3.80 ± 0.27		3.5 ± 0.35
Range		(5.08–7.12)	(2.73–4.41)		(2.54–4.06)
PCW		2.73 ± 0.19	1.97 ± 0.12		1.93 ± 0.17
Range		(2.37–3.22)	(1.69–2.2)		(1.53–2.37)
Wing height		n/a	1.24 ± 0.12		n/a
Range		n/a	(0.85–1.69)		n/a

from *Chaetodon unimaculatus* that had 8 SV and 9 SV morphotypes were not significantly different. *Kudoa* sp. from *Sillago ciliata* differed significantly between the 5 SV and 6 SV morphotype with the 6 SV morphotype having a larger width and thickness, but smaller valve breadth.

Histology and spore ratios

Histological sections of brain tissue stained with Giemsa and eosin revealed multiple different spore morphotypes i.e. spores with different numbers of spore valves, originating from a single plasmodium in both *Kudoa chaetodoni* n. sp. and *Kudoa* sp. from *Sillago ciliata* (Fig. 4). Histological sections from *Kudoa lethrini* n. sp. did not reveal the minor morphotype (6 SV).

Kudoa chaetodoni n. sp. had an almost equal proportion of 8 SV and 9 SV morphotypes. *K. lethrini* n. sp. had almost complete domination of the 7 SV morphotype over 6 SV. *Kudoa* sp. had 5 SV dominate over 6 SV at 6:1 in *Sillago ciliata* and 2:1 in *Sillago maculata*.

Sequence alignment

The sequence of *Kudoa ciliatae* 18S rDNA derived in this study was almost identical to that of sequence

from GenBank (see Kent *et al.* 2001, GenBank Accession number AF378348), but the new sequence provided an extra 700 base pairs (to the existing 880 bp) and resolved a number of indeterminate bases in the original sequence. Therefore, the sequence generated in this study was used for phylogenetic analyses and submitted to GenBank (Accession number DQ519390).

Phylogenetic analysis

Figure 5 shows a maximum likelihood analysis. Most clades were consistent in all analyses except for the placement of *K. ciliatae* Lom, Rohde & Dykova, 1992, *K. diana* Dykova, Avila & Fiala, 2002, *K. paniformis* Kabata and Whitaker, 1981, and *K. miniauriculata* Whitaker, Kent & Sakanari, J. A., 1996. *Kudoa ciliatae* was either found branching before the clade of *K. alliararia* Kovaleva, Shulman & Yakovlev, 1979, *K. clupeidae* Hahn, 1917, *K. diana*, *K. funduli* Hahn, 1915, *K. miniauriculata*, *K. paniformis*, and *K. rosenbuschi* Gelormini, 1943 (in maximum likelihood analyses); or forming a clade with *K. ovivora* Swearer & Robertson, D. R., 1999 (Bayesian, parsimony, and neighbour joining analyses). *Kudoa diana*, *K. paniformis*, and *K. miniauriculata* always formed a clade but with different topologies depending on the analysis. The clades

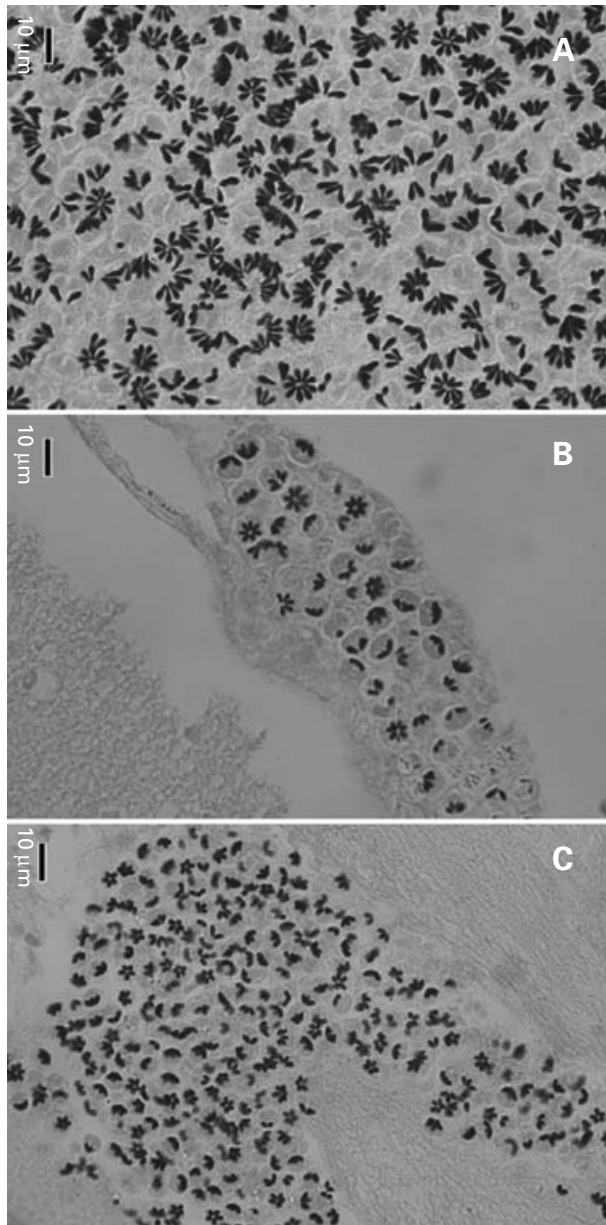


Fig. 4. Giemsa and eosin-stained histological section of pseudocyst. (A) *Kudoa chaetodonti* n. sp. (B) *Kudoa lethrini* n. sp. (C) *Kudoa* sp.

including these species always had low bootstrap confidence levels or low clade credibilities.

The 5 species infecting the brain formed a single clade. The 2 species that infect cardiac tissue, *K. hypoepicardialis* Blaylock, Bullard & Whipps, 2005 and *K. shiomitsui* Egusa & Shiomitsu, 1983, also formed a clade. There were 3 distinct groups containing multiple species that infect skeletal muscle and a further 2 skeletal muscle species, *K. thalassomi* Adlard, Bryant, Whipps & Kent, 2005 and *K. quadricornis* Whipps, Adlard, Bryant & Kent, 2003, that fell outside all other groups. The smooth muscle representatives, *K. ciliatae* and *K. dianaae*, did not form a clade. *Kudoa ovivora*, the only representative from gonadal tissue, did not form a clade with any small group of species or tissue type.

Only 2 clades of more than 1 species were associated with a single fish family, the Merlucciidae (*Kudoa alliaria* Kovaleva, Shulman & Yakovlov, 1979 and *K. rosenbuschi* Kovaleva, Shulman & Yakovlov, 1979); and Scombridae (*K. grammatorcyni* Adlard, Bryant, Whipps & Kent, 2005 and *K. scomberomori* Adlard, Bryant, Whipps & Kent, 2005). There are 10 fish families that have more than 1 *Kudoa* infection where the *Kudoa* species do not form clades.

Patterns in the analysis of the geographical distribution (Fig. 5) amongst the species varied depending on the scale considered. Two clades, 1 from Argentina and 1 from Australia, were associated with a single country. Resolution improved when looking at divisions based on the Atlantic/East Pacific and Indo/West Pacific giving 8 separate clades.

DISCUSSION

Characterization of new species

The morphological and molecular studies revealed 3 morphologically novel species from brain tissue. Histological examination of each of the species shows spore valve number plasticity comparable to that reported for *K. monodactyli* Gunter, Whipps, Cribb & Adlard, 2006 and *Kudoa permulticapsula* Whipps, Adlard, Bryant & Kent 2003.

Kudoa chaetodonti n. sp. is distinct from all other known species of multivalvulidan myxosporeans in having spores containing mainly 8 or 9 spore valves. This is the first myxosporean described from *Chaetodon unimaculatus*. Two multivalvulidans have been reported from chaetodontids previously, *Kudoa muscularis* (Cheung *et al.* 1983) from the muscle of *Chaetodon collare* Bloch, 1787 from the Philippine Islands and *Kudoa* sp. from muscle of a *C. xanthurus* Bleeker, 1857 of unknown provenance (Voelker, 1978). Molecular data are not available for these species. *Kudoa chaetodonti* n. sp. is most closely related to other species that infect brain tissue differing from *Kudoa* sp. from *Sillago ciliata* and *K. yasunagai* by 0.1% (2 bp), from *K. lethrini* n. sp. by 0.9% (14 bp) and from *K. neurophila* Grossel, Dykova, Handler & Munday, 2003 by 1.5% (23 bp).

Kudoa lethrini n. sp. is the second myxosporean species that has 7 spore valves. It is distinct from the other species, *Kudoa yasunagai*, in having lateral apical extensions at the distal edge of each valve similar to those of *K. miniauriculata*, a 4 SV species that infects the muscle of *Sebastes paucispinis* Ayres, 1854 (see Whitaker *et al.* 1996). The measurements of spores of *K. lethrini* n. sp. are distinct from those of *K. yasunagai* but this comparison is compromised by the fact that the measurements given by Hsieh and Chen (1984) for *K. yasunagai* were taken from spores preserved in formalin which shrinks spores (Parker and Warner, 1970). This is the first record of

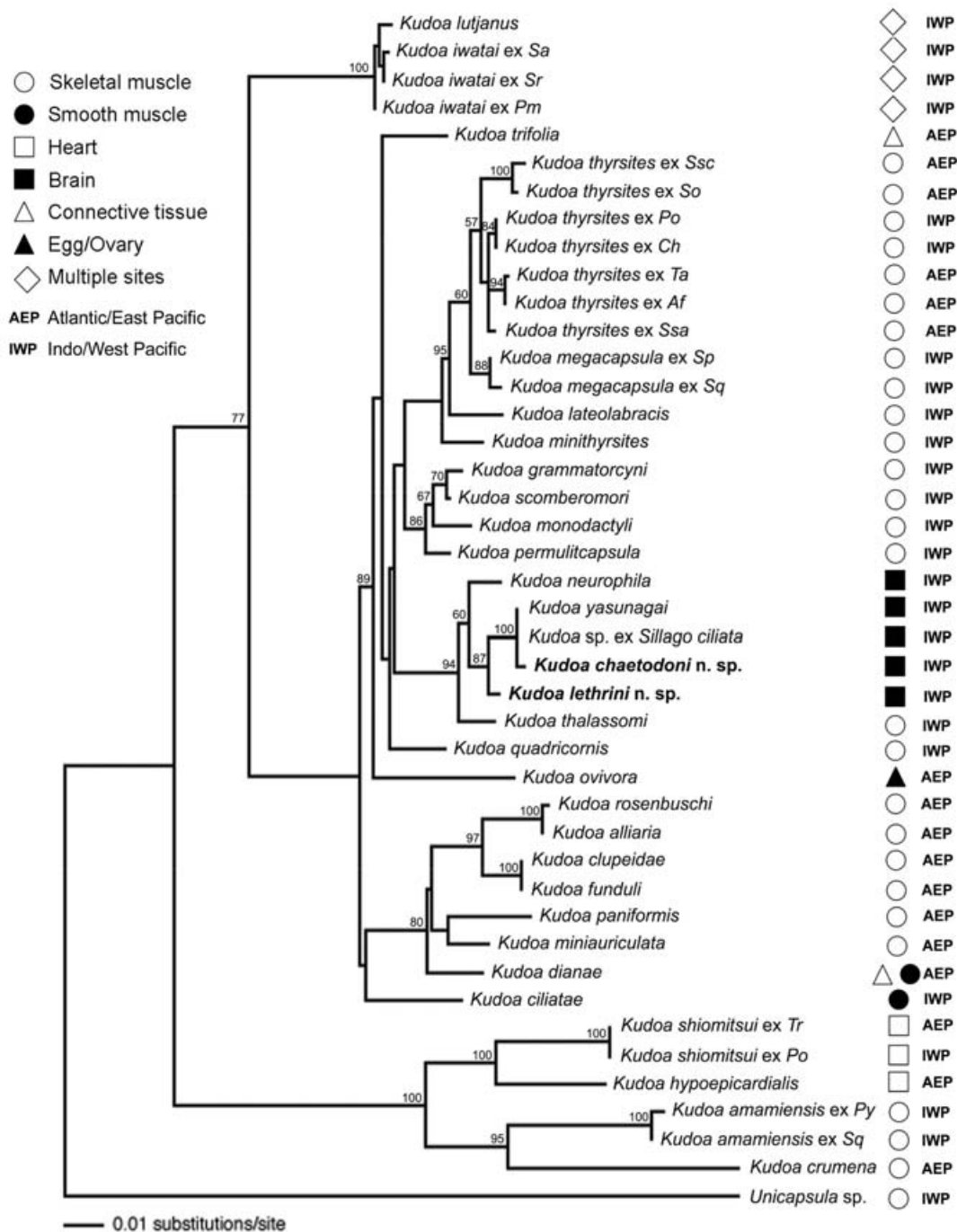


Fig. 5. Maximum likelihood analysis tree using the GTR + I + G model determined by the Akaike Information Criterion of kudoid 18S rDNA sequences. Bootstrap confidence levels greater than 50 shown at nodes. Symbols indicate the tissue type and geographical region from which each species was isolated. Where species have been sequenced from multiple hosts, see Table 2 for full host names.

Kudoa from the family Lethrinidae. The other 7 SV species, *K. yasunagai* was recorded from *Lateolabrax japonicus* Cuvier, 1828 (Lateolabracidae) and *Paralichthys olivaceus* Temminck & Schlegel, 1846 (Paralichthyidae).

In 18S rDNA sequence, *K. lethrini* n. sp. is most closely related to *Kudoa* sp. from *Sillago ciliata* and *K. yasunagai*, only differing by 0.8% (12 bp). *K. lethrini* n. sp. differs from the other brain-inhabiting

species by 0.9% (14 bp) from *K. chaetodoni* n. sp., and 1.2% (19 bp) from *K. neurophila*.

Kudoa sp. from *Sillago ciliata* differs from all but 1 (*K. neurophila*) of the 4 known 5 SV species of *Kudoa* by being localized in the brain (see Table 4). Its spores are distinctly larger than those of *K. neurophila* and *K. shulmani* Naidjenova & Zaika, 1970, and distinctly smaller than those of *K. cutanea* Kovaljova & Gajevskaja, 1984 and *K. muscularis* Cheung,

Table 4. *Kudoa* sp. data collated with the other known 5 spore valve *Kudoa* species (PCL = Polar capsule length, PCW = Polar capsule width.)

	<i>Kudoa</i> sp.	<i>K. neurophila</i>	<i>K. cutanea</i>	<i>K. muscularis</i>	<i>K. shulmani</i>
Width	9.96	7.73	12–13.3	10.0	7–8
Length	7.31	6.13	Not stated	5.5	Not stated
PCL	3.5	3.78	6.65	3.5	2–2.5
PCW	1.93	2.27	3–3.4	1.5	Not stated
Target tissue	Brain tissue	CNS	Sub-epidermal	Somatic muscle	Somatic muscle
Host	<i>Sillago ciliata</i>	<i>Latris lineata</i>	<i>Echiodon</i> sp.	<i>Chaetodon collare</i>	<i>Nemipterus japonicus</i>
Locality	Moreton Bay	Tasman Sea	South Atlantic	Indo-Pacific	Indian Ocean
Source	This study	Grossel <i>et al.</i> (2003)	Kovaljova and Gajevskaja (1984)	Cheung <i>et al.</i> (1983)	Naidjenova and Zaika (1970)

Table 5. Comparison of morphometrics of *Kudoa yasunagai* and *Kudoa* sp. from *Sillago ciliata* from formalin preserved spores

	<i>Kudoa yasunagai</i>	<i>Kudoa</i> sp.
Width	11.7	10.72
Range	(9.35–13.94)	(9.62–11.91)
Thickness	8.32	9.82
Range	(7.14–10.20)	(8.12–11.20)
Length	6.21	7.14
Range	(4.25–7.31)	(6.18–7.68)
Polar capsule length	3.64	2.65
Range	(3.40–4.25)	(2.23–2.98)
Polar capsule width	2.45	1.54
Range	(2.21–2.89)	(1.12–1.85)

Nigrelli & Ruggieri, 1983. The polar capsule length is similar to those of *K. neurophila* and *K. muscularis*, larger than that of *K. shulmani*, and smaller than that of *K. cutanea*. The polar capsule width is smaller than that of *K. neurophila* and *K. cutanea* but larger than that of *K. muscularis*.

Kudoa sp. from *Sillago ciliata* differs in 18S rDNA sequence from *K. chaetodoni* n. sp. by 0.1% (2 bp), *K. lethrini* n. sp. by 0.8% (12 bp), and *K. neurophila* by 1.4% (21 bp). However, the 18S rDNA sequence was identical to that of *K. yasunagai* (which has 7 SV) from the brain of *Paralichthys olivaceus*.

The morphometric data from the 7 SV *K. yasunagai* compared with that from *Kudoa* sp. from *Sillago ciliata* (see Table 5) shows significant differences, particularly in the proportions that reflect spore shape. Finding identical 18S rDNA sequences for myxosporeans that have dramatically different morphology and occur in widely disparate hosts is a surprising and unique outcome. Hsieh and Chen (1984) mentioned the existence of exceptional spores with 6 or 8 shell valves in the original description of *K. yasunagai*, which blurs the distinction, at least on polar capsule count, between it and *Kudoa* sp. from *Sillago ciliata*, which has both 5 SV and 6 SV morphotypes. Although spore valve number does not reflect directly the genetic affinities between groups of kudoid species (see Whipps *et al.* 2004), to identify *Kudoa* sp. as *K. yasunagai* on the basis of the 18S

rDNA sequence would be unsatisfactory as it would imply that almost no aspect of the morphology of kudoid spores was a reliable indicator of identity. Thus, we take the conservative approach and hypothesize that the morphological difference between these forms is indeed an indication of species-level separation and that the 18S rDNA is incapable of distinguishing some combinations of species of Kudoidae. The obvious next step is to investigate gene regions that offer higher rates of mutation and would thus provide higher resolution.

The paucity of morphological characters (at light microscope level) drives us to rely on molecular characters to establish individual taxa. However, this study has uncovered the likely limit of resolution in 18S rDNA by showing that morphologically distinct forms (*Kudoa* sp. from *Sillago ciliata* and *K. yasunagai*) shared 100% homology in that region. In contrast, analysis of 18S rDNA of *Kudoa thyrssites* Gilchrist, 1924 (see Whipps and Kent, 2006) shows that species identified as *K. thyrssites* can have significant variation in 18S rDNA correlated with the provenance of the isolate. We consider it likely that the variation in 18S rDNA shown by *K. thyrssites* isolates reflects the presence of a complex of species. The greatest reported difference between *K. thyrssites* 18S rDNA sequences (0.9%, 14 base pairs) was between that from *Scomber scombrus* Linnaeus, 1758 from England and that from *Paralichthys olivaceus* from Japan. This is the same level of difference we report here between the 2 morphologically distinct species *K. chaetodoni* n. sp. (8/9 SV) and *K. lethrini* n. sp. (7 SV with lateral apical extensions). The contrast between the complex of *K. thyrssites* and the molecular identity of *Kudoa* sp. from *Sillago ciliata* and *K. yasunagai* shows 2 extremes with regard to the analysis of morphology and genotype in the Kudoidae. This complexity provides a challenge for species identification of kudoids until further molecular data are available.

Tissue tropism and genetic relatedness

The need to correlate genetic relationships with characters other than spore valve number has been

clear since Whipps *et al.* (2004) showed that this character did not correlate with genetic relatedness within the Kudoidae. Our phylogenetic analyses support that conclusion in that species with different spore valve number such as *K. permulticapsula* (13 SV) and *K. scomberomori* (6 SV) are closely related, whereas others with the same number of spore valves (4 SV) such as *K. amamiensis* Egusa & Nakajima, 1980 and *K. iwatai* Egusa & Shiomitsu, 1983 are relatively distant.

Abollo *et al.* (2005) noted that since the majority of DNA sequences generated for *Kudoa* species are from those that infect muscle, it was necessary to include more species from different host tissues in order to assess the importance of tissue specificity. Our study has introduced 3 new isolates infecting the brain of fish hosts. In all the analyses, the 3 brain-dwelling isolates formed a strongly supported clade with the other available species from that tissue, *K. neurophila* and *K. yasunagai*. Furthermore, *K. hypoepicardialis* and *K. shiomitsui*, the only 2 kudoids known to infect cardiac muscle, also formed a clade in all analyses. This result supports the hypothesis that the tissue tropism of some kudoids correlates with genetic relatedness, but there are also significant departures from it. For example, *Kudoa diana* and *K. ciliatae* which infect smooth muscle are apparently unrelated, although the position of *K. diana* had low clade credibility and low bootstrap confidence values. Twenty-seven of the 43 18S species analysed were from skeletal muscle. The phylogenetic analyses revealed 3 major clades of muscle-infecting species and 3 species falling outside these clades. It is possible that this multiplicity of clades results from muscle being the ancestral site of infection. However, multiple skeletal muscle clades could equally reflect a series of separate radiations in this site.

Kudoa iwatai and *K. lutjanus* Wang, Huang, Tsai, Cheng, Tsai, Chen, Chen, Chiu, Liaw, Chang & Chen, 2005 have both been reported as infecting several tissue sites. *K. iwatai* was reported to infect brain, eye, intestinal tract, heart, kidney, muscle, mouth, nerve axon, ovary, and peritoneum (Diamant *et al.* 2005). Wang *et al.* (2005) reported that *K. lutjanus* infects brain, eye, muscle and intestinal organs. Thus, some species have the ability to infect almost any site. The apparent close relationship between *K. iwatai* and *K. lutjanus* suggests that there may be an 'opportunistic' clade.

Geographical distribution and genetic relatedness

Genetic relationships did not correlate well with geographical distribution. The only clades associated with a single region are that of *K. alliaris* and *K. rosenbuschi* from Argentina and *K. grammatorcyni*, *K. monodactyli*, *K. permulticapsula*, and *K. scomberomori* all from the east coast of Australia.

Otherwise, all the major clades incorporate species from multiple localities, despite sampling being concentrated in a few key regions of the world. Local concentration of data (due to the location of research groups) is likely to result in false geographical patterns but the reverse appears to be the case. There are 8 clades that correspond to the major biogeographical divisions in the Indo/West Pacific and Atlantic/East Pacific but there is so much mixing that this appears to have little significance.

Host specificity and genetic relatedness

Although some kudoids have been recorded from several fish families, and even from different fish orders, most have been recorded from a single host species or family. The findings in this study were consistent with this pattern; all 4 species studied here were found in 1 family; *Kudoa chaetodonti* n. sp. from a single chaetodontid, *K. ciliatae* was recorded from 3 sillaginid species, *K. lethrini* n. sp. was recorded from 2 lethrinid species, and *Kudoa* sp. from 2 sillaginids. This specificity cannot be considered definitive as sister host families in the geographical regions where infections were recorded are incompletely sampled. Specificity to a single host family, has previously been demonstrated experimentally for the bivalvulidan myxosporean, *Myxobolus cerebralis* Wolf & Markiw, 1984, by El-Matbouli *et al.* (1999). This species was able to establish in Salmonidae whereas Adrianichthyidae, Cyprinidae, Poeciliidae, and frogs were refractory to infection.

There are 5 cases of individual fish species being infected by 2 or more kudoids for which molecular data are available for both species. *Paralichthys olivaceus* is infected by *K. shiomitsui* (heart), *K. thyrsites* (muscle), and *K. yasunagai* (brain); *Pempheris ypsilychnus* Mooi & Jubb, 1996 is infected by *K. amamiensis* (muscle), and *K. minithyrsites* Whipps, Adlard, Bryant, Lester, Findlay & Kent, 2003 (muscle); *Scomberomorus commerson* Lacepède, 1800 is infected by *K. permulticapsula* (muscle) and *K. scomberomori* (muscle); *Seriola quinqueradiata* is infected by *K. amamiensis* and *K. megacapsula*; and *Sillago ciliata* is infected by *K. ciliatae* (smooth muscle of the gut wall) and *Kudoa* sp. (brain). In all 5 cases, relationships reflected site of infection rather than host identity. *Kudoa amamiensis* and *K. minithyrsites* from *Pempheris ypsilychnus* are both found in muscle-dwelling clades, but in clades of different origins. Including these examples, at the fish family level, there are 10 examples where tissue tropism relatedness is supported over host identity with the families: Carangidae being infected by *K. amamiensis*, *K. megacapsula* Yokoyama & Itoh, 2005, and *K. quadricornis*; Clupeidae being infected by *K. clupeidae* and *K. thyrsites*; Labridae being infected by *K. ovivora* and *K. thalassomi*; Merlucciidae being infected with *K. alliaris*, *K. paniformis*, and

K. rosenbuschi; Scombridae being infected with *K. crumena* Iversen & Van Meter, 1967, *K. grammatorcyni*, *K. permulticapsula*, *K. scomberomori*, *K. thyrsites*; Sparidae being infected by 2 split clade isolates of *K. iwatai*; and Tetraodontidae being infected with *K. diana*e and *K. shiomitsui*.

It is likely that host identity contributes to the parasite distribution observed, but these factors seem to have a lesser role than the tissue they infect. At present there is thus no evidence of any radiation of kudoids in association with fish host taxon.

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