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Phylogenetic characterisation of seven *Unicapsula* spp. (Myxozoa: Myxosporea: Multivalvulida) from commercial fish in southern China and Japan

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Research Article

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Abstract

The myxozoan genus *Unicapsula* Davis, 1924 (Myxosporea: Multivalvulida: Trilosporidae) is characterized as having one functional polar capsule (PC) and two rudimentary PCs in a three-valved myxospore. The plasmodia of *Unicapsula* spp. grow either in the myofibres or in the gills, oesophageal walls and urinary organs of marine fish. Few studies have investigated the taxonomy of *Unicapsula* spp. including the type species *Unicapsula muscularis*. Accordingly, the taxonomy of the genus was explored in the present study by using 15 new isolates of seven *Unicapsula* spp. (*U. muscularis*, *U. galeata*, *U. andersenae*, *U. pyramidata*, *U. pflugfelderi*, and two new species) that had formed pseudocysts in the trunk myofibres of commercial fish collected in southern China and Japan from November 2015 to January 2019. Two new species *Unicapsula trigona* n. sp., and *Unicapsula motomurai* n. sp. exhibited unique myxospore morphologies (semi-triangular and spherical myxospores, respectively) and 18S and 28S rDNA sequences that were distinct from those of the other *Unicapsula* spp. Phylogenetic analysis of the 18S and 28S rDNA sequences confirmed the monophyletic status of *Unicapsula*.

Introduction

The myxozoan genus *Unicapsula* Davis, 1924 (Myxosporea: Multivalvulida: Trilosporidae) is characterised as having a myxospore with three unequal shell valves (SVs) and polar capsules (PCs), of which one is prominent, while the two other PCs are rudimentary (Lom and Dyková, 2006; Fiala *et al.*, 2015). The genus was erected by Davis (1924), when he found plasmodia of *Unicapsula muscularis* Davis, 1924 in the myofibres of *Hippoglossus stenolepis* Schmidt, 1904 (Pacific halibut) from the northeastern Pacific Ocean, off the coast of North America. Davis (1924) referred to infected fish as 'wormy halibut', referring to their heavy parasite load. The second species to be described, *Unicapsula galeata* Naidjenova et Zaika, 1970, was discovered in the trunk muscle of the whitesaddle goatfish *Parupeneus ciliates* (Lacepède, 1802) more than 40 years later after the erection of the genus (Naidjenova and Zaika, 1970), and a total of only 13 species have been described to date (Schubert *et al.*, 1975; Alama-Bermejo *et al.*, 2009; Miller and Adlard, 2013; Tomochi *et al.*, 2014; Yokoyama *et al.*, 2014; Al-Jufaili *et al.*, 2016). As partially reflected in a limited number (only 13) of the currently recognized *Unicapsula* spp., Miller and Adlard (2013) suspected that the richness of *Unicapsula* spp. in fish off the coast of Australia was lower than that of *Kudoa* or other speciose myxosporean genera. Indeed, Miller and Adlard (2013) examined >4500 individuals of >500 species of teleost and elasmobranch fish collected from the water around Australia and found only three species, namely *Unicapsula pyramidata* (Naidjenova et Zaika, 1970), *Unicapsula seriolae* Lester, 1982, and *Unicapsula andersenae* Miller et Adlard, 2013, and only identified the parasites in seven teleost species.

In the present study, we report seven *Unicapsula* spp. from 15 commercial fish species in southern China and Japan, grown in the periphery of Pacific Ocean (South China Sea off southern China, Philippine Sea off southern Japan and Bering Sea off Alaska) and the East Atlantic Ocean off the coast of western Africa, on the basis of myxospore morphology and ribosomal RNA gene (rDNA) sequences. In addition to providing the first molecular-genetic characterization of *U. muscularis* and *U. galeata*, which are the type and the second-described species of the genus, respectively, the present study also provides multiple new hosts and geographical records for several species. This integrated taxonomic approaches could contribute to an improved understanding of the genus' global biodiversity and prevalence.

Materials and methods

Fish samples and parasitological examination

In total 22 fish species were examined in the present study. Between November 2015 and January 2019, 317 individual fish from 20 species were purchased from wet markets in

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Zhanjiang City, Guangdong Province, China and were examined parasitologically in Guangdong Ocean University. Of these 20 species, two frozen imported fish species, the royal threadfin *Pentanemus quinquarius* (Linnaeus, 1758) and West African goatfish *Pseudupeneus prayensis* (Cuvier, 1829), had been captured from the East Atlantic Ocean, off the West African coast between Senegal and Angola (based on the species' known distribution), although exact locations were unknown. In addition to those 20 species, two individuals of the golden threadfin breams *Nemipterus virgatus* (Houttuyn, 1782) were purchased from a local fish market in Kochi, Japan, in August 2016 and were transported on ice to Yamaguchi University, and several frozen fillets of the arrow-tooth flounder *Atheresthes stomias* (Jordan et Gilbert, 1880), which had been imported from the Bering Sea, off the coast of Alaska, USA, were purchased in February 2017 from a local fish market near Yamaguchi University.

The 22 putative host fish species were identified using DNA barcoding of the mitochondrial cytochrome *c* oxidase subunit I gene (*cox-1*), as described by Ward *et al.* (2005) and Zhang and Hanner (2011). Details of amplification and sequencing of *cox-1* fragments are provided in the following section. The current taxonomic status of the fish was consulted to Prof. Hiroyuki Motomura from Kagoshima University Museum, Japan.

In the Guangdong Ocean University and Yamaguchi University laboratories, the collected fish were thawed (if necessary) and dissected, and the gills, viscera and brain were removed for microscopic examination. The fish fillets were either examined on the day of their arrival or frozen until examination. To check for the presence of myxosporean cysts or pseudocysts, thin slices of the trunk muscle were placed in physiological saline, pressed between glass plates and examined under a dissection microscope. The commercially packaged fish fillets were examined in a similar manner. The tissue-embedded myxosporean plasmodia were then divided into two groups, and fixed in either 10% neutral-buffered formalin solution, for morphological examination or 70% ethanol, for molecular analysis.

For morphological examination, the myxospores were released from the formalin-fixed tissues using fine forceps, observed using a microscope equipped with differential interference contrast imaging, and photographed at a magnification of ×800. Measurements were conducted using digital photographs and the guidelines of Lom and Arthur (1989). All measurements are expressed in μm unless otherwise stated, and are reported as ranges with means in parentheses.

In addition, the parasite specimens in fixatives were deposited in the Meguro Parasitological Museum, Tokyo, Japan (collection nos. 21381, and 21500–21514).

DNA extraction, amplification and sequencing

To determine the species identities of the putative host fish, the mitochondrial *cox1* region was sequenced for each specimen, according to Ward *et al.* (2005) and Zhang and Hanner (2011). Briefly, small pieces of the ethanol-preserved fish tissue were washed thrice in sterile pure water, and DNA was extracted using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to manufacturer instructions. Polymerase chain reaction (PCR) amplification of the *cox1* region was performed in a 20-μl reactions that contained DNA polymerase, Blend Taq-Plus- (TOYOBO, Dojima Hama, Osaka, Japan), and either of two primer pairs, FishF1 (5′- TCA ACC AAC CAC AAA GAC ATT GGC AC-3′) and FishR1 (5′- TAG ACT TCT GGG TGG CCA AAG AAT CA-3′) (Ward *et al.*, 2005) or FishF2_t1 (5′- TGT AAA ACG ACG GCC AGT CRA CYA AYC AYA AAG AYA TYG GCA C-3′) and FR1d_t1 (5′- CAG GAA ACA GCT ATG ACA CYT CAG GGT GWC CGA ARA AYC ARA A-3′) (Zhang and Hanner, 2011). The PCR was performed using the following conditions: 3 min at 94°C; followed by 40 cycles of 30 s at 94°C, 1 min at 52°C, and 1 min at 72°C; and 7 min at 72°C.

Parasite DNA was extracted from the plasmodia of each source as outlined above, and overlapping rDNA fragments were PCR amplified as described by Tomochi *et al.* (2014). The resulting PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly using the primers described by Li *et al.* (2013) and Tomochi *et al.* (2014). For the arrow-tooth flounder isolate (i.e. *U. muscularis*), two new forward primers were designed for the 5′-terminus of the 28S rDNA using a partially sequenced fragment of the isolate: KUDOA28S3F (5′- CAG GTA AGA TAA CCC GCT GAA C -3′) and KUDOA28S4F (5′- GGC TTT GAC AGA CGT ACG TG -3′). These two primers worked better for the *U. muscularis* isolate than KUDOA28S2F (5′- AGG CAA GAC TAC CTG CTG AAC -3′), which was suitable for amplifying 28S rDNA from the other isolates. When direct sequencing results were not satisfactory, purified PCR products were cloned into the pTA2 plasmid vector (TArget Clone™; TOYOBO), as described previously (Tomochi *et al.*, 2014). Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.) and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

The resulting nucleotide sequences were submitted to the DDBJ/EMBL/GenBank databases under the accession nos. LC484867–LC484883 (fish *cox-1*) and accession nos. LC316969 and LC474121–LC474141 (*Unicapsula* spp.).

Phylogenetic analysis

For phylogenetic analysis, the newly obtained 18S and 28S rDNA sequences of *Unicapsula* spp. and related sequences from the DDBJ/EMBL/GenBank databases were aligned using CLUSTAL W (Thompson *et al.*, 1994), with subsequent manual adjustment. Two sets each of nucleotide sequences of the 18S and 28S rDNA were prepared. The first set of short sequences was used to cover as much data as possible from different *Unicapsula* spp., and the second set of long sequences was used to more accurately estimate phylogenetic relationships of different *Unicapsula* spp. The accession numbers of the sequences analysed are provided in the figures showing phylogenetic trees. Regions that were poorly aligned and characters that were missing from any of the sequences were excluded from subsequent analyses. A total of 722 and 1621 characters, of which 150 and 367 were variable, respectively, remained for subsequent analysis for the 18S rDNA regions, and 540 and 2156 characters, of which 257 and 660 were variable, respectively, remained for subsequent analysis for the 28S rDNA regions. Maximum likelihood (ML) analysis was performed using PhyML (Guindon and Gascuel, 2003; Dereeper *et al.*, 2008), which is available on the 'phylogeny.fr' website (http://www.phylogeny.fr/). The probabilities of the inferred branch were assessed by using approximate likelihood-ratio test (aLRT), which is an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel, 2006).

Results

Parasitological examination

The microscopic examination of trunk muscle slices from 317 fish samples, representing 22 potential host species, revealed plasmodia and myxospores from several myxosporean genera (i.e.

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Unicapsula, *Kudoa*, *Myxobolus* and *Myxidium*) in 55 fish samples (15 species from 10 families), 83 fish samples (nine species from eight families), 42 fish samples (one species from one family) and 19 fish samples (three species from three families), respectively. Most of the fish species ($n = 18$) were only infected by a single myxosporean species. However, there were four exceptions: six myxosporean species were detected in the longfinned mullet *Moolgarda perusii* (Valenciennes, 1836), and two myxosporean species in the dotted gizzard shad *Konosirus punctatus* (Temminck et Schlegel, 1846), royal threadfin, and arrow-tooth flounder. In the latter two cases, the infection was due to different combinations of *Kudoa* spp. and *Unicapsula* spp.

As mentioned above, pseudocyst-forming plasmodia of *Unicapsula* spp. were found in the trunk muscle of 55 fish samples (Table 1), including 32 samples of the Japanese threadfin bream *Nemipterus japonicus* (Bloch, 1791), which were infected with *U. pyramidata*. The fish samples varied in the prevalence and infection intensity of *Unicapsula* plasmodia.

Morphological and genetic myxospore characterization

Myxospores in pseudocysts from the trunk muscles of the 15 fish host species had three unequal SVs and three PCs, of which two were rudimentary and one was prominent and functional with a polar filament. These observed morphological features of the myxospores were well coincident with the definition of the genus *Unicapsula* (Lom and Noble, 1984; Lom and Dyková, 2006; Alama-Bermejo *et al.*, 2009; Fiala *et al.*, 2015). Myxospore formation in plasmodia in all host fish was well synchronized. Myxospores observed in the present study were classified according to the shape and dimension of myxospores, localization and dimension of prominent PCs, and myxospore ornamentation (Figs 1 and 2). Lastly, seven *Unicapusla* spp., including two new species, were differentiated by sequencing the 18S and 28S rDNA (Table 1).

Unicapsula andersenae *(Miller et Adlard, 2013)*

This species was detected in six fish species: tigertooth croaker (Sciaenidae), silver croaker (Sciaenidae), donkey croaker (Sciaenidae), whipfin silver-biddy (Gerreidae), broadbanded velvetchin (Hapalogenyidae) and speckled tonguesole (Cynoglossidae), as shown in Table 1. Plasmodia in the myofibres were elongated, like fine threads, had tapering ends and ranged from 0.5 to 2.5 mm in length.

Bilaterally symmetrical, almost subspherical myxospores ($n =$ 33 from three fish hosts), which measured 4.6–7.0 (5.4) in length by 4.3–6.5 (5.2) in width, with a functional semi-spherical PC, which measured 1.9–2.8 (2.3) in diameter, and two smaller PCs, which measured 0.4–1.0 (0.6) in diameter (Figs 1A–F and 2A). Turns of polar filaments in prominent PCs were not discernible. The dimensions of myxospores, functional PCs and other morphological features were consistent with the known morphology of *U. andersenae* (Table 2).

Both 18S and 28S rDNA sequences were successfully obtained for six isolates (DDBJ/EMBL/Genbank accession nos. LC474121–LC474127). These sequences were sorted into three genotypes: (A) two isolates (Li1-1 and Li4-1) from the tigertooth croaker and donkey croaker; (B) one isolate (Li3-4) from the whipfin silver-biddy; and (C) three isolates (Li1-6, Li1-9 and Li4-2) from the silver croaker, speckled tonguesole and broadbanded velvetchin (see Table 3). Pairwise comparisons revealed that the 18S rDNA of the three groups differed by 0.69% (7/1736)–0.98% (17/1736) nucleotide substitutions with/without two insertion/deletions (indels) and that the 28S rDNA of the three groups differed by 1.78% (49/2752)–3.35% (92/2750) nucleotide substitutions with one to three indels. Similar genetic segregation was previously reported for the 18S and 28S rDNA sequences of Australian *U. andersenae* isolates (Table 3). The genotype A 28S rDNA sequences (Li1-1, Li4-1, CMW-2003, RJG323 and RDA832) exhibited 99.04–99.68% similarity over 628-bp length, whereas the genotype C sequences (Li1-6, Li1-9 and Li4-2, RDA3589 and RDA3271) exhibited 99.52–99.84% similarity. However, the two genotypes were less similar to each other, ranging 96.34% (605/628)–97.77% (614/628) similarity.

Remarks: This species was described by Miller and Adlard (2013) from five fish species in the families Sciaenidae, Sparidae, Polynemidae, Lutjanidae and Sillaginidae (all in the Order Perciformes) and was reported to form pseudocysts in host trunk muscles. Although the significance of genetic segregation is unknown, this phenomenon was again observed in Chinese isolates of *U. andersenae* (present study), which exhibited the morphological and genetic characteristics of the species described by Miller and Adlard (2013). Six fish species of the families Sciaenidae, Gerreidae, Hapalogenyidae and Cynoglossidae are new host records, and South China Sea, off Guangdong, China, is a new geographical record.

Unicapsula pyramidata *(Naidjenova et Zaika, 1970)*

This species was highly prevalent in the Japanese threadfin bream *Nemipterus japonicus* (Bloch, 1791) and fork-tailed threadfin bream *Nemipterus furcosus* (Valenciennes, 1830) samples that originated from the South China Sea, off the coast of Guangdong, China. The species intensively formed pseudocysts (0.8–2 mm in length with tapering ends) in host myofibres (Table 1). One of four fork-tailed threadfin bream samples was parasitised by both *U. pyramidata* and *U. galeata*, which had plasmodia of 1.43–1.91 (1.67) mm × 0.06–0.08 (0.07) mm ($n = 7$) and 0.69–1.41 (1.01) mm × 0.05–0.10 (0.07) mm ($n = 10$), respectively.

Bilaterally symmetrical triangular myxospores, which each possessed a blunt apical SV ($n = 15$ from a fork-tailed threadfin bream sample), measured 5.3–6.8 (5.8) in length by 6.3–8.4 (7.4) in width, with a functional semi-spherical PC, which measured 2.1–2.6 (2.3) in diameter, and two smaller PCs, which measured 0.6–0.8 (0.7) in diameter (Figs 1G and 2B). Two posterior SVs had pointed distal ends with caudal filamentous appendages. These morphological characters were consistent with descriptions of *U. pyramidata* from *Scolopsis monogramma* in the Coral Sea, off the coast of Lizard Island, Great Barrier Reef, Australia (Miller and Adlard, 2013); *Nemipterus japonicus* in Ha Long Bay, South China Sea (Tomochi *et al.*, 2014); and *Nemipterus japonicus* in the Indian Ocean (Naidjenova and Zaika, 1970) (Table 2).

The 18S and 28S rDNA nucleotide sequences of the *U. pyramidata* isolates from Japanese threadfin bream and fork-tailed threadfin bream (LC474128–LC474130) were highly similar to the deposited sequences of Australian and Vietnamese isolates, with few nucleotide substitutions and indels. Interestingly, a serial rDNA nucleotide sequence (including the internal transcribed spacer (ITS) regions, 5246-bp) of an isolate from a Japanese threadfin bream only differed by four substitutions and three indels and was comparable in lengths to the Vietnamese isolates (AB971675 and AB971676).

Remarks: The new *U. pyramidata* isolates from the Japanese threadfin bream and fork-tailed threadfin bream from the South China Sea were highly similar, in terms of myxospore morphology and rDNA nucleotide sequence identity, to isolates collected from the waters around Australia and Vietnam (Miller and Adlard, 2013; Tomochi *et al.*, 2014). The fork-tailed threadfin bream is a new host record for *U. pyramidata*.

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Table 1. Fish samples examined and detected *Unicapsula* spp.

Fish species (common name, scientific name and family)		Host ID	Collection date	Locality	Parasite species	Prevalence[a] (%)	DDBJ / EMBL /GenBank accession no.	
							18S rDNA	28S rDNA
Percifomes								
Tigertooth croaker	*Otolithes ruber* (Block et Schneider, 1801): Sciaenidae	Fish1-1	8 Apr 16	South China Sea, off Guangdong, China	*U. andersenae*	2 / 3 (67)	LC474121	
Silver croaker	*Pennahia argentata* (Houttuyn, 1782): Sciaenidae	Fish1-6	25 Dec 16		*U. andersenae*	1 / 15 (6.7)	LC474122	
Donkey croaker	*Pennahia anea* (Bloch, 1793): Sciaenidae	Fish4-1	28 Dec 18		*U. andersenae*	1 / 2 (50)	LC474123	
Whipfin silver-biddy	*Gerres filamentosus* Cuvier, 1829: Gerreidae	Fish3-4	30 Sep 18		*U. andersenae*	1 / 5 (20)	LC474124	
Broadbanded velvetchin	*Hapalogenys analis* Richardson, 1845: Hapalogenyidae	Fish4-2	14 Jan 19		*U. andersenae*	1 / 2 (50)	LC474125	
Japanese threadfin bream	*Nemipterus japonicus* (Bloch, 1791): Nemipteridae	Fish1-itoyori	26 Nov 15	South China Sea, off Guangdong, China	*U. pyramidata*	32 / 50 (64)	LC474128	
Fork-tailed threadfin bream	*Nemipterus furcosus* (Valenciennes, 1830): Nemipteridae	Fish3-3	28 Apr 18		*U. pyramidata*	2 /4 (50)[b]	LC474129	LC474130
					U. galeata	1 /4 (25)[b]	–	–
Golden threadfin bream	*Nemipterus virgatus* (Houttuyn 1782): Nemipteridae	Itoyoridai	17 Aug 16	Philippine Sea, off Kochi, Japan	*U. trigona* n. sp.	2 / 2 (100)	LC316969	
West African goatfish	*Pseudupeneus prayensis* (Cuvier, 1829): Mullidae	Fish2-2	3 Jan 18	Southeast Atlantic Ocean, off African coast	*U. pflugfelderi*	3 / 30 (10)	LC474131	LC474132
Royal threadfin	*Pentanemus quinquarius* (Linnaeus, 1758): Polynemidae	Fish2-1	8 Jan 18		*U. motomurai* n. sp.	2 / 40 (5)	LC474133	LC474134
Clupeiformes								
Blacktip sardinella	*Sardinella melanura* (Cuvier, 1829): Clupeidae	Fish1-3	2 Feb 17	South China Sea, off Guangdong, China	*U. galeata*	4 / 18 (22.2)	LC474135	LC474136
Common hairfin anchovy	*Setipinna tenuifilis* (Valenciennes, 1848): Engraulidae	Fish2-3	7 Nov 17		*U. galeata*	1 / 22 (4.5)	LC474137	LC474138
Hamilton's thryssa	*Thryssa hamiltonii* Gray, 1835: Engraulidae	Fish2-4	30 Nov 17		*U. galeata*	1 / 10 (10)	LC474139	LC474140
Pleuronectiformes								
Speckled tonguesole	*Cynoglossus puncticeps* (Richardson, 1846): Cynoglossidae	Fish-1-9	2 Feb 17		*U. andersenae*	1 / 2 (50)	LC474126	LC474127
Arrow-tooth flounder	*Atheresthes stomias* (Jordan et Gilbert, 1880): Pleuronectidae	Aburakarei	13 Feb 17	Bering Sea, off Alaska, USA	*U. muscularis*	– (100)[c]	LC474141	

[a]No. of positive fish samples / no. of examined fish samples (%).
[b]Coinfection by *U. pyramidata* and *U. galeata* was observed for a single fish. The rDNA sequences were not obtained for *U. galeata*.
[c]One package with several fillets.

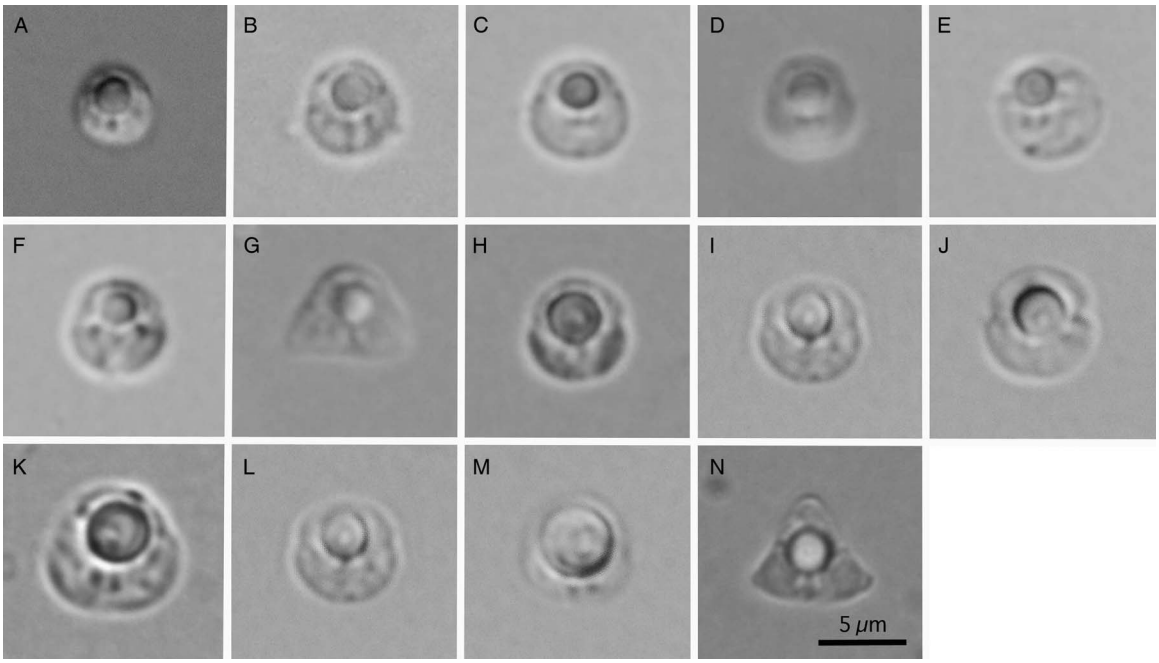

Fig. 1. Microscopic view of myxospores of *Unicapsula* spp. collected in the present study. (A–F) *Unicapsula andersenae* (A, isolate Li1-1; B, Li1-6; C, Li4-1; D, Li3-4; E, Li4-2; and F, Li1-9); (G) *Unicapsula pyramidata* (Li3-3); (H–J) *Unicapsula galeata* (H, Li1-3; I, Li2-3; and J, Li2-4); (K) *Unicapsula muscularis* (Aburakarei); (L) *Unicapsula pflugfelderi* (Li2-2); (M) *Unicapsula motomurai* n. sp. (Li2-1); and (N) *Unicapsula trigona* n. sp. (Itoyoridai). All photographs at the same magnification (scale bar in N).

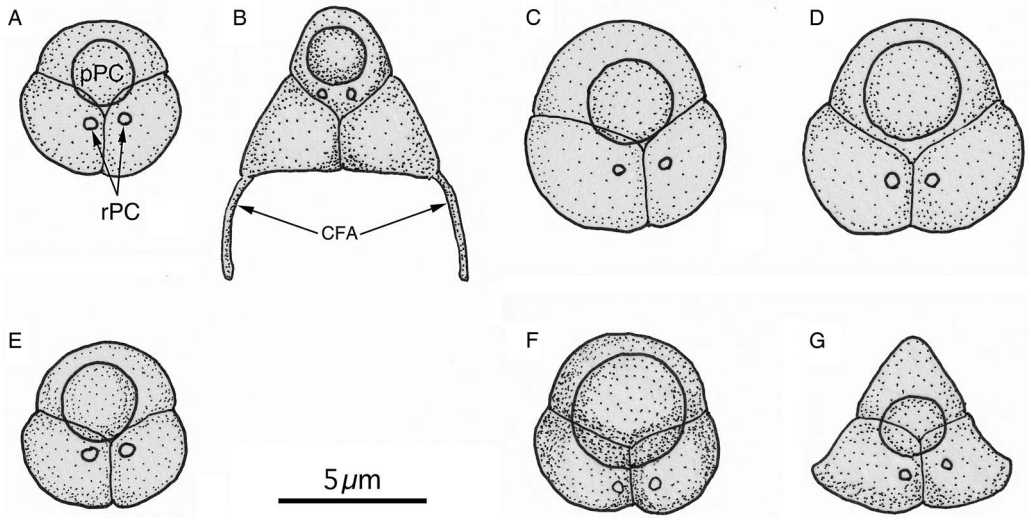

Fig. 2. Stylized diagrams of *Unicapsula* spp. collected in the present study. (A) *Unicapsula andersenae*: (B) *Unicapsula pyramidata*; (C) *Unicapsula galeata*; (D) *Unicapsula muscularis*; (E) *Unicapsula pflugfelderi*; (F) *Unicapsula motomurai* n. sp.; and (G) *Unicapsula trigona* n. sp. *Unicapsula* spores consist of three shell valves with one prominent PC (pPC) and two rudimentary PCs (rPC) as examples are shown in (A). *Unicapsula pyramidata* myxospores have caudal filamentous appendages (CFA).

Unicapsula galeata *(Naidjenova et Zaika, 1970)*

The plasmodia of this species were found in the trunk muscles of four of 18 (22.2%) blacktip sardinella samples, one of 22 common (4.5%) hairfin anchovy samples and one of 10 (10.0%) Hamilton's thryssa samples fished from the South China Sea, off the coast of Guangdong, China. As mentioned above, one of the four fork-tailed threadfin bream samples fished in the same sea area harboured plasmodia of both *U. galeata* and *U. pyramidata*.

Thread-like plasmodia, which had tapering ends, were found in trunk muscle myofibres and measured 1.06–1.73 (1.34) mm in length and 0.04–0.18 (0.10) mm in width ($n = 14$). Bilaterally symmetrical subspherical myxospores (Figs 1H–J and 2C), which were 5.6–6.7 (6.1) in length and 5.6–6.5 (6.1) in width, without caudal filamentous appendages ($n = 37$). A single functional and semi-spherical PC was 2.5–3.3 (2.8) in diameter and two smaller PCs were 0.5–0.9 (0.7) in diameter.

The 18S and 28S rDNA nucleotide sequences of isolates obtained from a common hairfin anchovy sample (Li2-3) and Hamilton's thryssa sample (Li2-4) were identical over 1692-bp and 2713-bp, respectively (LC474137–LC474140), whereas the 28S rDNA nucleotide sequence of an isolate from a blacktip sardinella sample (Li1-3) exhibited two nucleotide substitutions (LC474135 and LC474136). These sequences were distinct from the six *Unicapsula* spp. for which nucleotide data is available from DNA databases, as well as from three species that are characterized by the present study, based on the frequent occurrence of nucleotide substitutions and indels.

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Table 2. Morphological characteristics of *Unicapsula* spp. myxospores recorded from this and previous studies (measurements in μm)

Species	Isolate name	Fish host species (common name and scientific name)		Locality	Reference	N^a	Spore width	Spore length	Prominent PC (diameter)	Rudimentaryal PC (diameter)	Polar filament length	Length of caudal appendages
Unicapsula andersenae Miller et Adlard, 2013												
		Japanese meagre	*Argyrosomus japonicus*	CS	Miller and Adlard (2013)	130	4.7–6.5 (5.7)		1.4–2.9 (2.3)	0.6–1.5 (0.9)	–	No
	Li1-1	Tigertooth croaker	*Otolithes ruber*	SC	Present study	16	4.6–5.1 (4.9)	4.3–5.1 (4.6)	1.9–2.6 (2.2)	0.4–1.0 (0.6)	–	No
	Li1-6	Silver croaker	*Pennahia argentata*	SC	Present study	7	5.4–7.0 (6.1)	5.1–6.5 (5.9)	2.3–2.8 (2.5)	–	–	No
	Li3-4	Whipfin silver-biddy	*Gerres filamentosus*	SC	Present study	10	5.5–6.1 (5.7)	5.5–6.1 (5.7)	2.1–2.7 (2.3)	0.5–0.8	–	No
	Li4-1	Donkey croaker	*Pennahia anea*	SC	Present study	20	4.9–5.8 (5.5)	5.2–5.8 (5.5)	1.9–2.2 (2.0)	0.3–0.5 (0.3)	–	No
	Li4-2	Broadbanded velvetchin	*Hapalogenys analis*	SC	Present study	20	4.9–5.9 (5.4)	4.9–5.8 (5.5)	1.9–2.2 (2.1)	–	–	No
Unicapsula pyramidata (Nadjenova et Zaika, 1970)												
		Japanese threadfin bream	*Nemipterus japonicus*	IO	Naidjenova and Zaika (1970)	–	5	5–6	3	–	–	Yes
		Monogrammed monocle bream	*Scolopsis monogramma*	CS	Miller and Adlard (2013)	30	4.7–5.5 (5.1)	6.2–7.9 (7.0)	1.8–2.5 (2.3)	0.8–1.3 (1.0)	–	3.6–5.3 (4.5)
		Japanese threadfin bream	*Nemipterus japonicus*	SC	Tomochi *et al*. (2014)	24	5.5–6.4 (5.9)	5.6–9.6 (7.4)	2.0–2.4 (2.2)	0.4–0.8	–	7.2–7.4
	Fish3-3	Fork-tailed threadfin bream	*Nemipterus furcosus*	SC	Present study	15	5.3–6.8 (5.8)	6.3–8.4 (7.4)	2.1–2.6 (2.3)	–	–	Yes
Unicapsula galeata (Naidjenova et Zaika, 1970)												
		Whitesaddle goatfish	*Parupeneus ciliates*	IO	Naidjenova and Zaika (1970)	–	5	5–6	3	–	–	No
	Fish1-3	Blacktip sardinella	*Sardinella melanura*	SC	Present study	17	5.6–6.5 (6.0)	5.6–6.5 (6.0)	2.6–3.3 (2.9)	–	–	No
	Fish2-3	Common hairfin anchovy	*Setipinna tenuifilis*	SC	Present study	20	5.6–6.7 (6.2)	5.6–6.5 (6.1)	2.5–3.2 (2.7)	0.5–0.9 (0.7)	–	No
	Fish2-4	Hamilton's thryssa	*Thryssa hamiltonii*	SC								
Unicapsula muscularis Davis, 1924												
		Pacific halibut	*Hippoglosus stenolopis*	BS	Davis (1924)	–	6		3	–	–	No
	Aburakarei	Arrow-tooth flounder	*Atheresthes stomias*	BS	Present study	25	6.3–7.8 (7.1)	5.8–7.6 (6.5)	3.0–3.7 (3.3)	0.5–0.8 (0.7)	9.2–14.6 (11.8)	No
Unicapsula pflugfelderi Alama-Bermejo *et al*., 2009												
		Sand steenbras	*Lithognathus mormyrus*	MS	Alama-Bermejo *et al*. (2009)	30	4.6–6.1 (5.4)	5.2–6.7 (6.0)	2.2–2.9 (2.6)	0.8–1.0 (0.9)	6.4–9.5 (8.2)	No
		Picarel	*Spicara smaris*	MS		30			2.2–2.9 (2.5)	0.8–1.1 (0.9)	6.1–9.0 (7.5)	No

(*Continued*)

Table 2. (*Continued.*)

Species	Isolate name	Fish host species (common name and scientific name)		Locality	Reference	N^a	Spore width	Spore length	Prominent PC (diameter)	Rudimentaryl PC (diameter)	Polar filament length	Length of caudal appendages
					Alama-Bermejo *et al.* (2009)		4.7–5.7 (5.2)	5.3–6.6 (6.0)				
	Fish2-2	West African goatfish	*Pseudupeneus prayensis*	EAO	Present study	20	5.1–6.9 (5.9)	5.5–6.7 (6.1)	2.2–2.9 (2.5)	0.8–0.9 (0.8)	–	No
Unicapsula motomurai n. sp.												
	Fish 2-1	Royal threadfin	*Pentanemus quinquarius*	EAO	Present study	20	6.1–7.5 (6.7)	6.1–7.4 (6.6)	3.8–4.4 (4.1)	0.6–0.9 (0.7)	–	No
Unicapsula trigona n. sp.												
	Itoyoridai	Golden threadfin bream	*Nemipterus virgatus*	PS	Tomochi *et al.* (2014)	20	4.4–5.3 (4.9)	5.8–7.8 (6.7)	2.2–3.0 (2.5)	1.1–1.7 (1.4)	–	No
Unicapsula seriolae Lester, 1982												
		Yellowtail amberjack	*Seriola lalandi*	CS	Lester (1982)	10	4.9–6.3 (5.6)		2.1–3.5 (2.9)	–	14–21 (18.5)	No
		Yellowtail amberjack	*Seriola lalandi*	CS	Miller and Adlard (2013)	30	5.2–6.6 (6.1)		2.2–3.9 (3.4)	–	–	No
		Greater yellowtail	*Seriola dumerili*	JS (farmed)	Tomochi *et al.* (2014)	20	5.9–7.4 (6.9)	6.3–7.4 (6.9)	3.4–3.8 (3.6)	0.7–1.0 (0.9)	20.3–26.6 (24.5)	No
Unicapsula setoensis Tomochi *et al.*, 2014												
		Yellowfin goby	*Acanthogobius flavimanus*	ISJ	Tomochi *et al.* (2014)	20	5.6–6.9 (6.2)		1.9–2.5 (2.1)	1.1–1.7 (1.4)	9.4–13.8 (11.7)	No
Unicapsula marquesi Diebakate *et al.*, 1999												
		Giant African threadfin	*Polydactylus quadrifilis*	EAS	Diebakate *et al.* (1999)	–	6.1	7.2	3	–	–	No
Unicapsula chirocentrusi Sarkar, 1984												
			Chirocentrus dorab	IO	Sakar (1984)	–	6.0–6.9 (6.4)		3.2–3.8	–	–	No
Unicapsula maxima Sarkar, 1999												
		Ganges Jew fish	*Nibea coibor*[b]	IO	Sakar (1999)	–	10.0–14.4 (12.3)	9.0–11.0	3.2–4.5 (3.8)	–	–	No
Unicapsula fatimae Al-Jufaili *et al.*, 2016												
		White-spotted spinefoot	*Siganus canaliculatus*	AS	Al-Jufaili *et al.* (2016)	41	5.6–6.6 (6.2)	6.1–7.4 (6.8)	2.3–2.9(2.7)	–	11.7–20.0 (15.5)	No
Unicapsula pacifica Aseeva et Krasin, 2001												
		Giant grenadier	*Albatrossia pectoralis*[c]	OS	Aseeva and Krasin (2001)	–	7.3–8.6	3.9–4.0	–	–	No	No
Unicapsula schulmani Aseeva et Krasin, 2001												
		Giant grenadier	*Albatrossia pectoralis*[c]	OS	Aseeva and Krasin (2001)	–	7.8–10.3		2.8–3.8	–	–	No

AS, Arabian Sea; BS, Bering Sea; CS, Coral Sea (Australia); EAO, East Atlantic Ocean; IO, Indian Ocean; ISJ, Inland Sea of Japan; JS, Japan Sea; MS, Mediterranean Sea; OS, Okhotsk Sea; PS, Philippine Sea. off Kochi, Japan; and SC, South China Sea. PC, polar capsule; PF polar filament; and CA, caudal appendages.

[a]Number of spores measured. '–' means no data.

[b]*Nibea coibor* (Hamilton, 1822); Syn. *Pseudosciaena coibar* (Hamilton, 1822)

[c]*Albatrossia pectoralis* (Gilbert, 1892); Syn. *Coryphaenoides pectoralis* (Gilbert, 1892)

Table 3. Nucleotide differences in the 18S and 28S rDNA of *Unicapsula andersenae isolates from* different locations

(1) 18S rDNA[a]																										
Host fish (family) locality and isolate names[b]	Accession no.	Length (bp)[c]	192	200	201	202	204	205	209	211	643	675	697	905	1048	1105	1219	1319	1436	1624	1625	1638	1643	1646	1670
Pennahia anea (Sciaenidae) CN_Li4-1	LC474123	1738	C	T	G	T	A	A	A	T	G	C	C	A	T	T	G	G	C	T	T	A	T	T	A
Otolithes ruber (Sciaenidae) CN_Li1-1	LC474121	1738	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Argyrosomus japonicus (Sciaenidae) AU	AY302725	1679	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Acanthopagrus australis (Sparidae) AU_ RJG323	KF184378	1388	·	·	A	·	·	·	·	C	A	·	·	·	·	·	·	·	·						
Eleutheronema tetradactylum (Polynemidae) AU_ RDA832	KF184382	1027									A	·	·	·	·	·	·	·	·						
Gerres filamentosus (Gerreidae) CN_Li3-4	LC474124	1738	T	·	·	·	·	·	G	·	A	·	·	G	·	G	·	·	G	·	·	·	·	·	G
Pennahia argentata (Sciaenidae) CN_Li1-6	LC474122	1736	·	C	A	–	T	T	·	·	A	T	T	·	G	·	·	A	·	A	C	G	–	C	G
Cynoglossus puncticeps (Cynoglossidae) CN_Li1-9	LC474127	1715	·	C	A	–	T	T	·	·	A	T	T	·	G	·	Y	A	·	A	C	G	–	C	G
Hapalogenys analis (Hapalogenydae) CN_Li4-2	LC474125	1715	·	C	A	–	T	T	·	·	A	T	T	·	G	·	·	A	·	A	C	G	–	C	G
Lutjanus russellii (Lutjanidae) AU_ RDA3589	KF184380	1025									A	T	T	·	G	·	·	A	·						
Sillago ciliata (Sillaginidae) AU_ RDA3271	KF184381	1017									A	T	T	·	G	·	·	A	·						

(*Continued*)

Table 3. (*Continued.*)

(2) 28S rDNA[a]																														
Host fish (family) locality, and isolate names[b]	Accession no.	Length (bp)[c]	2	28	61	66	138	139	140	187	298	447	459	475	476	483	487	491	492	505	507	528	532	551	553	556	558	559	562
Pennahia anea (Sciaenidae) CN_Li4-1	LC474123	2752	A	C	A	A	T	C	G	A	A	C	A	C	T	T	G	A	G	A	G	A	A	G	T	C	A	C	C
Otolithes ruber (Sciaenidae) CN_Li1-1	LC474121	1994	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Argyrosomus japonicus (Sciaenidae) AU_ CMW-2003	AY302727	679					·	·	·	·	·	·	·	·	·	·	A	·	A	·	·	·	·	C	·	·	·	·	Y
Acanthopagrus australis (Sparidae) AU_ RJG323	KF184374	628					·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	G	T	·
Eleutheronema tetradactylum (Polynemidae) AU_ RDA832	KF184375	628					·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	C	·	·	·	·	·
Gerres filamentosus (Gerreidae) CN_Li3-4	LC474124	2750	·	T	·	·	·	·	·	G	T	T	·	·	·	·	·	·	·	·	A	T	T	C	A	T	A	T	T
Pennahia argentata (Sciaenidae) CN_Li1-6	LC474122	2753	T	·	·	·	C	T	A	·	·	·	G	T	C	C	·	G	A	T	·	·	·	C	·	·	·	T	T
Cynoglossus puncticeps (Cynoglossidae) CN_Li1-9	LC474127	2718			·	·	C	T	A	·	·	·	G	T	C	C	·	G	A	W	·	·	·	C	·	·	·	·	·
Hapalogenys analis (Hapalogenydae) CN_Li4-2	LC474125	2753	T	·	T	C	C	T	·	·	·	·	G	T	C	C	·	G	A	T	·	·	·	C	·	·	·	·	·
Lutjanus russellii (Lutjanidae) AU_ RDA3589	KF184372	628					C	T	A	·	·	·	G	T	C	C	·	G	A	·	·	·	·	C	·	·	·	·	·
Sillago ciliata (Sillaginidae) AU_ RDA3271	KF184373	628					C	T	A	·	·	·	G	T	C	C	·	G	A	·	·	·	·	C	·	·	·	·	·

Host fish (family) locality, and isolate names[b]	Accession no.	Length (bp)[c]	563	564	565	569	570	572	574	576	590	599	600	614	615	618	641	654	657	668	669	690	704	705	709	715	718	745
Pennahia anea (Sciaenidae) CN_Li4-1	LC474123	2752	C	T	A	G	G	T	C	G	A	A	T	T	G	A	G	A	C	C	C	A	C	T	C	G	G	T
Otolithes ruber (Sciaenidae) CN_Li1-1	LC474121	1994	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Argyrosomus japonicus (Sciaenidae) AU_ CMW-2003	AY302727	679	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·
Acanthopagrus australis (Sparidae) AU_ RJG323	KF184374	628	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	T	·	·	·		
Eleutheronema tetradactylum (Polynemidae) AU_ RDA832	KF184375	628	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·		
Gerres filamentosus (Gerreidae) CN_Li3-4	LC474124	2750	·	–	–	T	A	C	T	C	G	C	·	·	A	·	A	T	·	T	·	G	·	A	·	A	A	C
Pennahia argentata (Sciaenidae) CN_Li1-6	LC474122	2753	T	·	·	·	·	·	·	·	G	·	A	A	·	T	·	T	T	·	·	·	·	A	·	·	·	·
Cynoglossus puncticeps (Cynoglossidae) CN_Li1-9	LC474127	2718	T	·	·	·	·	·	·	·	G	·	A	A	·	T	·	T	T	·	T	·	·	A	·	·	·	·
Hapalogenys analis (Hapalogenydae) CN_Li4-2	LC474125	2753	T	·	·	·	·	·	·	·	G	·	A	A	·	T	·	T	T	·	·	·	·	A	·	·	·	·
Lutjanus russellii (Lutjanidae) AU_ RDA3589	KF184372	628	·	·	·	·	·	·	·	·	G	·	C	A	·	T	·	T	T	·	·	·	·	A	·	·		
Sillago ciliata (Sillaginidae) AU_ RDA3271	KF184373	628	T	·	·	·	·	·	·	·	G	·	C	A	·	C	·	T	T	·	·	·	·	A	·	·		

[a]Nucleotide position relative to the 5′-terminus of the 18S and 28S rDNA sequences of *Unicapsula andersenae* Li4-1 (LC474123). Dots denote that a base was identical to the base of the uppermost nucleotide sequence and blanks indicate no available data. "– " indicates a nucleotide deletion.
[b]Locality is expressed by country name (CN, China; and AU, Australia).
[c]Lengths of the 18S and 28S rDNA sequences from the DDBJ/EMBL/GenBank databases are expressed in base pairs (bp).

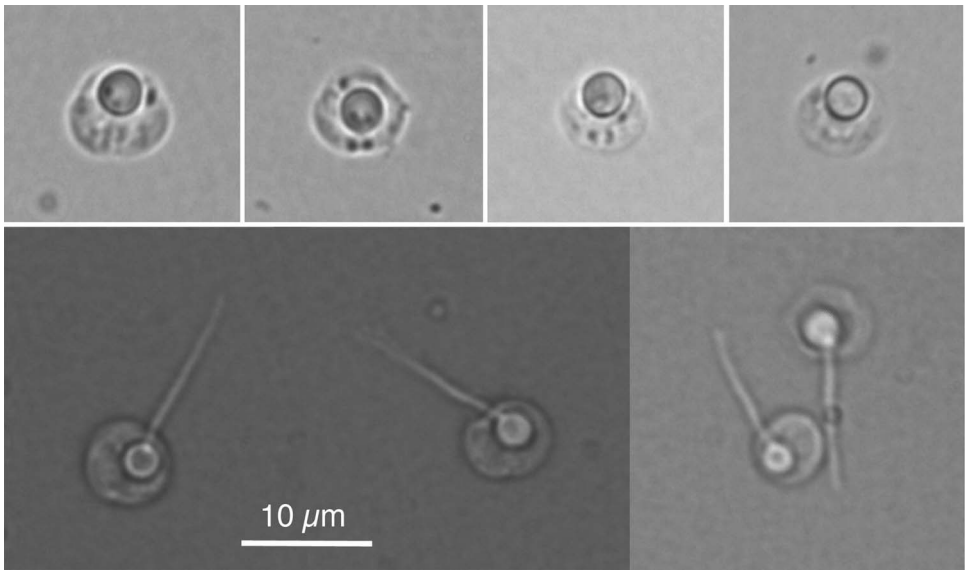

Fig. 3. Microscopic view of myxospores of *Unicapsula muscularis* (isolate Aburakarei) collected in the present study.

Remarks: This species was originally described from the trunk muscle of the whitesaddle goatfish *Parupeneus ciliates* (Lacepède, 1802) (syn. *Pseudopeneus pleurotaenia* and *Parupenes ciliates*), which was collected from the Indian Ocean by Naidjenova and Zaika (1970). The myxospore morphology, dimensions (5 × 5–6 μm), and prominent PC diameter (3 μm) of that isolate are consistent with those of myxospores obtained in the present study (Figs 1H–J and 2C; Table 2). The whitesaddle goatfish is widely distributed in the Indo-Pacific Ocean, which ranges from the western Indian Ocean to Japan and to northern Australia. The marine distributions of the fish host species identified by the present study (i.e. blacktip sardinella, hairfin anchovy and Hamilton's thryssa) overlap with that of the *U. galeata* type host. Although it is necessary to genetically confirm the conspecificity of *U. galeata* from its type host at the type locality and the *U. galeata* isolates analysed by the present study, it is possible (1) that *U. galeata* is capable of using fish hosts from different families (e.g. Mullidae, Clupeidae, Engraulidae and Nemipteridae), as demonstrated for *U. andersenae*, which was recorded from members of the Sciaenidae, Sparidae, Polynemidae, Lutjanidae, Silaginidae, Cynoglossidae, Hapalogenyidae and Gerreidae (Miller and Adlard, 2013; present study), and (2) that *U. galeata* is widely distributed in the Indo-Pacific Ocean, along with its host fish, as demonstrated for *U. pyramidata* (Miller and Adlard, 2013; Tomochi *et al.*, 2014; present study).

Unicapsula muscularis *(Davis, 1924)*

This species was found in imported arrow-tooth flounder, *Atheresthes stomias* (Jordan et Gilbert, 1880) that was purchased from a fish market near Yamaguchi University. The fish originated in the Bering Sea, off the coast of Alaska, USA. Fish that were packed similarly and purchased on different occasions were consistently infected with plasmodia of *Unicapsula* and *Kudoa* species. Then, we supposed a high prevalence of these two multivalvulid species in the trunk muscles of arrow-tooth flounder, as recorded by Davis (1924) in the trunk muscles of the Pacific halibut *Hippoglosus stenolopis* Schmidt, 1904, from the Pacific Ocean off the coast of North America. In the myofibres of arrow-tooth flounder, *Unicapsula* plasmodia were 2.71–9.94 (6.65) mm in length ($n = 15$), whereas the *Kudoa* plasmodia were 5.25–13.78 (7.87) mm in length ($n = 15$). No host responses, such as leukocyte accumulation or tissue degeneration, were observed.

Bilaterally symmetrical, almost subspherical myxospores ($n = 25$) measured 5.8–7.6 (6.5) in length by 6.3–7.8 (7.1) in width, with a functional semi-spherical PC measuring 3.0–3.7 (3.3) in diameter, and two smaller PCs measuring 0.5–0.8 (0.7) by 0.5–0.6 (0.5) (Figs 1K, 2D and 3). Turns of polar filaments in prominent PCs were not discernable, and the polar filaments ($n = 10$) were 9.2–14.6 (11.8) in length. The dimensions of the myxospores and functional PCs of the present specimens were consistent with those of *U. muscularis* (Table 2), which is the type species of the genus. However, the species' original description was concise, and no additional description has been made on the species' myxospore morphology.

A serial rDNA nucleotide sequence (5117-bp) was obtained from the *U. muscularis* plasmodia recovered from the arrow-tooth flounder. The sequence contained 1706-bp partial 18S rDNA, 300-bp ITS-1, 157-bp 5.8S rDNA, 253-bp ITS-2 and 2701-bp partial 28S rDNA sequences (DDBJ/EMBL/GenBank accession no. LC474141). The 18S rDNA nucleotide sequence of *U. muscularis* was most similar to sequences of *Unicapsula trigona* n. sp., which was erected in the present study [LC316969; 93.13% (1586/1703) with three indels], *U. pyramidata* [AB971675 and AB971676; 92.78% (1580/1703) with three indels] and *U. setoensis* [AB971679; 92.72% (1580/1704) with two indels], followed by sequences from another *Unicapsula* spp. The 28S rDNA nucleotide sequence of *U. muscularis* was similar to that of the *Unicapsula* spp. mentioned above, but by less than 91%.

Remarks: Although *U. muscularis* is well-known as the type species of the genus (Lom and Dyková, 2006) and is the cause of 'wormy halibut' (Davis, 1924), little is known about its parasitism and biology. After our first observation of multivalvulid parasitism in commercially available arrow-tooth flounder, we examined several packages of the imported fish on different occasions and constantly found the trunk muscle myofibres of the host species contained *Unicapsula* and *Kudoa* plasmodia. This infection status is very similar to the infection of these two species in the Pacific halibut reported by Davis (1924). Morphological characteristics of *Unicapsula* and *Kudoa* myxospores observed in the arrow-tooth flounder are also similar to those of these two species found in the Pacific halibut reported by Davis (1924). Based on these circumstantial supports, the *Unicapsula* species found in the arrow-

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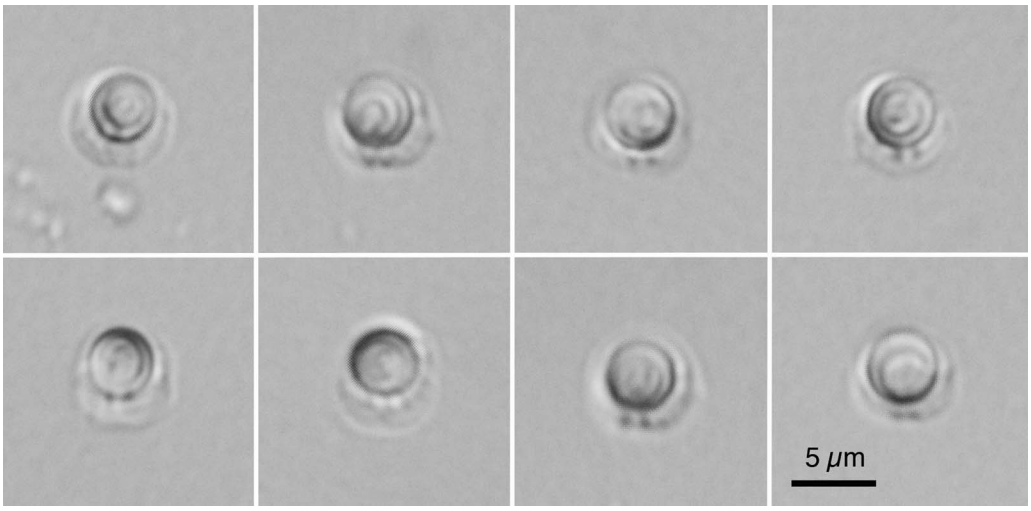

Fig. 4. Microscopic view of myxospores of *Unicapsula motomurai* n. sp. (isolate Li2-1) collected in the present study.

tooth flounder is speculated to be *U. muscularis* described from the Pacific halibut *Hippoglosus stenolopis* Schmidt, 1904, from the Pacific Ocean off the coast of North America. The rDNA sequences of *U. muscularis* was similar to but distinct from, those of congeners with available rDNA sequences, such as *U. andersenae*, *U. fatimae*, *U. pflugfelderi*, *U. pyramidata*, *U. seriolae*, *U. setoensis* and *Unicapsula* spp., for which rDNA nucleotide sequences were newly obtained in the present study (*U. galeata*, *U. trigona* n. sp. and *U. motomurai* n. sp.).

Unicapsula pflugfelderi *(Alama-Bermejo* et al.*, 2009)*

This species was found extensively in the trunk muscle myofibres of three imported samples of the West African goatfishes *Pseudupeneus prayensis* (Cuvier, 1829). The pseudocysts ranged 0.5–2 mm in length and exhibited tapering ends.

Bilaterally symmetrical, almost subspherical myxospores (n = 20), which measured 5.1–6.9 (5.9) in length by 5.5–6.7 (6.1) in width, with a functional semi-spherical PC, which measured 2.2–2.9 (2.5) in diameter and two smaller PCs, which measured 0.8–0.9 (0.8) in diameter (Figs 1L and 2E). Turns of polar filaments in prominent PCs were not discernable.

Partial 18S rDNA (1690-bp) and partial 28S rDNA (2702-bp) sequences were obtained for the present isolate (DDBJ/EMBL/GenBank accession nos. LC474131 and LC474132). Parts of these sequences were highly similar to the partial 18S and 28S rDNA nucleotide sequence of *U. pflugfelderi*; 100% over 726-bp (AM931470) and 722-bp (AM931471), excluding primer-annealing sequences and 98.65% (586/594) with the 28S rDNA sequence (AM831468).

Remarks: As indicated by Miller and Adlard (2013), it seems impossible to differentiate *U. pflugfelderi* and *U. andersenae* on the basis of morphology when using light microscopy. However, the species can be easily distinguished on the basis of their 18S and 28S rDNA nucleotide sequences. These two species are currently distributed in different oceans. The West African goatfish is the new host record for *U. pflugfelderi*.

Unicapsula motomurai *n. sp. (Myxozoa: Myxosporea: Multivalvulida)*

This species was found in the trunk muscles of two of 40 (5%) samples of the royal threadfins *Pentanemus quinquarius* (Linnaeus, 1758), which had been imported from the Southeast Atlantic Ocean, off the coast of West African, between Senegal and Angola. However, the exact fishing point of the examined fish was unknown. The species possessed remarkably large functional PCs, relative to the myxospore dimensions.

Description (Figs 1M, 2F and 4; Table 2)

Thread-like plasmodium with tapering ends in the myofibres of trunk muscles, measuring 0.66–1.42 (1.01) mm in length, and 0.04–0.09 (0.06) mm in width (n = 9). Bilaterally symmetrical subspherical myxospores (n = 20), measuring 6.1–7.5 (6.7) in length by 6.1–7.4 (6.6) in width, with a functional semi-spherical PC measuring 3.8–4.4 (4.1) in diameter and two smaller PCs measuring 0.6–0.9 (0.7) in diameter.

The partial 18S rDNA nucleotide sequence of 1709-bp length and partial 28S rDNA of 2702-bp length (DDBJ/EMBL/GenBank accession nos. LC474133 and LC474134, respectively) were most similar to the 18S and 28S rDNA sequences of *U. pflugfelderi*: 99.10% (1654 / 1669) and 96.15% (2596 / 2700) with two indels, respectively.

Taxonomic summary

Host: *Pentanemus quinquarius* (Linnaeus, 1758), royal threadfin (Actinopteryii: Perciformes: Polynemidae).

Locality: Southeast Atlantic Ocean, off the coast of West Africa.
Site of infection: Pseudocysts in trunk muscle myofibres.

Materials deposited: Hapantotype no. 21510, Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: Two of 40 examined fish, but the high intensity in infected fish.

Etymology: The species name is dedicated to Prof. Hiroyuki Motomura (Kagoshima University Museum), who helped identify the fish samples used in the present study, especially threadfins, based on his comprehensive research and review of family Polynemidae (Motomura, 2004).

Remarks: The most conspicuous traits of this new species are its relatively large dimensions and the ratio of its prominent PCs [3.8–4.4 μm; average 4.1 μm (n = 20)] to its myxospores. Similar feature is observed for *U. chirocentrusi* Sarkar, 1984 (prominent PCs, 3.2–3.8 μm) from the trunk muscle of *Chirocentrus dorab* (Forsskål, 1775) in the Indian Ocean; for *U. maxima* Sarkar, 1999 (prominent PCs, 3.2–4.5 μm; average 3.8 μm) from the

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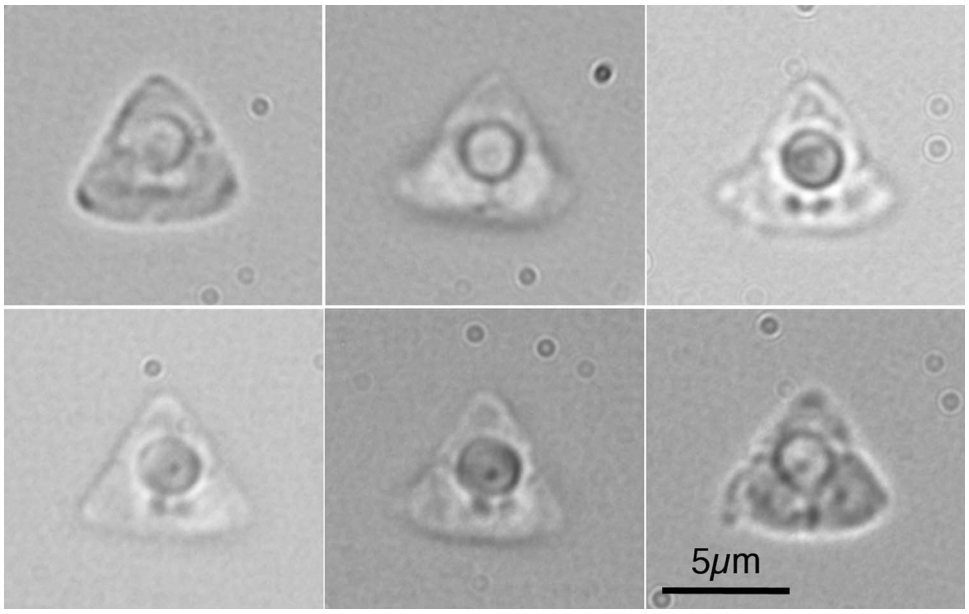

Fig. 5. Microscopic view of myxospores of *Unicapsula trigona* n. sp. (Itoyoridai) collected in the present study.

renal parenchyma of *Nibea coibor* (Hamilton, 1822) [syn. *Pseudosciaena coibar* (Hamilton, 1822)] in the Indian Ocean; and for both *U. pacifica* Aseeva et Krasin, 2001 (prominent PCs, 3.9–4.0 μm) and *U. schulmani* Aseeva et Krasin, 2001 (prominent PCs, 2.8–3.8 μm) in the urinary bladder of *Albatrossia pectoralis* (Gilbert, 1892) [Syn. *Coryphaenoides pectoralis* (Gilbert, 1892)] from the Okhotsk Sea (Table 2). All these species mentioned above were recorded from fish hosts in the Indo-Pacific Ocean and Okhotsk Sea, different from the royal threadfin with *U. motomurai* n. sp., which were found to be distributed in the Eastern Atlantic Ocean. The organ/tissue specificity of *U. maxima* and *U. schulmani* is distinct from other species that form pseudocysts in trunk muscle myofibres. Furthermore, *Unicapsula* spp., except for *U. chirocentrusi,* possess myxospores that are larger than those of *U. motomurai* n. sp. (⩾7.3 μm *vs* ⩽7.5 μm), which reduces the ratio of prominent PCs to myxospores (Table 2). The myxospore dimensions of *U. chirocentrusi* are almost similar to those of *U. motomurai* n. sp. However, the dimensions of prominent PCs are smaller (3.2–3.8 μm *vs* 3.8–4.4 μm). Since the present study generated sufficiently long 18S and 28S rDNA nucleotide sequences for *U. motomurai* n. sp., molecular-genetic characterization, along with an intensive morphological comparison with *Unicapsula* spp. (which possess morphologically similar myxospores), enables us to identify the new species.

The new species described here, *U. motomurai* n. sp., is the third *Unicapsula* species, after *U. pflugfelderi* and *U. marquesi* Diebakate *et al.*, 1999, to be reported from fish distributed in the Eastern Atlantic Ocean.

Unicapsula trigona *n. sp.* *(Myxozoa: Myxosporea: Multivalvulida)*

The thorough examination of almost all trunk muscles of two golden threadfin bream samples, with body weights of 917 g and 1174 g, revealed five and 19 plasmodia, respectively. The shortest plasmodium measured 0.73 mm in length and 0.14 mm in width, whereas the longest one measured 6.98 mm in length and 0.17 mm in width. Triangular myxospores resembled the myxospore morphology of *U. pyramidata*. However, the two species could be differentiated by morphological and genetic differences.

Description (Figs 1N, 2G and 5; Table 2)

Thread-like plasmodium with tapering ends in trunk muscle myofibres, measuring 0.73–6.98 (2.56) mm in length and 0.09–0.29 (0.16) mm in width ($n = 11$). Bilaterally symmetrical triangular myxospores with a fairly pointed apical corner and rounded caudal corners, formed by an apical SV and two caudal SVs, respectively; 4.4–5.3 (4.9) in length and 5.8–7.8 (6.7) in width, without caudal filamentous appendages ($n = 20$). A functional semi-spherical PC measuring 2.2–3.0 (2.5) in diameter, and two smaller PCs measuring 1.1–1.7 (1.4) in diameter, located closely at the centre of myxospores.

The rDNA sequence of the present isolate was 5240-bp in length, including 1708-bp partial 18S rDNA, 404-bp ITS-1, 156-bp 5.8S rDNA, 270-bp ITS2 and 2702-bp partial 28S rDNA sequences (DDBJ/EMBL/GenBank accession nos. LC316969). On the basis of both morphological and genetic characteristics, this new species was most similar to *U. pyramidata*: 18S rDNA, 99.24% (1695/1708); 5.8S rDNA, 99.36% (155/156); and 28S rDNA, 98.74% (2664/2698), when compared with a deposited rDNA sequence of *U. pyramidata* (AB971676; 5249-bp). Identical nucleotides occurred at less than 70% of sites for both the ITS-1 and ITS-2 regions, with multiple indels over 52-bp and 38-bp lengths, respectively.

Taxonomic summary

Host: *Nemipterus virgatus* (Houttuyn, 1782), golden threadfin bream (Actinopteryii: Perciformes: Nemipteridae).

Locality: Philippine Sea, off the coast of Kochi Prefecture, Japan. Site of infection: Pseudocysts in the trunk muscle myofibres.

Materials deposited: Hapantotype no. 21381, Meguro Parasitological Museum, Tokyo, Japan.

Prevalence: Two of two fishes examined, with low intensity (several plasmodia/fish) as far as examined.

Etymology: The species name refers to its triangular myxospore shape.

Remarks: Of the 13 known and two novel *Unicapsula* spp., only *U. pyramidata* and *Unicapsula trigona* n. sp. possess triangular myxospores. There are, however, several morphological differences between them, and *U. trigona* n. sp. is characterized by its lack of

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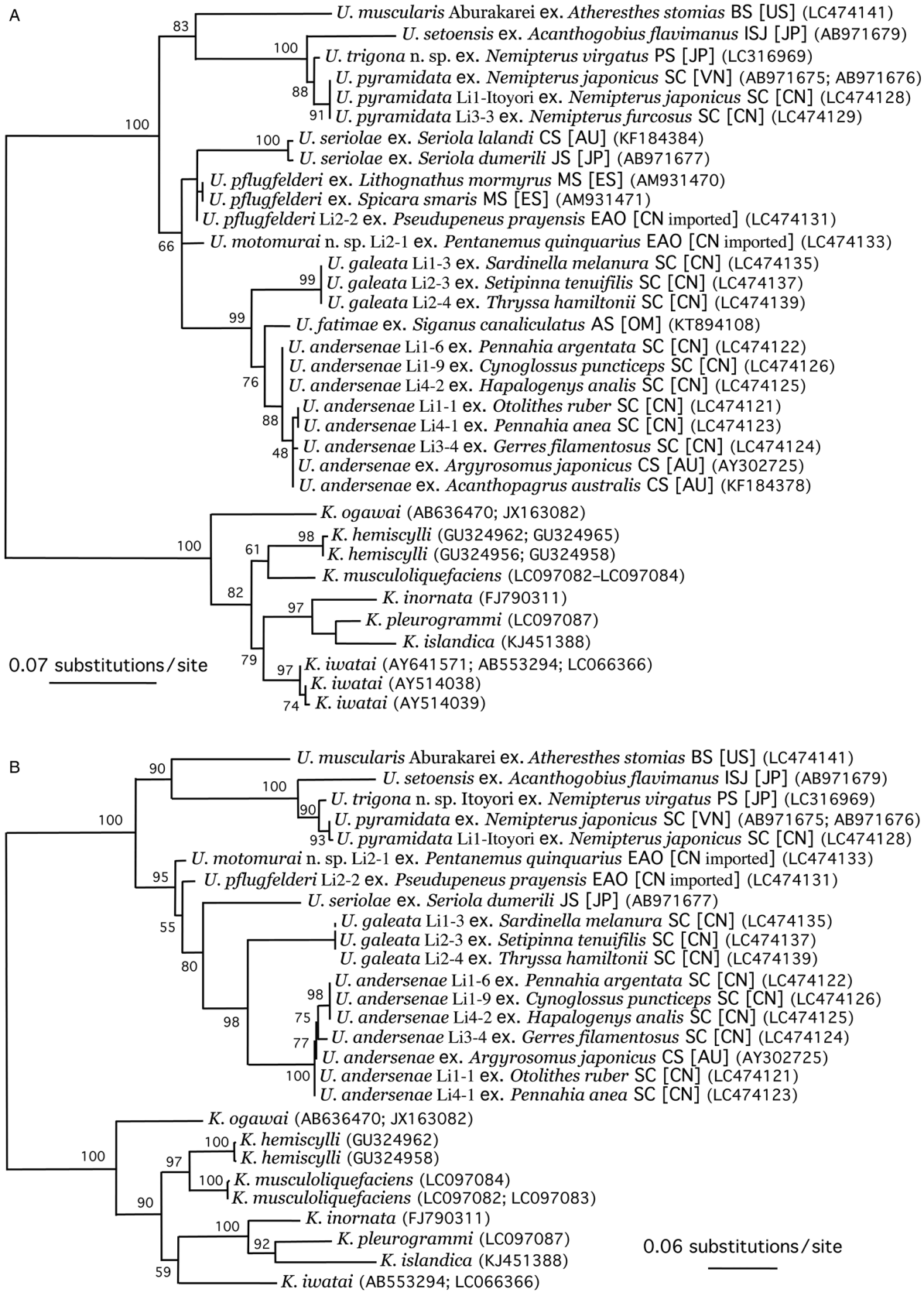

Fig. 6. Maximum likelihood phylogenetic tree of 18S rDNA sequence (A: 722 characters, and B: 1621 characters) from *Unicapsula* spp. Each species name is followed by the name of its fish host, the sea [country] from which it was collected and DDBJ/EMBL/GenBank accession number in parentheses. Abbreviation of sea names: AS, Arabian Sea; BS, Bering Sea; CS, Coral Sea around Australia; EAO, East Atlantic Ocean; ISJ, Inland Sea of Japan; JS, Japan Sea; MS, Mediterranean Sea; PS, Philippine Sea off Kochi, Japan; and SC, South China Sea. Abbreviation of country names: AU, Australia; CN, People's Republic of China; ES, Spain; JP, Japan; OM, Oman; US, United States of America; and VN, Vietnam.

caudal filamentous appendages, pointed apical SV corner, rounded posterior SV corners and the localization of prominent PCs at the centre of myxospores. This morphological differentiation is supported by genetic differences in the rDNA nucleotide sequences: long 28S rDNA sequences showed 98.74% similarity over 2698-bp, and short 28S sequences, limited to the

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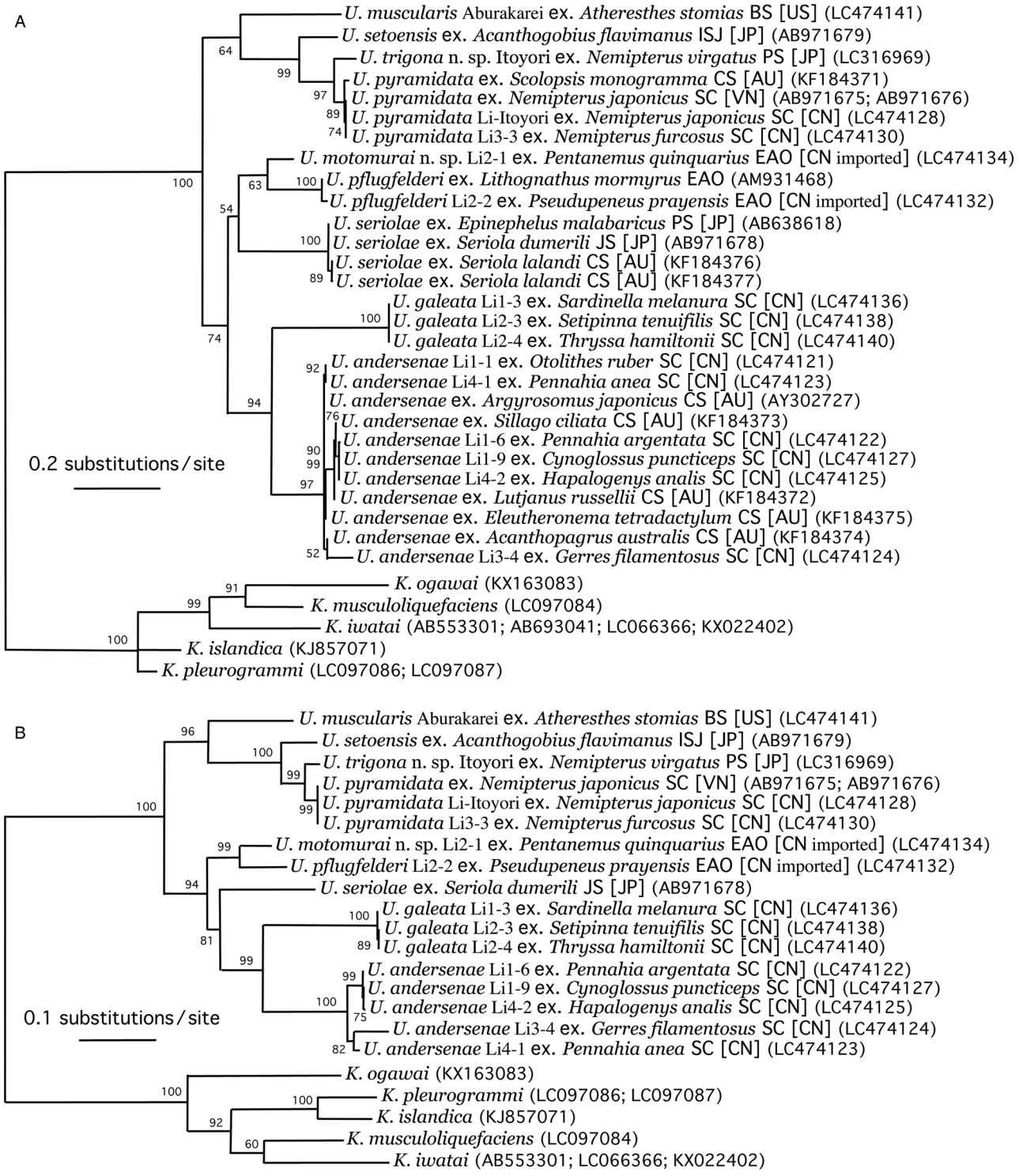

Fig. 7. Maximum likelihood phylogenetic tree of 28S rDNA sequence (A: 540 characters, and B: 2156 characters) from *Unicapsula* spp. Labelling of each isolate and abbreviations of sea and country names are similar to those described in the legend for Fig. 6.

initial 800-bp from the 5′-terminus, showed 96.63% similarity. In addition, the myxospores of *U. marquesi* exhibit a mitre-like shape with two prominent posterior SVs that lack caudal filamentous appendages and the species forms elongated cysts in the gill filaments of the giant African threadfin *Polydactylus quadrifilis* (Cuvier, 1829) in the East Atlantic Ocean, off the coast of Senegal. Even though molecular data for *U. marquesi* have yet to be deposited at DNA databases, the unique myxospore morphology and tissue specificity differentiate the species from the rest of *Unicapsula* spp., including *U. trigona* n. sp.

Phylogenetic analyses

In the present study, long nucleotide sequences of the 18S and 28S rDNA were obtained for all 15 of the new *Unicapsula* isolates. The phylogenetic trees constructed from short sequences (722 and 540 characters of the 18S and 28S rDNA, respectively; Figs 6A and 7A) depict the phylogenetic relationships of almost all the *Unicapsula* nucleotide sequences available from DNA databases, and the newly obtained rDNA sequences coincide well with the previously deposited ones. However, the phylogenetic trees constructed from long sequences (1621 and 2156 characters of the

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18S and 28S rDNA, respectively; Figs 6B and 7B) might reflect more accurate molecular relationships among the *Unicapsula* spp. Indeed, the topologies of the phylogenetic trees based on the long 18S rDNA sequences and long 28S rDNA sequences were almost identical, whereas those of phylogenetic trees based on the short 18S rDNA sequences and short 28S rDNA sequences were different. Based on the data analysed in the present study, it appears that there is little intraspecific genetic diversity in *Unicapsula* spp., except for *U. andersenae*, which exhibited frequent nucleotide variation in both the 18S and 28S rDNA (Table 3). We could not find currently phylogenetic clustering of species based on myxospore shape (spherical *vs.* triangular), host classification or geographical distribution.

Discussion

The type species for the genus *Unicapsula* Davis, 1924 is *U. muscularis* specified by Davis (1924) and accepted by Lom and Noble (1984) and Lom and Dyková (2006). The lack of additional taxonomic study, such as morphological observation, of the species after its original description by Davis (1924) is evident. However, the present study successfully characterised *U. muscularis*, both morphologically and molecular-genetically, that was detected in the myofibres of arrow-tooth flounder from the northeastern Pacific Ocean, off the coast of North America, although the isolation source was not from the type host for *U. muscularis*.

As shown in Table 2, 13 *Unicapsula* spp. have been recorded to date, and the present study adds two new species (i.e. *U. trigona* n. sp. and *U. motomurai* n. sp.). According to Lom and Noble (1984), the genus *Unicapsula* is defined as a group of multivalvulid species with subspherical myxospores with three unequal SVs; one small SV that covers a single spherical PC, and two larger, bilaterally and symmetrically arranged SVs that contain two PC rudiments. The distinct morphology of *Unicapsula* myxospores enabled the easy identification of the genus. However, species-level identification based mainly on myxospore morphology was rather difficult. This taxonomic status might be reflected in a limited number of specific descriptions in the genus, as well as in a limited number of reports of fish infected with *Unicapsula* spp. in the world. Specific differentiation of the morphologically simplest myxospores of *Unicapsula* spp. emphasizes the importance of molecular taxonomic approaches (Whipps *et al.*, 2004; Alama-Bermejo *et al.*, 2009; Miller and Adlard, 2013; Tomochi *et al.*, 2014; Al-Jufaili *et al.*, 2016). In this sense, the present study successfully provides sufficient molecular data (i.e. 18S and 28S rDNA sequences) for 15 isolates of seven *Unicapsula* spp.

For the amplification of 18S and 28S rDNA sequences from of *Unicapsula* spp., primer combinations designed for *Kudoa* spp., which are closely related multivalvulid myxosporeans, are useful with a few exceptions: the primer for the 5′-terminus of the 18S rDNA was not useful for any *Unicapsula* spp. (Tomochi *et al.*, 2014), and the primer for the 5′-terminus of the 28S rDNA of *U. muscularis* (present study). The molecular characterisation of 15 new isolates of seven *Unicapsula* spp. and of the deposited rDNA sequences of several other *Unicapusla* spp. (i.e. *U. andersenae*, *U. fatimae*, *U. pflugfelderi*, *U. pyramidata*, *U. seriolae*, and *U. setoensis*), allowed us to analyse the interspecific and intraspecific phylogenetic relationships of 10 *Unicapsula* spp. (using 18S rDNA; Fig. 6) and 9 *Unicapsula* spp. (using 28S rDNA; Fig. 7). As suspected, the phylogenetic trees based on the shorter 18S and 28S rDNA sequences exhibited different topological relationships among certain species (e.g. those of *U. seriolae* and *U. motomurai* n. sp. with other species). Meanwhile, the phylogenetic trees that were based on the longer 18S and 28S rDNA sequences possessed nearly identical topologies. Although the monophyly of all described *Unicapsula* spp. has been demonstrated in the previous studies (Miller and Adlard, 2013; Tomochi *et al.*, 2014; Fiala *et al.*, 2015), the genus has at least two major clades: (I) *U. muscularis*, *U. setoensis*, *U. trigona* n. sp. and *U. pyramidata*; and (II) *U. motomurai* n. sp., *U. pflugfelderi*, *U. seriolae*, *U. galeata*, *U. fatimae* and *U. andersenae*. The division of the genus into two these major clades is not related to either myxospore shape (spherical *vs* triangular) or to geographical distribution. The present study segregated *U. andersenae* isolates from South China Sea fish into three genetic groups (Table 3), as previously reported by studies in the Coral Sea around Australia (two genotypes; Miller and Adlard, 2013). With the exception of *U. andersenae*, which may actually be a species complex, like *Kudoa thyrsite* (Whipps and Kent, 2006; Burger and Adlard, 2011; Kasai *et al.*, 2016), little intraspecific variation was observed in the rDNA sequences of the 15 isolates. This may be partly due to the limited number of isolates analysed. However, *U. galeata* and *U. pyramidata* from different fish hosts or geographical distribution exhibited few to zero nucleotide substitutions.

Studies of the economic impacts of *Unicapsula* infection to commercial or recreational fish are currently limited to knowledge on *U. muscularis* and *U. seriolae*. *Unicapsula muscularis* is known to form macroscopic pseudocysts in the trunk muscle of the Pacific halibut, which is called 'wormy halibut' when infected (Davis, 1924), and *U. seriolae* is known to cause postmortem myoliquefaction in yellowtail amberjack *Seriola lalandi* Valenciennes, 1833, fished from the waters around Australia (Lester, 1982). As far as could be observed by the microscopic analysis of wet specimens, no inflammatory cell accumulation was observed around myofibres with *Unicapsula* pseudocysts, nor was postmortem myoliquefaction in fish samples examined in the present study. As mentioned in Tomochi *et al.* (2014) and Ohnishi *et al.* (2018), the parasitism of aquacultured greater amberjack *Seriola dumerili* (Risso, 1810) by *U. seriolae* is considered responsible for sporadic outbreaks of food-borne disease, which is characterized by clinical diarrhoea and vomiting after the consumption of raw greater amberjack. Greater yellowtail and other *Seriola* spp. are popular with Japanese people, and raw muscle slices are often served as sashimi or sushi. Consequently, Japanese aquaculture is expanding dramatically (Nakada, 2008). Like *Kudoa* food poisoning outbreaks, which are caused by *K. septempunctata* in aquacultured olive flounders (Kawai *et al.*, 2012; Sugita-Konishi *et al.*, 2014), aquaculture provides intensive infection in fish (exceeding a certain threshold number of myxospores for food poisoning), which might be responsible for the increase in food-borne diseases associated with the consumption of raw greater amberjack during the last decade. Even though the infection of arrow-tooth flounder (and probably Pacific halibut) by *U. muscularis* is intensive enough to exceed such threshold myxospore numbers per gram muscle, commercially available fish in Japan are mainly frozen and imported from North America and are absolutely free from the risk of food-borne myxosporean diseases, since myxospores are inactivated by freezing (Kawai *et al.*, 2012; Sugita-Konishi *et al.*, 2014).

The present study has added two new species, *U. trigona* n. sp. and *U. motomurai* n. sp., to the list of *Unicapusla* spp., and the genus now contains 15 species, of which the rDNA nucleotide sequences of 10 species have been characterised. Additional morphological and genetic characterisation of *U. chirocentrusi*, *U. maxima*, *U. pacifica*, *U. schulmani* and *U. marquesi* is needed to better understand the biodiversity and evolutionary history of the genus.

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Conflict of interest. None.

Ethical standards. Not applicable.

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