First taxonomic description of multivalvulidan myxosporean parasites from elasmobranchs: *Kudoa hemiscylli* n.sp. and *Kudoa carcharhini* n.sp. (Myxosporea: Multivalvulidae)

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SUMMARY

Myxosporean parasites are significant parasites of fishes not only for their apparent high diversity but also for their potential impact on fish health and/or marketability. Regardless, our knowledge of most myxosporeans, especially those found in elasmobranch hosts, is superficial. A study of multivalvulidan diversity in a range of elasmobranchs from Queensland, Western Australia and the Northern Territory (Australia) was conducted to address this knowledge gap. Specimens were collected from a total of 3 orders, 9 families and 31 species of elasmobranchs. Myxosporean infections referable to the genus *Kudoa* were discovered in host muscle and characterized morphologically and genetically. Both small subunit (SSU) and large subunit (LSU) rDNA sequences were used in molecular phylogenetic analyses. *Kudoa* spp. infected 27 of the 31 species of elasmobranchs examined, representing new records of this parasite genus in 26, of the 27, host species. Kudoids were observed in all 3 orders, and 7 out of the 9 families of elasmobranchs investigated. This paper reports the first 2 multivalvulidan species to be formally described from elasmobranchs, *Kudoa hemiscylli* n.sp. characterized from *Hemiscyllium ocellatum* (and 8 other host species) and *Kudoa carcharhini* n. sp. characterized from *Carcharhinus cautus* (and 2 other host species). Phylogenetic analyses revealed that kudoids from elasmobranchs form a separate lineage to those of teleosts, but are anchored within the overall kudoid clade.

Key words: Multivalvulida, Myxosporea, Kudoidae, *Kudoa*, diversity, phylogeny, elasmobranchs.

INTRODUCTION

Myxosporeans are overwhelmingly parasites of teleost fishes (Yokoyama, 2003). In addition, they have been reported from hosts that include invertebrates, reptiles, amphibians (some listed by Kent *et al*. 2001); elasmobranch fishes (some listed by Kudo 1920; O'Donoghue and Adlard, 2000; Benz and Bullard, 2004); octopus (Yokoyama and Masuda, 2001); moles (Friedrich *et al*. 2000); shrews (Prunescu *et al*. 2007); and in waterfowl (Bartholomew *et al*. 2008). Myxozoans are significant parasites not only for their apparent high diversity, but also for their potential impact on host organism health and/or marketability (Egusa, 1986; Moran *et al*. 1999; Kent *et al*. 2001). Approximately 2200 myxozoan species (Phylum Myxozoa) are currently recognized, with most of the diversity within the Class Myxosporea (Lom and Dyková, 2006). Over 2000 species are reported from the Order Bivalvulida, whereas only about 80 species are reported from the Order Multivalvulida.

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This study focuses on elasmobranchs, the cartilaginous fishes belonging to the Class Chondrichthyes, subclass Elasmobranchii, which includes the sharks and rays (Compagno *et al*. 2005). There are about 1100 living species worldwide, with new species still being discovered (*ibid*.). Elasmobranchs play an important role in marine ecosystems, since they are high-order predators of a variety of other species (Last and Stevens, 2009; Compagno *et al*. 2005). They also currently provide about 1% of world fisheries landings, or some 700 000 to 800 000 tonnes per year, with this figure likely to increase as the capture fisheries for wild teleost stocks decline (Compagno *et al*. 2005).

There are 124 previous records of myxosporeans from elasmobranchs, with Stroffregen and Anderson (1990) suggesting that myxosporeans of elasmobranchs are found rarely. Records include members of both the myxosporean orders, the Bivalvulida represented by the genera *Ceratomyxa, Chloromyxum, Sinuolinea, Sphaerospora, Myxidium, Leptotheca*, while the Multivalvulida is represented by the genera *Kudoa* and *Unicapsula*. Only 2 records of multivalvulidans from elasmobranchs have been reported, an undescribed *Kudoa* sp. from the skeletal muscle of *Hemiscyllium ocellatum* (see Heupel and

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Table 1. This table shows the elasmobranch species examined, the locality in which the host were collected, and the prevalence of infection with *Kudoa*

	Host Species	Host Family	Sample Locality	Number Sampled	Prevalence N (%)
Order Carcharhiniformes	*Carcharhinus amblyrhynchos*	Carcharhinidae	Lizard Island	9	4 (44·4)
	Carcharhinus amboinensis	Carcharhinidae	Moreton Bay	5	4 (80)
	Carcharhinus cautus	Carcharhinidae	Moreton Bay	8	7 (87·5)
	Carcharhinus dussumieri	Carcharhinidae	Darwin	1	1 (100)
	Carcharhinus leucas	Carcharhinidae	Moreton Bay	3	1 (33·3)
	Carcharhinus limbatus	Carcharhinidae	Moreton Bay	11	8 (72·7)
	Carcharhinus melanopterus	Carcharhinidae	Heron Island	12	12 (100)
	"	"	Lizard Island	9	9 (100)
	Carcharhinus obscurus	Carcharhinidae	Moreton Bay	4	1 (25)
	Carcharhinus sorrah	Carcharhinidae	Moreton Bay	2	1 (50)
	Negaprion acutidens	Carcharhinidae	Heron Island	7	7 (100)
	Rhizoprionodon acutus	Carcharhinidae	Moreton Bay	2	1 (50)
	Rhizoprionodon taylori	Carcharhinidae	Moreton Bay	5	0
	Triaenodon obesus	Carcharhinidae	Lizard Island	1	1 (100)
	"	"	Townsville	1	1 (100)
	Hemigaleus australiensis	Hemigaleidae	Moreton Bay	17	14 (82·3)
	Hemipristis elongata	Hemigaleidae	Moreton Bay	2	0
	Sphyrna lewini	Sphyrinidae	Moreton Bay	5	2 (40)
Order Rajiformes	*Dasyatis fluviorum*	Dasyatidae	Moreton Bay	13	13 (100)
	Neotrygon kuhlii	Dasyatidae	Moreton Bay	26	0
	"	"	Lizard Island	8	3 (37·5)
	Himantura fai	Dasyatidae	Heron Island	3	1 (33·3)
	Himantura granulata	Dasyatidae	Lizard Island	5	1 (20)
	Pastinachus astrus	Dasyatidae	Heron Island	3	2 (66·7)
	"	"	Lizard Island	1	0
	Taeniura lymma	Dasyatidae	Lizard Island	3	3 (100)
	Aetobatus narinari	Myliobatidae	Heron Island	1	0
	Aptychotrema rostrata	Rhinobatidae	Moreton Bay	5	5 (100)
	Glaucostegus typus	Rhinobatidae	Heron Island	11	11 (100)
	"	"	Moreton Bay	2	2 (100)
	Rhynchobatus sp.	Rhynchobatidae	Moreton Bay	1	0
Order Orectolobiformes	*Chiloscyllium punctatum*	Hemiscylliidae	Moreton Bay	26	26 (100)
	Hemiscyllium ocellatum	Hemiscylliidae	Heron Island	8	8 (100)
	Orectolobus maculatus	Orectolobidae	Moreton Bay	29	28 (96·5)
	Orectolobus ornatus	Orectolobidae	Moreton Bay	30	30 (100)
	Orectolobus hutchinsi	Orectolobidae	Western Australia	5	2 (40)

Bennett, 1996), and a suspected *Unicapsula* sp. in the muscle of *Carcharhinus melanopterus* (see Stroffregen and Anderson, 1990).

MATERIALS AND METHODS

Collection and dissection

Elasmobranchs were collected in collaboration with the Queensland Shark and Ray Research Group from 4 locations along the Queensland coastline of Australia; Moreton Bay (27°50′S, 152°50′E), southern Great Barrier Reef (surrounding Heron Island: 23°27′S, 151°55′E), central Great Barrier Reef (surrounding Chicken Reef, off Townsville: 18°40′S, 147°44′E) and northern Great Barrier Reef (surrounding Lizard Island: 14°40′S, 145°27′E); from one site off Perth, Western Australia (31°57′S, 115°51′E); and from one site off Darwin, Northern Territory (12°28′S, 130°51′E) from 2007 to 2009 (Table 1). Individuals were collected either by gill netting, seine netting or line fishing. Muscle samples were taken from each specimen by dissecting 3 muscle blocks (each 20 mm^3) from 1 side of each fish. Muscle blocks were taken at locations dorsal to the midline, with 1 sample taken in each third of the specimen's length. A subsample (approximately 5 mm^3) of each was then examined microscopically in the laboratory

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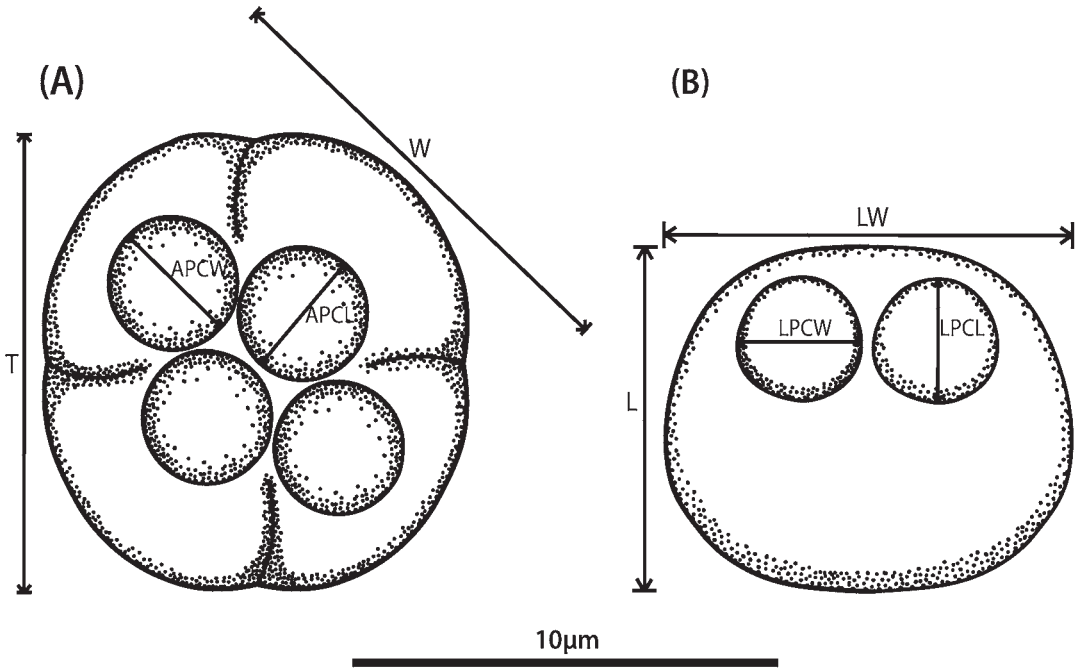

Fig. 1. Stylised line diagrams of *Kudoa hemiscylli* n.sp. spore showing the morphological characters measured. (A) Apical view of spore: W, apical spore width; T, thickness; APCW, apical polar capsule width; APCL, apical polar capsule length. (B) Lateral view of spore: LW, lateral spore width; L, spore length; LPCW, lateral polar capsule width; LPCL, lateral polar capsule length. Scale bar = 10 μm.

to determine infection status. Samples were prepared using a standard wet mount preparation based on methods described by St-Hilaire *et al*. (1997). Slides were then viewed using light microscopy at 400× magnification. Samples containing myxosporeans were preserved with a third in each of 90% ethanol, 10% neutral buffered formalin (NBF) fixative and the remainder frozen at −20 °C or −70 °C, for molecular, histological and morphological analysis of spores, respectively.

Morphological analysis of kudoids

Frozen or fresh tissue samples were used for all morphological analyses. Morphometrics of spores followed the guidelines proposed by Lom and Arthur (1989) for species descriptions of Myxosporea with further recommendations from Burger *et al*. (2008). Digital images were taken using a Nikon Digital Sight DS-L1 (Nikon Corporation, Japan) camera/capture image device mounted on an Olympus BH2 compound microscope. A minimum of 30 different spores were photographed in both apical and lateral view for each myxosporean isolate. Measurements were taken from digital images of the width (W), thickness (T), apical polar capsule length (APCL), apical polar capsule width (APCW), lateral polar capsule length (LPCL), lateral polar capsule width (LPCW), and length (L) of a spore (see Fig. 1). A principle component analysis (PCA) was conducted using PAlaeontological STatistics (PAST) version 1.74 (Hammer *et al*. 2001) to compare spore measurements of each kudoid isolate.

SSU rDNA and LSU rDNA extraction, amplification and sequencing

DNA of kudoids was extracted from the muscle of 12 host species including: *Aptychotrema rostrata* (Shaw, 1794); *Glaucostegus typus* (Bennett, 1830); *Dasyatis fluviorum* Ogilby, 1908; *Neotrygon kuhlii* (Műller and Henle, 1841); *Taeniura lymma* (Forsskål, 1775); *Hemiscyllium ocellatum* (Bonnaterre, 1788); *Orectolobus hutchinsi* Last and Chidlow, 2006; *Orectolobus maculatus* (Bonnaterre, 1788); *Orectolobus ornatus* (de Vis, 1883); *Carcharhinus amboinensis* (Műller and Henle, 1839); *Carcharhinus cautus* (Whitley, 1945); and *Carcharhinus limbatus* (Műller and Henle, 1839).

DNA was extracted from a 2 mm^3 section of ethanol-preserved tissue using a QIAgen DNeasy™ Kit (Qiagen Inc., Valencia, California) according to the manufacturer's protocol. The small subunit (SSU) and large subunit (LSU) ribosomal DNA (rDNA) was amplified by PCR. Primers specific to SSU and LSU rDNA sequence conserved among multivalvulid family members and universal primers (Table 2) were used for amplification. PCR fragments were sequenced using primer combinations for SSU: 18e-Mbseq1r and Kud6F-18R and for LSU: Kt28S1F-28S1R. Standard 25 μl Hotmaster Taq (Eppendorf, Hamburg, Germany) PCR

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Table 2. Primers specific to SSU and LSU rDNA sequence of the order Multivalvulida

Primer	Sequence	Position	Source
18e	5′-CTG GTT GAT CCT GCC AGT	1 SSU[a]	Hillis & Dixon (1991)
Kud6F	5′-TCA CTA TCG GAA TGA ACG	478 SSU[a]	Whipps *et al.* (2003*a*)
Mbseq1r	5′-CAA TCC TAT CAA TGT CTG GAC CTG	1160 SSU[a]	Burger *et al.* (2007)
18R	5′-CTA CGG AAA CCT TGT TAC G	1740 SSU[a]	Whipps *et al.* (2003*b*)
Kt28S1F	5′-CAA GAC TAC CTG CTG AAC	~150 LSU[a]	Whipps *et al.* (2004)
28S1R	5′-GTG TTT CAA GAC GGG TGG	~950 LSU[a]	Whipps *et al.* (2004)

[a] Position relative to universal primer 18e.

reactions were performed using 2 μl of template DNA, as described by Burger *et al.* (2007). PCR reactions were performed in a cp2-01 thermocycler (Corbett Research, Sydney, Australia) following guidelines of Burger *et al.* (2007).

Amplified PCR products were purified by standard submarine agarose gel electrophoresis using a PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) or QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California). Sequencing reactions were performed in both directions following standard sequencing protocol for ABI Big Dye® Terminator (Applied Biosystems) as described by Gunter *et al.* (2006).

Phylogenetic analysis

Sequences were aligned using BioEdit version 7.0.5.3 (Hall, 1999), together with other *Kudoa* spp. SSU and LSU sequences available from GenBank using ClustalW (Thompson *et al.* 1994). The alignment was then checked and adjusted by eye where required. *Unicapsula* sp. a multivalvulidan in the family Trilosporidae and the only other recognized family in the Myxosporea (see Whipps *et al.* 2004), was used as an outgroup for both analyses.

Neighbour-joining, Parsimony and Maximum Likelihood analyses were performed using PAUP* 4.0b10 (Swofford, 2002) and Bayesian analysis conducted using MrBayes 3.0B4 (Heulsenbeck and Ronquist, 2001). The Neighbour-joining and Parsimony phylogenetic relationships were tested by bootstrapping with 1000 replicates. Optimum evolutionary models were used for maximum likelihood analyses as determined by Modeltest 3.7 (Posada and Crandall, 1998). Two models were used: GTR + 1 + G as determined by Akaike information analyses of the sequence data and TrN + 1 + G selected by hierarchiacal likelihood ratio tests. Bayesian analysis was conducted with 2 million generations of Markov chain Monte Carlo analysis, a set of 4 simultaneous chains with a burn-in of 3000 trees and saving current trees to file every 100 generations (as described by Burger *et al.* 2007).

Histology

Tissue samples preserved in 10% formalin were sectioned using standard histological methods. Alternating samples were stained either with Giemsa and eosin or with haematoxylin and eosin.

Slides were examined using compound light microscopy at 100×, 200×, and 400× magnification. Digital images of myxosporean spores were taken at all magnifications using a Nikon Digital Sight DS-L1 (Nikon Corporation, Japan). Type specimens of *Kudoa hemiscylli* n.sp. and *Kudoa carcharhini* n.sp. were deposited in the collections of the Queensland Museum, Brisbane, Australia.

RESULTS

A total of 209 muscle samples from 284 examined (73·6%), were infected with myxospores typical of the genus *Kudoa* Meglitsch, 1947. Only end-stage infections were observed, with kudoids found in all 3 orders, 7 out of 9 families, and 27 of the 31 host species (Table 1). Table 1 shows the hosts examined, the locality in which the host was collected, and the prevalence of infection for each kudoid parasite.

Characterization of elasmobranch-infecting myxosporean species

Phylum Myxozoa
Class Myxosporea
Order Multivalvulida
Family Kudoidae Meglitsch, 1960
Genus *Kudoa* Meglitsch, 1947

Kudoa hemiscylli n.sp.
(Table 3; Fig. 2A–C)

Description. Spores (Fig. 2A and B), in apical view are rounded to subquadrate, in lateral view ellipsoid. Valves, 4 equal-sized, suture lines visible, shell distal margins tapering to a point. Polar capsules, spherical, 1 per valve, located in anterior position of shell valves, occupy approximately 50% of shell valve length in apical view. Occasionally, spores with 5 equal polar capsules and spore valves were observed. Spores of uniform development (i.e. no extra-sporogonic stages observed) only found in muscle of host, with no infections observed in other organs (i.e. not found in gall bladder, brain, heart, or liver). Spore measurements ($n = 30$) are shown in Table 3.

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Table 3. Mean spore dimensions in μm ± S.D. with range in parentheses for respective isolates from each host-parasite combination ($n = 30$)

	Kudoa carcharhini n.sp ex. *Carcharhinus cautus*	*Kudoa hemiscylli* n.sp. ex. *Hemiscyllium ocellatum*	*Kudoa hemiscylli* n.sp. ex. *Dasyatis fluviorum*	*Kudoa hemiscylli* n.sp. ex. *Glaucostegus typus*	*Kudoa hemiscylli* n.sp. ex. *Orectolobus ornatus*
Spore Apical Width -1	10·14 ± 10·49 (9·53–11·29)	9·921 ± 0·43 (9·07–10·81)	10·16 ± 0·48 (9·02–11·27)	10·82 ± 0·40 (10–11·67)	10·4 ± 0·38 (9·74–11·13)
-2	10·23 ± 0·40 (9·52–11·02)	10·08 ± 0·38 (9·24–11.00)	10·02 ± 0·46 (8·33–11·00)	10·25 ± 0·47 (9·65–11·23)	9·97 ± 0·53 (9·00–11·00)
-3	10·09 ± 0·45 (9·26–10·82)	10·18 ± 0·33 (9·31–10·92)	9·98 ± 0·51 (8·67–11·00)	10·14 ± 0·40 (9·64–11·02)	10·00 ± 0·52 (8·50–11·00)
Spore Apical Thickness -1	9·79 ± 0·55 (7·76–10·59)	9·51 ± 0·38 (8·72–10·12)	9·67 ± 0·57 (8·67–11·45)	10·47 ± 0·32 (9·52–10·95)	9·87 ± 0·36 (9·39–10·43)
-2	9·92 ± 0·43 (8·36–10·63)	9·88 ± 0·36 (8·72–10·46)	9·60 ± 0·55 (9·00–10·33)	10·05 ± 0·39 (8·86–10·42)	9·87 ± 0·42 (9·00–10·67)
-3	9·86 ± 0·43 (8·57–10·45)	9·87 ± 0·45 (7·76–10·59)	9·53 ± 0·41 (8·50–10·33)	9·94 ± 0·45 (8·24–10·45)	9·76 ± 0·42 (8·67–10·67)
Polar Capsule -1	2·77 ± 0·25 (2·47–3·18)	2·48 ± 0·19 (2·09–3·14)	2·72 ± 0·27 (2·08–3·12)	2·89 ± 0·19 (2·62–3·33)	2·64 ± 0·18 (2·43–3·13)
Apical Length -2	2·82 ± 0·24 (2·43–3·23)	2·82 ± 0·25 (2·35–3·13)	2·55 ± 0·18 (2·17–3·00)	2·82 ± 0·18 (2·43–3·23)	2·54 ± 0·14 (2·17–3·00)
-3	2·84 ± 0·24 (2·41–3·14)	2·86 ± 0·22 (2·47–3·18)	2·56 ± 0·19 (2·17–3·00)	2·77 ± 0·16 (2·46–3·09)	2·55 ± 0·19 (2·17–3·00)
Polar Capsule -1	2·82 ± 0·31 (2·12–3·53)	2·46 ± 0·16 (2·09–2·97)	2·77 ± 0·24 (2·25–3·12)	2·86 ± 0·19 (2·38–3·33)	2·29 ± 0·15 (2·09–2·61)
Apical Width -2	2·89 ± 0·27 (2·38–3·33)	2·77 ± 0·19 (2·35–3·22)	2·56 ± 0·17 (2·17–3·00)	2·85 ± 0·19 (2·33–3·33)	2·58 ± 0·13(2·33–2·83)
-3	2·78 ± 0·26 (2·31–3·23)	2·82 ± 0·18 (2·25–3·13)	2·57 ± 0·16 (2·33–3·00)	2·80 ± 0·18 (2·35–3·23)	2·60 ± 0·18 (2·17–3·00)
Spore Lateral Width -1	9·77 ± 0·49 (8·47–10·59)	9·63 ± 0·36 (9·07–10·47)	9·76 ± 0·35 (9·02–10·40)	10·48 ± 0·47 (9·52–11·43)	9·71 ± 0·35 (9·04–10·43)
-2	10·01 ± 0·40 (9·06–10·87)	9·86 ± 0·45 (8·86–10·89)	9·49 ± 0·35 (8·83–10·33)	10·04 ± 0·40 (8·87–10·87)	9·47 ± 0·49 (8·67–10·50)
-3	9·83 ± 0·44 (8·57–10·49)	10·07 ± 0·49 (9·12–11·00)	9·34 ± 0·36 (8·50–10·00)	9·87 ± 0·44 (8·55–10·69)	9·63 ± 0·39 (9·00–10·67)
Spore Length -1	8·16 ± 0·56 (7·24–9·53)	7·59 ± 0·35 (6·98–8·20)	7·67 ± 0·54 (6·94–8·67)	8·17 ± 0·52 (7·38–10·24)	7·46 ± 0·45 (6·43–8·35)
-2	8·15 ± 0·59 (7·41–10·07)	8·14 ± 0·68 (7·22–9·39)	7·77 ± 0·24 (7·33–8·33)	8·14 ± 0·52 (7·47–9·82)	8·08 ± 0·37 (7·50–8·33)
-3	8·23 ± 0·52 (7·33–9·43)	8·31 ± 0·77 (7·31–9·96)	7·78 ± 0·22 (7·33–8·33)	8·23 ± 0·56 (7·29–10·23)	8·21 ± 0·57 (7·33–9·17)
Polar Capsule -1	2·86 ± 0·24 (2·47–3·53)	2·69 ± 0·32 (2·09–3·49)	2·90 ± 0·33 (2·25–3·47)	3·17 ± 0·24 (2·62–3·57)	2·50 ± 0·20 (2·09–2·78)
Lateral Length -2	2·96 ± 0·30 (2·48–3·51)	2·83 ± 0·29 (2·43–3·43)	2·52 ± 0·18 (2·17–3·00)	2·97 ± 0·28 (2·38–3·47)	2·55 ± 0·15 (2·33–3·00)
-3	2·92 ± 0·28 (2·41–3·52)	2·99 ± 0·26 (2·58–3·43)	2·49 ± 0·13 (2·17–2·83)	2·92 ± 0·20 (2·46–3·43)	2·61 ± 0·20 (2·17–3·00)
Polar Capsule -1	2·79 ± 0·30 (2·47–3·53)	2·55 ± 0·21 (2·09–3·14)	2·76 ± 0·29 (2·25–3·47)	2·99 ± 0·18 (2·62–3·57)	2·53 ± 0·20 (2·09–2·96)
Lateral Width -2	2·91 ± 0·25 (2·68–3·51)	2·78 ± 0·30 (2·42 –3·41)	2·51 ± 0·17 (2·17–3·00)	2·91 ± 0·19 (2·45–3·46)	2·51 ± 0·18 (2·33–3·00)
-3	2·85 ± 0·26 (2·45–3·43)	2·96 ± 0·24 (2·62–3·51)	2·50 ± 0·12 (2·33–2·67)	2·89 ± 0·22 (2·42–3·43)	2·65 ± 0·17 (2·33–3·00)

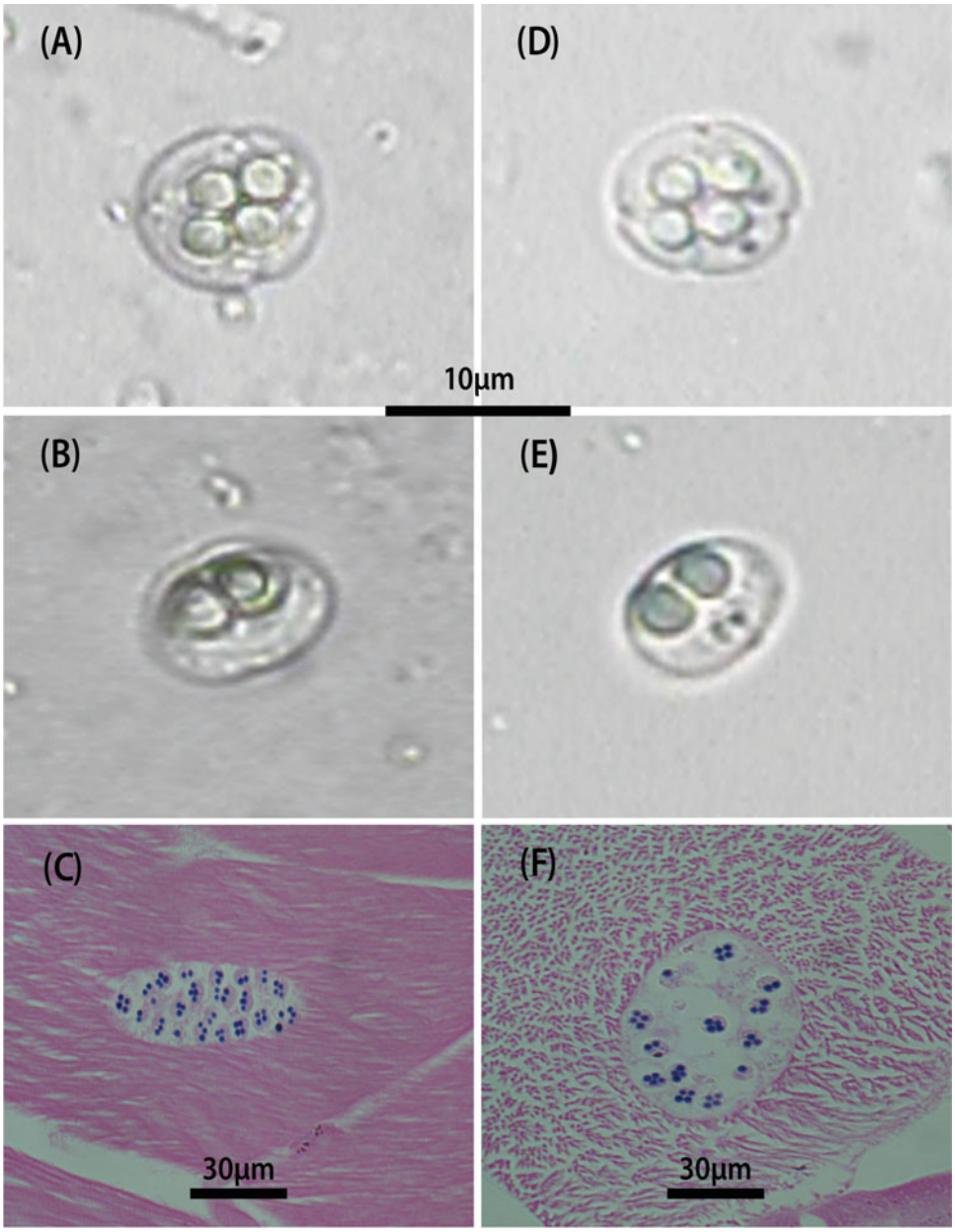

Fig. 2. Phase-contrast micrographs of fresh spore preparations and histology sections. *Kudoa hemiscylli* n. sp. ex. *Hemiscyllium ocellatum*: (A) Apical view. (B) Lateral view. (C) Giemsa and eosin-stained histological oblique section of pseudocyst. *Kudoa carcharhini* n. sp. ex. *Carcharhinus cautus*. (D) Apical view. (E) Lateral view. (F) Giemsa and eosin-stained histological transverse section of pseudocyst.

Type material: Syntypes G465391-G465393 (Giemsa and eosin-stained tissue sections) and G465394-G465395 (haematoxylin and eosin-stained tissue sections); Voucher G465396 (muscle tissue in absolute ethanol), deposited in Queensland Museum, Brisbane, Australia.

Type Host: *Hemiscyllium ocellatum* (Bonnaterre, 1788), Epaulette Shark (Elasmobranchii, Hemiscyllidae) adult.

Other Hosts: *Dasyatis fluviorum* Ogilby, 1908, Estuary stingray (Elasmobranchii, Dasyatidae) adult; *Neotrygon kuhlii* (Müller and Henle, 1841), Bluespotted stingray (Elasmobranchii, Dasyatidae) adult; *Taeniura lymma* (Forsskål, 1775), Bluespotted ribbontail ray (Elasmobranchii, Dasyatidae) adult; *Aptychotrema rostrata* (Shaw, 1794), East Australian shovelnose ray (Elasmobranchii, Rhinobatidae) adult; *Glaucostegus typus* (Bennett, 1830), Giant shovelnose ray (Elasmobranchii, Rhinobatidae) adult; *Orectolobus hutchinsi* Last and Chidlow, 2006, Western wobbegong (Elasmobranchii, Orectolobidae) adult; *Orectolobus maculatus* (Bonnaterre, 1788), Spotted wobbegong (Elasmobranchii, Orectolobidae) adult; and *Orectolobus ornatus* (de Vis, 1883), Ornate wobbegong (Elasmobranchii, Orectolobidae) adult.

Prevalence: *Hemiscyllium ocellatum* – 8 of 8 from North Heron Reef, Capricorn-Bunker Group;

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Dasyatis fluviorum – 13 of 13 from Moreton Bay; *Neotrygon kuhlii* – 3 of 34 (3 of 8 from off Lizard Island; 0 from 26 Moreton Bay); *Taeniura lymma* – 3 of 3 from off Lizard Island; *Aptychotrema rostrata* – 5 of 5 from Moreton Bay; *Glaucostegus typus* – 13 of 13 (11 of 11, North Heron Reef, Capricorn-Bunker Group; 2 of 2 from Moreton Bay); *Orectolobus hutchinsi* – 2 of 5 from off Perth, Western Australia; *Orectolobus maculatus* – 28 of 29 from Moreton Bay; and *Orectolobus ornatus* – 30 of 30 from Moreton Bay.
Type locality: North Heron Reef (23°27′S; 151°55′E), Capricorn-Bunker Group, Great Barrier Reef, Queensland, Australia.
Other Localities: Moreton Bay (27°50′S, 152°50′E), Brisbane, Queensland, Australia; off Lizard Island (14°40′S, 145°27′E), Great Barrier Reef, Queensland, Australia; and off Perth (31°57′S, 115°51′E), Western Australia, Australia.
Location in the host: Somatic muscle; pseudocysts not evident to the naked eye or under dissection microscope at a magnification of 40×.
Etymology: specific name refers to type host genus.

Taxonomic affinities. *Kudoa hemiscylli*. n.sp. is morphologically similar to *Kudoa carcharhini* n.sp., and is indistinguishable through comparison of morphometrics, and can only be separated through variation in SSU and LSU rDNA sequences. *Kudoa hemiscylli* n.sp. can be differentiated from most other similar shaped *Kudoa* species in having a larger spore size in terms of the width, thickness and length. Furthermore, it can be distinguished from similar-sized *K. crumena* (see Iversen and van Meter, 1967), *K. iwatai* (see Egusa and Shiomitsu, 1983), *K. alliaria* (see Kovaljova *et al*. 1979), in having an ellipsoid shape in lateral view rather than pyriform, and can be distinguished from *K. hypoepicardialis* (see Blaylock *et al*. 2004) in having spherical polar capsules, and occurs in muscle tissue, rather than heart tissue.

Remarks. Twenty-one SSU sequences of *Kudoa hemiscylli* n.sp. were isolated from 3 *Hemiscyllium ocellatum*, 4 *Dasyatis fluviorum*, 2 *Neotrygon kuhlii*, 2 *Taeniura lymma*, 1 *Aptychotrema rostrata*, 3 *Glaucostegus typus*, 2 *Orectolobus hutchinsi*, 1 *Orectolobus maculatus*, and 3 *Orectolobus ornatus*; and 8 LSU sequences isolated from 2 *Hemiscyllium ocellatum*, 2 *Dasyatis fluviorum*, 2 *Glaucostegus typus*, and 2 *Orectolobus ornatus*. For each isolate approximately 1500 bases of SSU rDNA and 790 bases of LSU rDNA were generated. The sequence of *Kudoa hemiscylli* n.sp. differs from other sequences of *Kudoa* spp. by 9-137 nucleotides in SSU and by 100-214 nucleotides in LSU, being most similar to *Kudoa carcharhini* n.sp.: 99·4% in SSU (GenBank Accession nos GU324968 – GU324972 from syntypes) and 87·4% in LSU (GenBank Accession nos GU446630 – GU446631 from syntypes) sequence.

Kudoa carcharhini n.sp.
(Table 3; Fig. 2D–F)

Description. Spores (Fig. 2D and E), in apical view are rounded to subquadrate, in lateral view ellipsoid. Valves, 4 equal-sized, suture lines visible, shell distal margins tapering to a point. Polar capsules, spherical, 1 per valve, located in anterior position of shell valves, occupy approximately 50% of shell valve length in apical view. Occasionally spores with 5 equal polar capsules and spore valves were observed. Spores of uniform development (i.e. no extra-sporogonic stages observed) only found in muscle of host, with no infections observed in other organs (i.e. not found in gall bladder, brain, heart, or liver). Spore measurements ($n = 30$) are shown in Table 3.

Type material: Syntypes G465397-G465399 (Giemsa and eosin-stained tissue sections) and G465400-G465401 (haematoxylin and eosin-stained tissue sections); Voucher G465402 (muscle tissue in absolute ethanol), deposited in Queensland Museum, Brisbane, Australia.
Type Host: *Carcharhinus cautus* (Whitley, 1945), Nervous shark (Elasmobranchii, Carcharhinidae) adult.
Other Hosts: *Carcharhinus amboinensis* (Műller and Henle, 1839), Pigeye shark (Elasmobranchii, Carcharhinidae) adult; and *Carcharhinus limbatus* (Műller and Henle, 1839), Black-tip Shark (Elasmobranchii, Carcharhinidae) adult.
Prevalence: *Carcharhinus cautus* – 7 of 8 from Moreton Bay; *Carcharhinus amboinensis* – 4 of 5 from Moreton Bay; *Carcharhinus limbatus* – 8 of 11 from Moreton Bay.
Type locality: Moreton Bay (27°50′S, 152°50′E), Queensland, Australia.
Location in the host: Somatic muscle; pseudocysts not evident to the naked eye or under dissection microscope at a magnification of 40×.
Etymology: specific name refers to the genus of the host.

Taxonomic affinities. *Kudoa carcharhini*. n.sp. is morphologically similar to *Kudoa hemiscylli* n.sp., and is indistinguishable through comparison of morphometrics, and can only be separated through variation in SSU and LSU rDNA sequences. *K. carcharhini* n.sp. can be differentiated from most other similar-shaped *Kudoa* species in having a larger spore size in terms of the width, thickness and length. Furthermore, it can be distinguished from similar-sized *K. crumena* (see Iversen and van Meter, 1967), *K. iwatai* (see Egusa and Shiomitsu, 1983), *K. alliaria* (see Kovaljova *et al*. 1979), in having an ellipsoid shape in lateral view rather than pyriform, and can be distinguished from *K. hypoepicardialis* (see Blaylock *et al*. 2004) in having spherical polar capsules, and occurs in muscle tissue, rather than in heart tissue.

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Remarks. Five SSU sequences were generated for *Kudoa carcharhini* n.sp. from 3 *Carcharhinus cautus*, 1 from *Carcharhinus amboinensis* and 1 from *Carcharhinus limbatus*; and 2 LSU sequences isolated from 2 *Carcharhinus cautus*. The sequence of *K. carcharhini* n.sp. differs from other aligned sequences of *Kudoa* spp. by 9-142 nucleotides in SSU and 100-202 nucleotides in LSU, and is most similar to *Kudoa hemiscylli* n.sp.: 99·4% in SSU (GenBank Accession nos GU324947 – GU324967 from syntypes) and 87·4% in LSU sequence (GenBank Accession nos GU446622 – GU446629 from syntypes).

Morphometric data of elasmobranch-infecting myxosporean species

We examined parasitic infections from 3 individuals of each of the following host species: *Hemiscyllium ocellatum; Dasyatis fluviorum; Glaucostegus typus; Orectolobus ornatus*; and *Carcharhinus cautus*. These represent 5 host families, from 3 elasmobranch orders. Table 3 lists the morphometric data from spores of infected individuals representing each host family. There was overlap in each of the measured characters between isolates. Principle component analyses (PCA) were conducted for both the apical and lateral views (Fig. 3A and B) which confirmed that there was no significant difference in morphology between isolates from different host species.

Molecular data of elasmobranch-infecting myxosporean species

Sequences of the partial SSU rDNA and partial LSU rDNA gene were generated for the kudoids from 12 (with between 1 and 4 isolates from each host species) and 5 (with 2 isolates from each host species) elasmobranch species, respectively. A BLAST search of each of the partial SSU rDNA fragments found that the closest related sequences were all kudoid myxosporeans from teleosts. A search of GenBank records of SSU rDNA found maximum homology of 95·5% with *Kudoa iwatai* (see Diamant *et al*. 2005), differing at approximately 61 base pairs along its length. Identical sequences of SSU and LSU were isolated from replicates of each host-parasite combination (i.e. no intra-isolate variation). However, significant variation (0·6–0·9% in SSU and 15·8% in LSU) occurred between isolates from the host genus *Carcharhinus* (order Carcharhiniformes) compared with isolates from the host orders Orectolobiformes and Rajiformes. In SSU an absolute difference of 9-13 nucleotides (Table 4), and LSU of 100 nucleotides (Table 5) occurred between the hosts from *Carcharhinus* and Orectolobiformes/Rajiformes. Within these groupings there was little variation, with absolute differences between host species being 0-3 base pairs in SSU and 7-24 base pairs in LSU.

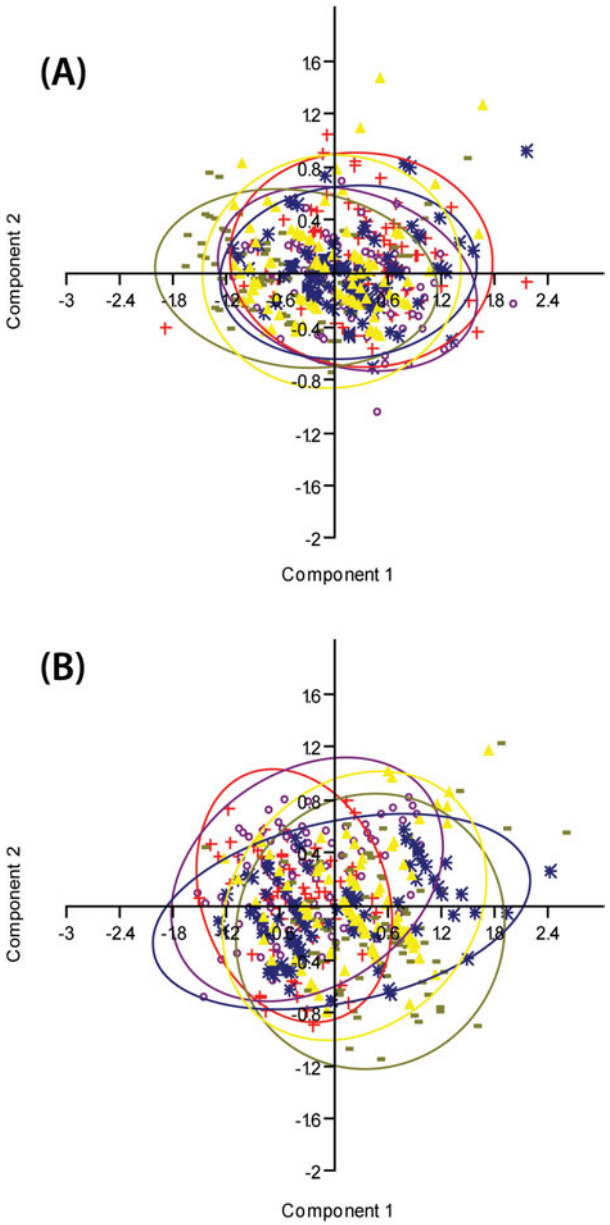

Fig. 3. Principle component analysis scatterplot with 95% confidence ellipses shown for each host-isolate combination. (A) PCA of apical view morphometrics. (B) PCA of lateral view morphometrics. For both analyses *Kudoa carcharhini* n.sp. ex. *Carcharhinus cautus* (triangles), *Kudoa hemiscylli* n.sp. ex. *Dasyatis fluviorum* (crosses), *Kudoa hemiscylli* n.sp. ex. *Glaucostegus typus* (dashes), *Kudoa hemiscylli* n.sp. ex. *Hemiscyllium ocellatum* (star) and *Kudoa hemiscylli* n.sp. ex. *Orectolobus ornatus* (circle).

The closest related myxosporean LSU sequence was that of *K. thalassomi* differing at least at 139 nucleotides (but LSU data for myxosporeans are sparse).

Phylogenetics

The kudoids sequenced from elasmobranch hosts group to the exclusion of all other kudoids, but are

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Table 4. Distance matrix of SSU rDNA sequences of a representative sample of elasmobranch kudoids (i.e. *Kudoa hemiscylli* n.sp. (*K. hemi*) and *Kudoa carcharhini* n.sp. (*K. carc*)) and *Kudoa iwatai* isolates

(Column 2 identifies host isolate. Lower triangle shows base pair differences over total of 1500. Upper triangle shows % difference. (Note isolate abbreviations: *Df-Dasyatis fluviorum; Nk-Neotrygon kuhlii; Tl-Taeniura lymma; Oh-Orectolobus hutchinsi; Oo-Orectolobus ornatus; Om-Orectolobus maculatus; Ho-Hemiscyllium ocellatum; Ar-Aptychotrema rostrata; Gt-Glaucostegus typus; Ca-Carcharhinus amboinensis; Cc-Carcharhinus cautus; Cl-Carcharhinus limbatus*).)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*K. hemi* ex. *Df*1		0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
2	*K. hemi* ex. *Nk*1	2		0	0	0	0	0	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
3	*K. hemi* ex. *Tl*1	2	0		0	0	0	0	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
4	*K. hemi* ex. *Oh*1	2	0	0		0	0	0	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
5	*K. hemi* ex. *Oo*1	2	0	0	0		0	0	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
6	*K. hemi* ex. *Om*	2	0	0	0	0		0	0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
7	*K. hemi* ex. *Ho*1	2	0	0	0	0	0		0·1	0·1	0·9	0·9	0·7	4·5	4·5	4·5
8	*K. hemi* ex. *Ar*	2	2	2	2	2	2	2		0·1	0·9	0·9	0·7	4·4	4·5	4·5
9	*K. hemi* ex. *Gt*1	1	1	1	1	1	1	1	1		0·8	0·9	0·6	4·5	4·5	4·5
10	*K. carc* ex. *Ca*1	13	13	13	13	13	13	13	13	12		0	0·2	4·4	4·5	4·4
11	*K. carc* ex. *Cc*1	13	13	13	13	13	13	13	13	12	0		0·2	4·5	4·5	4·5
12	*K. carc* ex. *Cl*	10	10	10	10	10	10	10	10	9	3	3		4·5	4·5	4·5
13	*K. iwatai* (iso. J)	63	63	63	63	63	63	63	61	61	62	62	63		0·3	0·2
14	*K. iwatai* (iso. RS2)	64	64	64	64	64	64	64	64	62	63	63	64	4		0·1
15	*K. iwatai* (iso. RS1)	63	63	63	63	63	63	63	63	61	62	62	63	3	1	

Table 5. Distance matrix of LSU rDNA sequences of elasmobranch kudoids (i.e. *Kudoa hemiscylli* n.sp. (*K. hemi*) and *Kudoa carcharhini* n.sp. (*K. carc*)) and *Kudoa thalassomi*

(Column 2 identifies host isolate. Lower triangle shows base pair differences over total of 790. Upper triangle shows % difference. (Note isolate abbreviations: Df-*Dasyatis fluviorum; Oo-Orectolobus ornatus; Ho-Hemiscyllium ocellatum; Gt-Glaucostegus typus; Cc-Carcharhinus cautus*).)

		1	2	3	4	5	6	7	8	9	10	11
1	*K. hemi* ex. *Ho*1		0	3·4	3·4	2·8	2·8	1·0	1·0	15·8	15·8	24·7
2	*K. hemi* ex. *Ho*2	0		3·4	3·4	2·8	2·8	1·0	1·0	15·8	15·8	24·7
3	*K. hemi* ex. *Gt*1	24	24		0	3·1	3·1	3·4	3·4	15·8	15·8	24·5
4	*K. hemi* ex. *Gt*2	24	24	0		3·1	3·1	3·4	3·4	15·8	15·8	24·5
5	*K. hemi* ex. *Df*1	20	20	22	22		0	2·8	2·8	15·8	15·8	24·4
6	*K. hemi* ex. *Df*2	20	20	22	22	0		2·8	2·8	15·8	15·8	24·4
7	*K. hemi* ex. *Oo*1	7	7	24	24	20	20		0	15·8	15·8	24·3
8	*K. hemi* ex. *Oo*2	7	7	24	24	20	20	0		15·8	15·8	24·3
9	*K. carc* ex. *Cc*1	100	100	100	100	100	100	100	100		0	26·2
10	*K. carc* ex. *Cc*2	100	100	100	100	100	100	100	100	0		26·2
11	*K. thalassomi*	140	140	140	140	139	139	139	139	147	147	

still nestled within the overall kudoid clade with the current data set of sequences available from GenBank. Maximum parsimony, Neighbour-joining, maximum likelihood and Bayesian analyses show similar tree topologies. Figure 4 shows the Bayesian inference analysis tree with bootstrap values of 50% or greater shown at the nodes for SSU rDNA sequences and Fig. 5 shows the Bayesian inference analysis tree for the LSU rDNA sequences.

Histology

Histological examination was conducted on infected *Hemiscyllium ocellatum, Carcharhinus cautus, Negaprion acutidens* and *Sphyrna lewini*. No obvious inflammation (as indicated by infiltration of haemocytes) was evident around pseudocysts (see Fig. 2C and F). The pseudocysts were not obvious to the naked eye or when viewed under a dissection microscope at 40× magnification. Spores were only visible under a compound microscope at higher magnifications (i.e. 200× and 400×). There was no apparent superficial variation in either the size or shape of the pseudocysts between the different hosts examined.

DISCUSSION

Infection of elasmobranchs with muscle-dwelling kudoids appears to be the norm, rather than the exception, in Australian waters; a phenomenon that may well be reflected in elasmobranch fauna globally.

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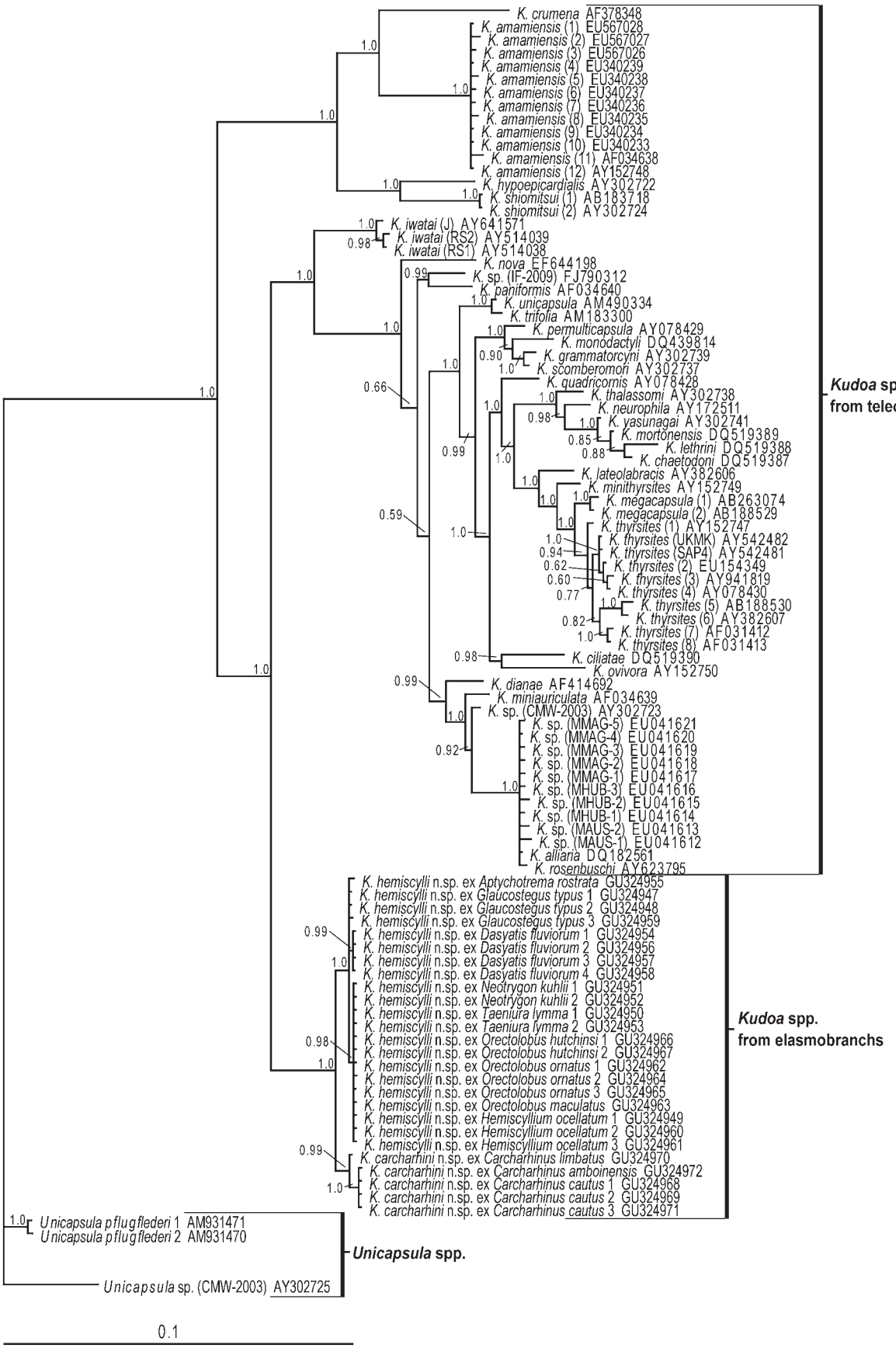

Fig. 4. Phylogenetic tree resulting from Bayesian inference analysis for the SSU rDNA dataset, conducted using 2 million generations of Markov chain Monte Carlo analysis, a set of 4 simultaneous chains with a burn-in of 3000 trees and saving current trees to file every 100 generations. Clade credibilities are indicated at branch nodes. GenBank Accession numbers follow the species name.

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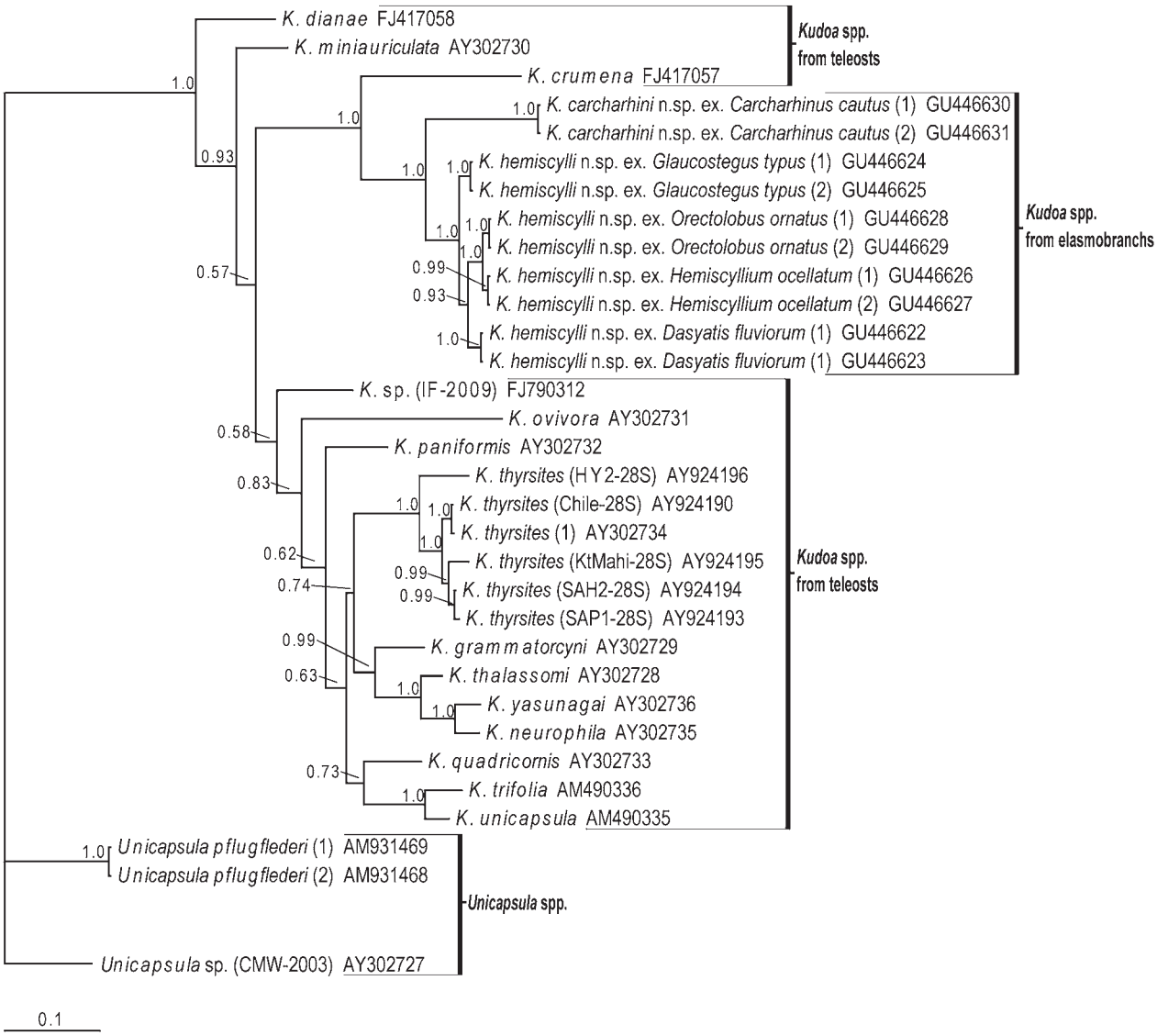

Fig. 5. Phylogenetic tree resulting from Bayesian inference analysis for the LSU rDNA dataset, conducted using 2 million generations of Markov chain Monte Carlo analysis, a set of 4 simultaneous chains with a burn-in of 3000 trees and saving current trees to file every 100 generations. Clade credibilities are indicated at branch nodes. GenBank Accession numbers follow the species name.

Our data record a prevalence of 73·6% of all adult individuals examined, representing infections in 27 (of 31) species, 7 (of 9) families, and all 3 orders of elasmobranchs. Formal descriptions of the first 2 kudoid myxosporeans from elasmobranchs are presented here, while prior to this study, only a single report of an unidentified kudoid from elasmobranchs (Heupel and Bennett, 1996) existed. It is now clear that the paucity of information on kudoid parasites of elasmobranchs represents a lack of research effort rather than an absence of parasitic fauna in these hosts. Nonetheless, the kudoids examined showed no major inflammatory response at late-stage infections in hosts, suggesting, like most members of the Myxosporea, that they cause little detrimental effect upon their host (Moran *et al*. 1999; Kent *et al*. 2001).

Characterization of species

A comparison of the kudoid spores from this study with similar 4-valved morphotypes revealed that those from elasmobranchs have generally larger spores with more spherical polar capsules than those of other kudoid species. However, morphological variation alone does not provide sufficient evidence of novelty since variability in spore morphometrics within a species has been demonstrated in many studies (Lom and Dyková, 1992; Moran *et al*. 1999). As such, molecular data were deemed critical to determine whether genotypic differences correlate with biological differences such as the identity of the host, and then assist in the proposal of new species. The recognition of novel species of parasite typically takes into account morphology

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(usually of primary importance), geography (where isolation can lead to allopatric speciation), host (where specificity can be a powerful indicator of novelty), and increasingly, molecular data which provide phenotype-independent characters for assessment. The morphology of *Kudoa hemiscylli* n.sp. and *K. carcharhini* n.sp. clearly offers no evidence for the presence of different species since they show no significant differences in either spore morphometrics or the gross size and shape of pseudocysts. The molecular data from the SSU rDNA sequence of 12 parasite isolates were targeted because they evolve slowly, are useful for examining evolutionary events (Hillis and Dixon, 1991), and have proven variable among myxozoans allowing both inter- and intraspecific relationships to be examined (Kent *et al*. 2001; Andree *et al*. 1999; Diamant *et al*. 2005; Whipps and Kent, 2006). Perhaps the most compelling reason for selection of SSU rDNA is that the majority of previous molecular studies of myxosporeans target this region and thus it provides a comparative tool for genetic assessment of species boundaries. In the absence of morphological differences these genetic data were used to identify DNA motifs that mapped to our concept of putative species. In addition, partial LSU rDNA sequences for 5 isolates were determined since it has been recognized that LSU rDNA can offer a higher level of taxonomic information than that of SSU rDNA sequence (see Burger and Adlard, 2010).

Our molecular results clearly indicate that the kudoid isolates we examined fell into 2 discrete genetic groups separated by 9-13 nucleotides in SSU rDNA and 100 nucleotides in LSU rDNA. Furthermore, these 2 genetic groups showed a within-group genetic variation of only 0-3 nucleotides in SSU and 7-24 nucleotides in LSU. Additionally, 1 genetic group (*K. hemiscylli* n.sp.) mapped only to hosts in the elasmobranch Orders Rajiformes and Orectolobiformes (i.e. *Aptychotrema rostrata, Glaucostegus typus, Dasyatis fluviorum, Neotrygon kuhlii, Taeniura lymma, Hemiscyllium ocellatum, Orectolobus hutchinsi, Orectolobus maculatus, Orectolobus ornatus*), while the other genetic group (*K. carcharini* n.sp.) was restricted to hosts in the genus *Carcharhinus* (i.e. *Carcharhinus cautus, Carcharhinus amboinensis* and *Carcharhinus limbatus*).

Further examination of geographical and host distribution data revealed that these genetic isolates from kudoids remained consistent within their host grouping both in sympatry (e.g. *K. hemiscylli* n.sp. from: *Aptychotrema rostrata; Glaucostegus typus; Orectolobus maculatus; Orectolobus ornatus*; and *Dasyatis fluviorum*; and *K. carcharini* n.sp. from: *Carcharhinus cautus; Carcharhinus amboinensis*; and *Carcharhinus limbatus* at Moreton Bay, Queensland) and in allopatry (e.g. *K. hemiscylli* n.sp. from *Glaucostegus typus* at both North Heron Reef and from Moreton Bay). Consequently, we consider that there exists clear evidence for the proposal of 2 new species of kudoid parasites from elasmobranchs, regardless of their morphological similarity.

Host specificity varies amongst kudoids, with kudoid infections being predominantly associated with a single host species or family; however, some show broader host specificity with infections across multiple families and even orders (e.g. *Kudoa thyrsites* see Whipps and Kent, 2006). *Kudoa carcharhini* n.sp. has been described from 1 genus of host (i.e. *Carcharhinus*), within, and possibly restricted to, a single host order, Carcharhiniformes. While *Kudoa hemiscylli* n.sp. has been recorded from 4 host families (i.e. Dasyatidae, Rhinobatidae, Hemiscyllidae and Orectolobidae), from 2 different host orders, Rajiformes and Orectolobiformes. However, to assess comprehensively the host specificity of these elasmobranch-infecting kudoids further host-parasite combinations need to be investigated. Once this is examined the distribution of kudoid parasites in elasmobranchs may then even inform our understanding of elasmobranch relatedness i.e. they may represent biological markers of their host's relatedness. Current elasmobranch phylogenies place the Rajiformes as a separate lineage to the Orectolobiformes and Carcharhiniformes, with the Orectolobiformes being a sister group to the Carcharhiniformes (Douady *et al*. 2003; Winchell *et al*. 2004). However, some conjecture still remains concerning the relationships of Orectolobiformes to Carcharhiniformes (Winchell *et al*. 2004).

The absence of kudoids in *Neotrygon kuhlii* individuals from Moreton Bay is intriguing. The sample size from this host from that site is relatively large (26) and provides a reasonable level of confidence in detection; at Lizard Island, 3 of 8 *N. kuhlii* were infected with *Kudoa hemiscylli*. What could drive such an apparently patchy distribution? Classically, we could explain it through geographical differences in the levels of encounter of *N. kuhlii* with infective stages of the parasite. However, individuals of *Glaucostegus typus, Dasyatis fluviorum* and *Orectolobus ornatus* collected from the same site in Moreton Bay at the same time showed kudoid infection prevalences of 100%. Does the pattern in *N. kuhlii* then reflect the development of an innate immunity in Moreton Bay populations of this species driven by high levels of transmission, or does it reflect a past mortality of infected individuals? Our data do not provide evidence either way nor indeed can we discount fine-scale patchiness in the encounter between *N. kuhlii* and infective stages of the parasite. This distributional pattern for *K. hemiscylli* remains intriguing and worthy of further investigation.

Phylogeny

Our molecular analyses included a broad range of multivalvulidans and were aimed first at confirming

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species boundaries then second to examine the phylogenetic relatedness between parasites from elasmobranchs and those reported from teleosts. The Neighbour-joining, parsimony, maximum likelihood and Bayesian analyses revealed similar topologies. The isolates from *K. hemiscylli* n.sp. and *K. carcharini* n.sp. clearly form a separate lineage to those of teleosts, but are anchored within the overall kudoid clade. From these data there is little value in speculating at the origins of muscle-dwelling kudoids, however, it is clear that radiation has occurred at a much higher level in teleosts than it has in elasmobranchs.

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