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Taxonomic significance of tunic spicules in photosymbiotic ascidians: a quantitative and molecular evaluation

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*Many didemnid ascidians have calcareous spicules in the tunic. Since the spicules of each species have a specific shape and size-range, they are often regarded as an important character for taxonomy. To evaluate the taxonomic significance of tunic spicules, a quantitative survey of spicule size and shape was combined with a molecular phylogeny inferred from the partial sequence of the cytochrome c oxidase subunit I (*COI*) gene in some groups of didemnid species that are supposedly closely related. This study revealed the presence of substantial intraspecific variations in the shape and size of tunic spicules. The spicules are, therefore, not always crucial features discriminating species, particularly among related species. Although tunic spicules are potentially valuable features for didemnid taxonomy, their intraspecific variation should be carefully considered before they are used as a key character for species identification.*

Keywords: calcareous spicules, *COI*, Didemnidae, size range, taxonomy, tunicate, Ascidiacea

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INTRODUCTION

Colonial organisms tend to have zooids with simple morphs and few characters are available to discriminate among species. Therefore, species identification is often difficult in the colonial organisms such as cnidarians and tunicates. Ascidian species of the family Didemnidae are always colonial, and some didemnid species inhabiting tropical waters harbour symbiotic cyanophytes, *Prochloron* and *Synechocystis* (see Lewin & Cheng, 1989). Faunal surveys were recently conducted of the photosymbiotic didemnids in the Ryukyu Archipelago and Bonin Islands, Japan (Hirose *et al.*, 2007 and some references therein) and encountered some species with taxonomic debates or problems. Several potentially cryptic species in *Didemnum molle* were also found; partial mitochondrial gene sequences discriminated four morphotypes, white, brown, grey, and large (Hirose *et al.*, 2009b). Moreover, six photosymbiotic didemnids from the Ryukyus were recently described as new species (Oka *et al.*, 2005; Hirose & Oka, 2008; Hirose *et al.*, 2009a; Hirose & Hirose, 2009), suggesting that there may be other undescribed species in this area.

Calcareous spicules of various shapes are contained in the tunic of many colonial ascidians of the families Didemnidae and Polycitoridae and some solitary ascidians of Pyuridae. Individual species have spicules of a specific shape and size-range that can be useful features for specific identification (e.g. Turon, 1986; Monniot *et al.*, 1991; Kott, 2001), especially among congeners with very similar appearances. For instance, the zooids of *Lissoclinum bistratum* are very similar in morphology to those of *L. timorense* (>*L. voeltzkowi*), but colonies of the former contain only globular spicules and colonies of the latter contain both globular and stellate spicules (see Kott, 2001). However, discrimination of species that closely resemble each other based on spicule shape is contested by some taxonomists. For example, for the *Lissoclinum* species mentioned above, Monniot & Monniot (2001) proposed that *L. timorense* is a junior synonym of *L. bistratum*, with the variability of spicules representing intraspecific variation. Similarly, *Tridemnum cyclops* can be discriminated from *T. paracyclops* by spicule size, colony size, and some other features of zooids and larvae (see Kott, 2001), although Monniot & Monniot (1987) assigned both species to *T. cyclops* and regarded the spicule differences as intraspecific variation. Kott (2004) described a new species of *Didemnum* from the north-east coast of the USA based in large part on some differences in spicules from other known species, but it was later proven to be a worldwide invader that happens to produce variable spicules (Lambert, 2009).

Here, we conducted a quantitative study of spicule shape and size in some photosymbiotic didemnid ascidian species. In combination with DNA barcodes, we evaluated the taxonomic significance of tunic spicules in some closely related species.

MATERIALS AND METHODS

Animals

Colonies of photosymbiotic ascidians were collected by snorkelling at shallow coral reefs in the Ryukyu Archipelago, Japan. Species collected were *Didemnum molle*, *Lissoclinum bistratum*, *L. timorense*, *Tridemnum cyclops*, *T. paracyclops*, and *T. nubilum* (Table 1). The identification

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of each species was primarily based on colony appearance in the field and was confirmed under a stereomicroscope, mainly following Kott (2001). The four morphotypes of *D. molle* were easily distinguished by colony colour and colony size (Hirose *et al.*, 2009b). The specimens were fixed in 10% formalin–seawater for microscopy and in 99% ethanol for DNA analysis. We used different specimens for spicule analyses and DNA sequences. At least two specimens from two or more sites for each species or morphotype were examined.

Microscopy

Tunic pieces containing spicules were cut off from the fixed colonies; the lateral side of the *D. molle* colonies and the colonial margins in the other species. The specimens were immersed in 2–3% sodium hypochlorite for two days. Following a rinse with distilled water, the spicules were air-dried on aluminium stubs 15 mm in diameter. The dried specimens were sputter coated with gold-palladium and observed under a JEOL JSM-6060LV scanning electron microscope (SEM) in low vacuum mode (~30 Pa). In *D. molle*, *T. cyclops*, and *T. paracyclops*, the diameter of spicules was measured from the digital images using ImageJ 1.41 (rsbweb.nih.gov). We examined 100 spicules for each specimen. For the stellate spicules of *T. cyclops* and *T. paracyclops*, we used the length between the bases of opposite cones as the spicule diameter, because the tips of the cones were sometimes broken. The variance in spicule diameter among specimens was tested using a Kruskal–Wallis test and Dunn's multiple comparison test, since some data were not normal. Statistical analyses were performed using Instat 3 for Macintosh (GraphPad Software).

Table 1. List of the specimens for SEM observation and molecular phylogeny.

Species (Morphotype)	Collection site	No. of colonies for SEM observation	Abbreviation (Accession No.) for DNA specimen*
***Didemnum molle* (brown)**	1	2	*D.m* B_Sera1 (AB433947)[a], *D.m* B_Sera2 (AB433948)[a], *D.m* B_Sera3 (AB433949)[a]
	2	2	*D.m* B_Seso1 (AB433952)[a], *D.m* B_Seso2 (AB433953)[a]
	3		*D.m* B_Ikei1 (AB433950)[a], *D.m* B_Ikei2 (AB433951)[a]
***Didemnum molle* (grey)**	4	2	*D.m* G_Bise1 (AB433943)[a], *D.m* G_Bise2 (AB433944)[a], *D.m* G_Bise3 (AB433945)[a], *D.m* G_Bise4 (AB433946)[a]
	5	2	*D.m* G_Ara (AB499970)[b]
***Didemnum molle* (white)**	6	2	*D.m* W_Zanpa (AB499971)[b]
	7	2	*D.m* W_Odo1 (AB433954)[a], *D.m* W_Odo2 (AB433955)[a], *D.m*W_Odo3 (AB433956)[a]
	3		*D.m* W_Ikei1 (AB433957)[a], *D.m* W_Ikei2 (AB433958)[a]
	5		*D.m* W_Ara (AB433959)[a]
	8		*D.m* W_Shira1 (AB433960)[a], *D.m* W_Shira2 (AB433961)[a]
	9		*D.m* W_Hira1 (AB433962)[a], *D.m* W_Hira2 (AB433963)[a]
***Didemnum molle* (large)**	1	2	*D.m* L_Sera1 (AB433964)[a], *D.m* L_Sera2 (AB433965)[a], *D.m* L_Sera3 (AB433966)[a]
	2	2	*D.m* L_Seso1 (AB433967)[a], *D.m* L_Seso2 (AB433968)[a], *D.m* L_Seso3 (AB433969)[a]
	10		*D.m* L_Ugachi (AB433970)[a]
	11		*D.m* L_Manza (AB433971)[a]
Tridemnum cyclops	4	2	
	13	2	*T. cyclops*_Maeda (AB499972)[b]
	9	2	*T. cyclops*_Hirakubo (AB433978)[a]
Tridemnum paracyclops	5	2	*T. paracyclops*_Ara beach (AB499973)[b]
	14	2	
	9	2	*T. paracyclops*_Hirakubo (AB499974)[b]
Tridemnum nubilum	4	4	*T. nubilum*_Bise (AB499975)[b]
	5	2	*T. nubilum*_Ara beach (AB499976)[b]
Lissoclinum bistratum	5		*L. bistratum*_Ara beach (AB433977)[a]
	4		*L. bistratum*_Bise (AB499978)[b]
	13	2	*L. bistratum*_Maeda (AB499979)[b]
	16	2	*L. bistratum*_Shinrihama (AB499980)[b]
Lissoclinum timorense	4		*L. timorense*_Bise (AB499981)[b]
	5	2	*L. timorense*_Ara each (AB499982)[b]
	7	2	*L. timorense*_Odo (AB499983)[b]

*, References: [a], Hirose *et al.* (2009b); [b], this study. Collection site: (1) Seragaki, Okinawajima Island (26°30′30″N 127°51′50″E); (2) Seokojima Island (26°30′00″N 127°51′52″E); (3) Ikei, Ikijima Island (26°22′55″N 127°59′55″E); (4) Bise, Okinawaji Island (26°42′40″N 127°52′30″E); (5) Ara beach, Kumejima Island (26°19′0″N 126°46′30″E); (6) Zanpa, Okinawajima Island (26°26′10″N 127°42′40″E); (7) Odo, Okinawajima Island (26°5′20″N 127°42′30″E); (8) Shiraho, Ishigakijima Island (24°20′55″N 124°15′22″E); (9) Hirakubo, Ishigakijima Island (24°36′50″N 124°19′0″E); (10) Ugachi, Okinawajima Island (26°26′00″N 127°44′00″E); (11) Manza, Okinawajima Island (26°26′30″N 127°50′00″E); (12) Ginoza, Okinawajima Island (26°29′N 128°0′E); (13) Maeda, Okinawajima Island (26°26′30″N 127°46′0″E); (14) Sumiyoshi, Iriomore Island (24°26′20″N 123°46′40″E); (15) Nyu, Fukui (35°42′41″N 135°58′10″E); (16) Shinrihama, Kumejima Island (26°21′0″N 126°42′50″E).

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DNA extraction, amplification, and sequencing

The preserved colonies were dissected under a stereomicroscope and about 30–40 zooids were suspended in 400 µl CTAB buffer (2% hexadecyltrimethyl ammonium bromide (CTAB), 1.0 M NaCl, 75 mM EDTA pH 8.0, and 35 mM Tris-HCl; pH 8.0) containing 0.1% sodium dodecyl sulphate (SDS) and 0.2% beta-mercaptoethanol, following the methods of Hirose *et al.* (2009b). The samples were incubated at 65°C for 1 h. Next, proteinase K was added to the samples to a final concentration of 0.1 mg ml^{-1}, and the samples were incubated overnight at 37°C. DNA was then extracted with phenol-chloroform as described by Sambrook *et al.* (1989). The PCR amplification of the cytochrome *c* oxidase subunit I (*COI*) was performed using EX *Taq* DNA polymerase (Takara) and a combination of the degenerate primers UroCox1-244F (5′-CATTTWTTTT GATTWTTTRGWCATCCNGA-3′) and UroCox1-387R (5′-G CWCYTATWSWWAAWACATAATGAAARTG-3′) (Hirose *et al.*, 2009b). The PCR amplification was performed under the following conditions: 94°C for 5 minutes; followed by 35 cycles of 94°C for 10 seconds, 40°C for 5 seconds, and 72°C for 2 minutes; with a final extension at 72°C for 7 minutes. For cloning, the TOPO TA cloning kit for sequencing (Invitrogen) was used. Cycle sequencing reactions were performed using DTCS Quick Start Master Mix (Beckman Coulter), and products were analysed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system. At least five clones were randomly selected for each sample and sequenced. The sequences determined in this study were deposited in GenBank, the European Molecular Biology Laboratory (EMBL), and the DNA Data Bank of Japan (DDBJ); Accession numbers are provided in Table 1.

Phylogenetic analysis

Phylogenetic trees were built separately for each genus. Initial sequence alignments were performed using CLUSTAL W 1.83 (Thompson *et al.*, 1994); alignments were then inspected by eye and manually edited. The following analyses were performed on the aligned DNA sequences of the partial *COI*: maximum likelihood (ML) using TREEFINDER version October 2008 (Jobb, 2008), and maximum parsimony (MP) and neighbour-joining (NJ) using PAUP* 4.0 beta10 (Swofford, 2003). To select an appropriate nucleotide substitution model, Modeltest v. 3.7 (Posada & Crandall, 1998) was used with PAUP. For data with all three codon positions from *Didemnum* spp., the Hasegawa, Kishino, Yano 85 (HKY85) model was selected as the best model based on Akaike's information criterion (AIC). For data with all three codon positions from *Lissoclinum* spp. and *Trididemnum* spp., transitional model (TIM) was selected under AIC. The MP trees were searched using a heuristic approach with 100 random initial trees. Statistical support for the ML, MP, and NJ trees was evaluated using a non-parametric bootstrap test with 1000 re-sampling events.

RESULTS AND DISCUSSION

Four morphotypes of *Didemnum molle*

Brown-, grey-, and white-type colonies of *Didemnum molle* were usually symmetrical domes of 2 cm or less in diameter; the brown- and grey-type colonies exhibited those respective pigmentation colours. Large-type colonies were irregular in shape, 5 cm or more in the long axis, and had grey patches. In all morphotypes, colonies always contained globular spicules and there were no differences in spicule shape among morphotypes (Figure 1). Spicule diameter varied considerably within each colony (Figure 2), and variation among specimens was significant (Kruskal–Wallis test, $P < 0.001$). A significant difference was found between some specimens in the same morphotype but not between combinations of different morphotypes (Dunn's multiple comparison test, $P > 0.05$).

A total of eleven haplotypes were found among the 31 specimens of *D. molle*. The aligned *D. molle* sequences had no gaps (insertion/deletion) and 68 variable sites over 401 bp. There were 64 parsimony-informative characters.

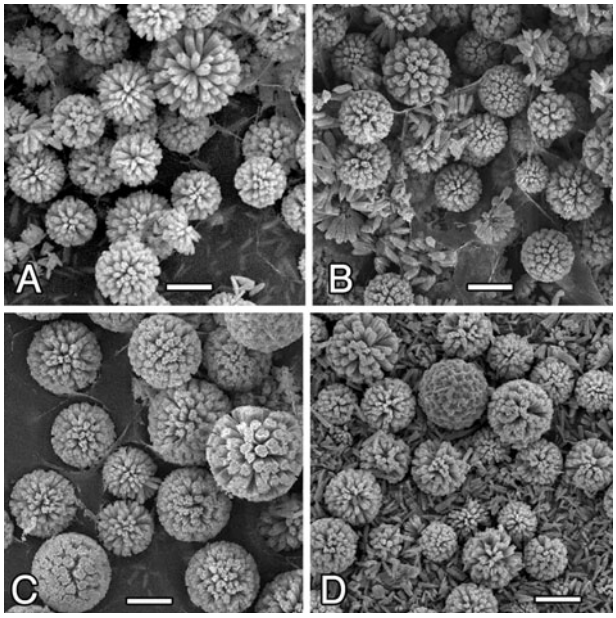

Fig. 1. Tunic spicules of the four morphotypes of *Didemnum molle*. (A) Brown type (Seragaki, Okinawajima Island); (B) grey type (Ara Beach, Kumejima Island); (C) white type (Odo, Okinawajima Island); (D) large type (Seragaki). Scale bars: 10 µm.

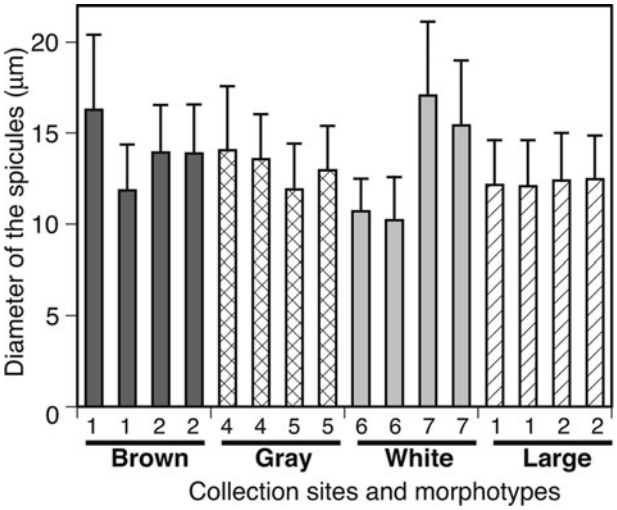

Fig. 2. Size variation of the tunic spicules of the four morphotypes of *Didemnum molle*. Each bar indicates the average of 100 spicules from one specimen with standard deviation. The numbers under the bars indicate the collection site (see Table 1).

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The ML tree of *Didemnum* spp. based on partial cytochrome *c* oxidase subunit I (*COI*) sequences using the HKY + I substitution model (log-likelihood = −1026.993) is presented in Figure 3. Because the topologies of the ML, MP, and NJ trees were nearly identical, the strict-consensus MP tree (tree length = 110, consistency index (CI) = 0.8818, retention index (RI) = 0.9772, rescaled index (RC) = 0.8617, and homoplasy index (HI) = 0.1182) and the NJ tree under the HKY85 + I substitution model (proportion of invariable sites = 0.6723, as estimated using the model) are not shown. Monophyly of the grey- and brown-types was supported by high bootstrap values and monophyly of each morphotype (grey, brown, and large) was also supported by high bootstrap values. However, monophyly of the white-type was supported by relatively high bootstrap values. Accordingly, the present results confirmed a previous study indicating potential speciation among morphotypes in *D. molle* (Hirose *et al.*, 2009b), but spicule morphology alone cannot be used to distinguish among these morphotypes.

Trididemnum cyclops and *T. paracyclops*

Trididemnum cyclops colonies were small oval cushions of about 5 mm in diameter, whereas *T. paracyclops* colonies were irregularly shaped sheets. Both species contained stellate spicules that were very similar in shape (Figure 4). Spicule diameter varied somewhat (Figure 5); while it was not significantly different within species, it did differ between *T. cyclops* and *T. paracyclops* (Dunn's multiple comparison test, $P < 0.001$).

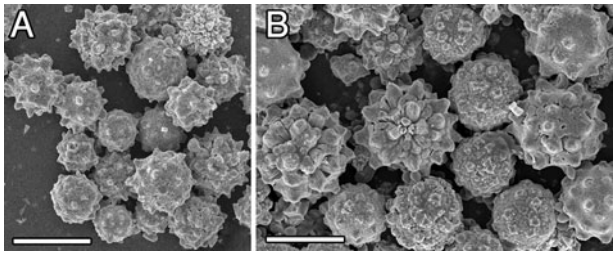

Fig. 4. (A) Tunic spicules of *Trididemnum cyclops* from Bise, Okinawajima Island and (B) *T. paracyclops* from Hirakubo, Ishigakijima Island. Scale bars: 50 μm.

A total of three haplotypes were found in two specimens each of *T. cyclops* and *T. paracyclops*. The partial *COI* sequences (401 bp) from two collection sites of *T. paracyclops* were identical. The aligned *T. cyclops* and *T. paracyclops* sequences had no gaps (insertion/deletion) and 53 variable sites over 401 bp. There were 44 parsimony-informative characters. The ML tree of *Trididemnum* spp. based on partial *COI* sequences under the TIM substitution model (log-likelihood = −1213.4027) is presented in Figure 6. Because the ML, MP, and NJ trees had nearly identical topologies, the strict-consensus MP tree (tree length = 171, CI = 0.9181, RI = 0.8955, RC = 0.8222, and HI = 0.0819) and NJ tree under the TIM substitution model are not shown. Monophyly of *T. cyclops* from Hirakubo (Ishigakijima Island) and *T. paracyclops* from two collection sites was supported by moderate bootstrap values, whereas monophyly of *T. cyclops* + *T. paracyclops* was supported by high bootstrap values.

Trididemnum paracyclops is distinguished from *T. cyclops* by characters such as larger colony size, larger zooids, and number of coils in the vas deferens (see Kott, 2001). However, Monniot & Monniot (1987, 1991) did not

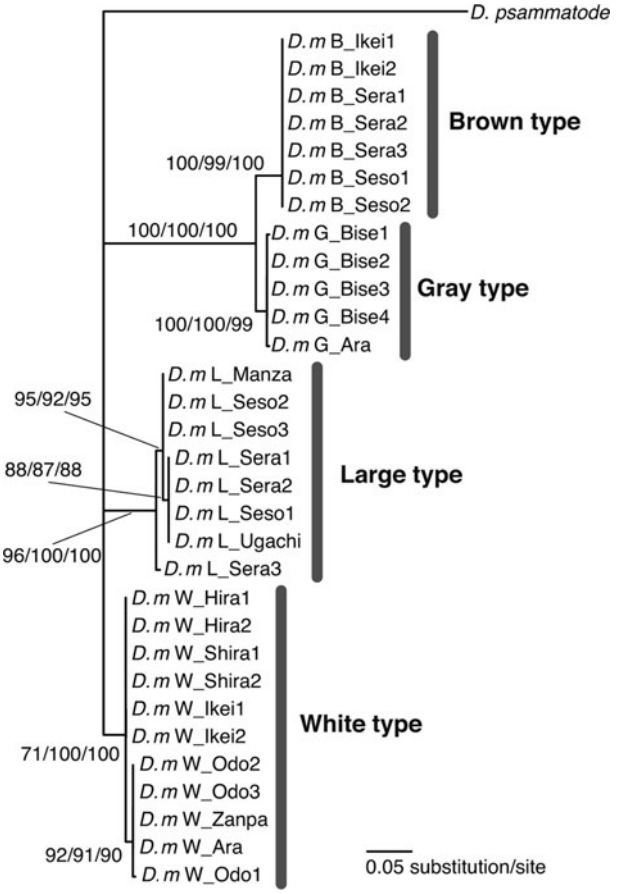

Fig. 3. Maximum likelihood tree of *Didemnum* spp. based on partial *COI* sequences. *Didemnum psammatode* (AB433972) was used as the outgroup. The HKY + I model was used for the analysis. Bootstrap probabilities larger than 50% are noted for the ML, MP, and NJ trees.

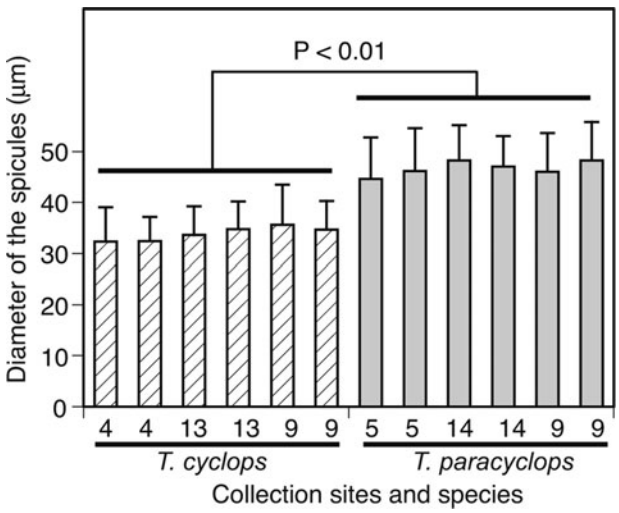

Fig. 5. Size variation of the tunic spicules of *Trididemnum cyclops* and *T. paracyclops*. Each bar indicates the average of 100 spicules from one specimen with standard deviation. The numbers under the bars indicate the collection site (see Table 1).

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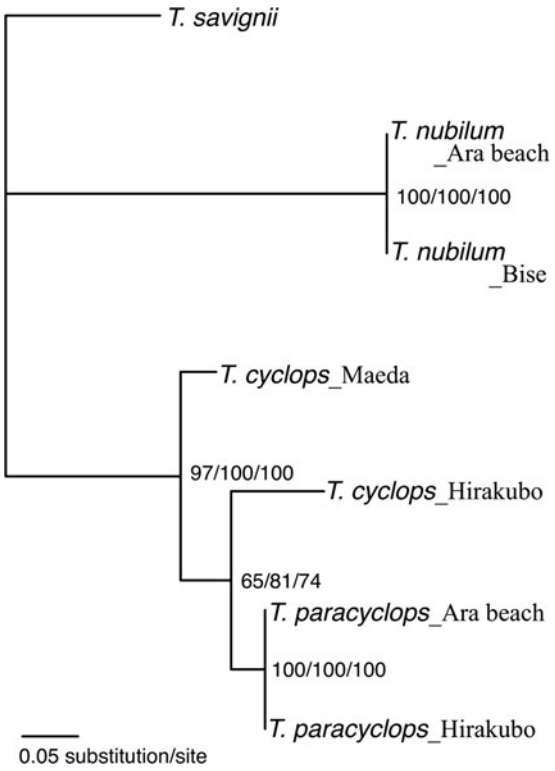

Fig. 6. Maximum likelihood tree of *Tridemnum* spp. based on partial *COI* sequences. *Tridemnum savignii* (AB499977) was used as the outgroup. The TIM model was used for the analysis. Bootstrap probabilities larger than 50% are noted for the ML, MP, and NJ trees.

discriminate between the two species in their faunal reports, and probably regarded these differences as intraspecific variation (see Kott, 2001). In the present study, the molecular phylogenies based on the *COI* sequence did not recognize two species, but a more extensive survey is required to conclude the taxonomic debate. Colony size was mainly used as the key character for species identification. Thus, neither colony size nor spicule size should be used to discriminate between these two species, even if they are two valid and distinct species.

Two types of spicules in *Tridemnum nubilum*

The tunic spicules of *T. nubilum* were always stellate but could be distinguished into two types. In Type-A spicules, the bases of the spicule cones fused with one another. In Type-B, the bases of the cones did not fuse, and there was a crack between the bases (Figure 7). The composition of the two types of spicules varied among colonies: more than 90% of spicules were Type-A in colonies from Bise, Okinawajima Island (N = 4), while about 65% were Type-B in colonies from Ara Beach, Kumejima Island (N = 2). On the other hand, the partial *COI* sequences (401 bp) from the two collection sites of *T. nubilum* were identical. Therefore, differences in the ratio of Type-A and Type-B spicules were probably due to intraspecific variation. It is unclear whether the difference in this ratio is caused by genetic variance, microhabitat, or other factors.

Lissoclinum bistratum and *L. timorense*

Both *Lissoclinum bistratum* and *L. timorense* formed sheet- or cushion-like colonies of irregular shapes. *Lissoclinum*

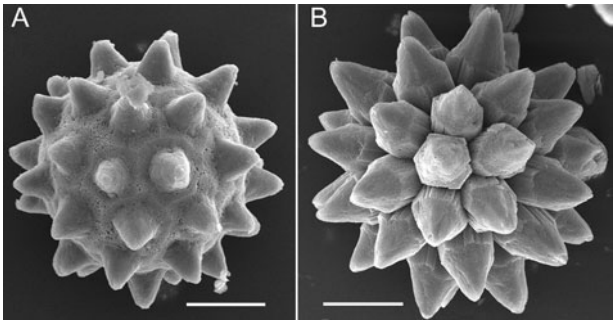

Fig. 7. Two types of stellate tunic spicules in *Tridemnum nubilum*. (A) The bases of the spicule cones fused with one another (Type-A); (B) the bases of cones not fused with one another (Type-B). Scale bars: 10 μm.

timorense colonies had linguiform projections of tunic around the colony periphery (Figure 8A&C) and sometimes on the colony surface, whereas such projections never occurred in *L. bistratum*. The tunic spicules of *L. bistratum* were always globular (Figure 8B). In contrast, those of *L. timorense* varied from globular to stellate. Spicules of typical shapes are shown in Figure 8D, E; some intermediate shapes were also observed. Therefore, we could not calculate the composition ratio for each specimen of *L. timorense*. It should be noted that the colonies of *L. timorense* always contained stellate spicules, which were never found in *L. bistratum*.

A total of five *COI* haplotypes were found among four specimens of *L. bistratum* and three specimens of *L. timorense*. The aligned *L. bistratum* and *L. timorense* sequences had no gaps (insertion/deletion) and 67 variable sites over 401 bp. There were 64 parsimony-informative characters. The ML tree under the TIM substitution model (log-likelihood = −1027.8732) is presented in Figure 9. Because the topologies of the ML, MP, and NJ trees were nearly identical, the strict-consensus MP tree (tree length = 171, CI = 0.9181, RI = 0.8955, RC = 0.8222, and HI = 0.0819) and the NJ tree under the TIM substitution model are not shown. The partial *COI* sequences of two distant collection sites of *L. timorense* from Ara Beach and Odo were identical. The difference of DNA sequences between *L. bistratum* from Ara beach and Bise was 2 bp over 401 bp, and the differences between the haplotype from the *L. timorese* (from Ara beach and Odo) and *L. bistratum* from Ara beach and Bise were 1 bp, respectively. Monophyly of *L. bistratum* from Ara Beach and Bise and *L. timorense* from Ara Beach and Odo was supported by high bootstrap values. The partial *COI* sequences of *L. bistratum* from Shinrihama and *L. timorense* from Bise were identical. The difference of DNA sequences between the haplotype and *L. bistratum* from Maeda was 2 bp over 401 bp. Monophyly of *L. bistratum* from Maeda and Shinrihama and *L. timorense* from Bise was supported by high bootstrap values. The sequence difference within each monophyletic group was very low (0–2 bp, 0–0.5%). On the other hand, the difference between the '*L. bistratum* from Ara Beach and Bise and *L. timorense* from Ara beach and Odo' and '*L. bistratum* from Maeda and Shinrihama and *L. timorense* from Bise' groups was high (64–65 bp, 16.0–16.2%).

The *COI* phylogeny of *L. bistratum*–*L. timorense* specimens discriminated two clades, but each clade included both *L. bistratum* and *L. timorense* (Figure 9). Therefore, the presence or absence of stellate spicules does not discriminate the

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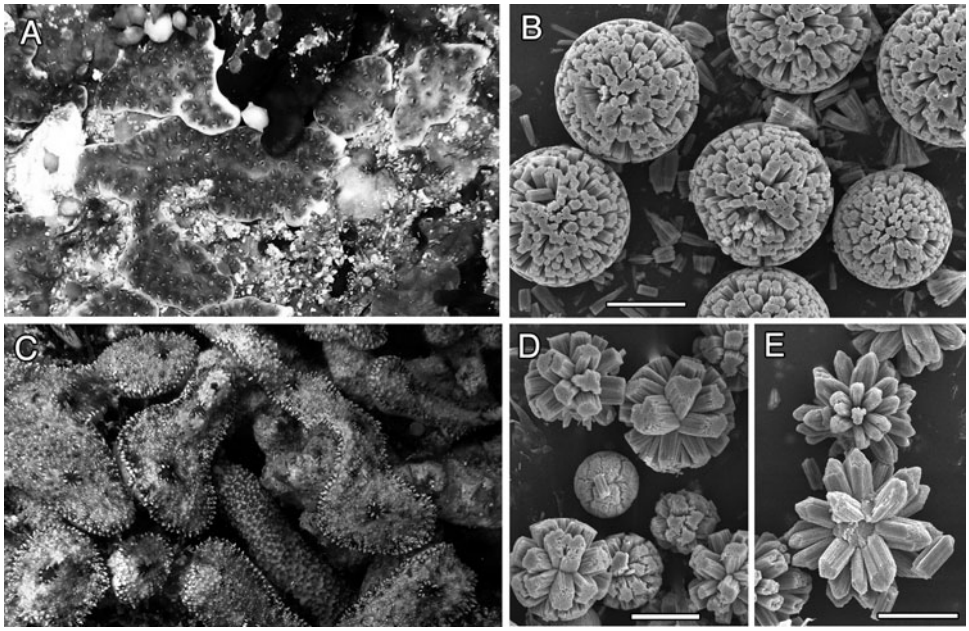

Fig. 8. Colonies of *Lissoclinum bistratum* (A) containing only spherical spicules (B), and those of *L. timorense* (C) containing both spherical (D) and stellate (E) spicules. Scale bar: 20 μm.

two clades of *L. bistratum*–*L. timorense*. Monniot & Monniot (2001) proposed that *L. timorense* and *L. voeltzkowi* are junior synonyms of *L. bistratum*, considering the large variability of spicules among colonies inhabiting the same site and the absence of distinct zooid morphology. On the other hand, it is possible that the two clades recognized in Figure 9 represent two distinct species, but we could not find any morphological features with which to distinguish them.

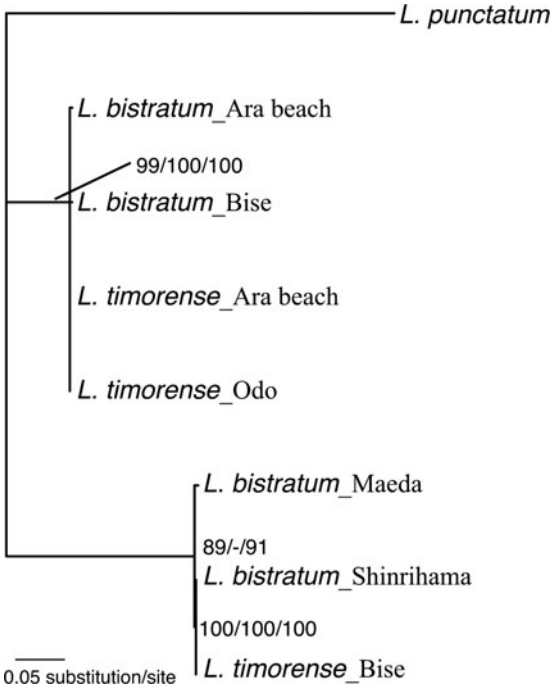

Fig. 9. Maximum likelihood tree of *Lissoclinum* spp. based on partial *COI* sequences. *Lissoclinum puctatum* (AB433976) was used as the outgroup. The TIM model was used for the analysis. Bootstrap probabilities larger than 50% are noted for the ML, MP, and NJ trees.

Spicules as a taxonomic character

Didemnid species, other than *Diplosoma* spp., usually possess calcareous spicules. There are various forms of spicules (e.g. globular, stellate, burr-like; see Kott, 2001), and each species has a particular type or types of spicules of a specific size range. Therefore, tunic spicules are undoubtedly an important character for taxonomy in didemnid ascidians. However, our results, from a combination of quantitative and molecular studies, revealed the presence of substantial intraspecies variation in shape and size of tunic spicules. A similar finding was reported for *Didemnum vexillum* (Lambert, 2009). Therefore, spicules are not always crucial features discriminating species, particularly among closely related species. For instance, the four morphotypes of *D. molle*, which were supported by the molecular phylogeny, possess spicules of almost the same shape and size. On the other hand, the size of stellate spicules differed significantly between *T. cyclops* and *T. paracyclops*, but the molecular phylogeny did not support this species level distinction. Phenotypic plasticity is a delicate problem, and the divergence of the spicule form and colony form may result from the different microhabitats in *Lissoclinum bistratum*–*L. timorense*. By contrast, Dias *et al.* (2009) showed the genetic differentiation between morphotypes from intertidal and subtidal zones in *Trididemnum orbiculatum*. We also encountered, albeit infrequently, some malformed spicules (Figure 10) as did Lambert (2009). The presence of malformed spicules indicates that ascidian colonies cannot always produce spicules accurately and that there could be variation in their shape and size. In the present study, we could not well quantify the shape of the spicules. The variability of the spicules causes the occurrence of intermediate forms, and they are difficult to be classified into discrete categories. Evaluation of shape parameters, such as ray number, may provide useful characters for taxonomy. In conclusion, tunic spicules are potentially valuable features for didemnid taxonomy, but intraspecific variation

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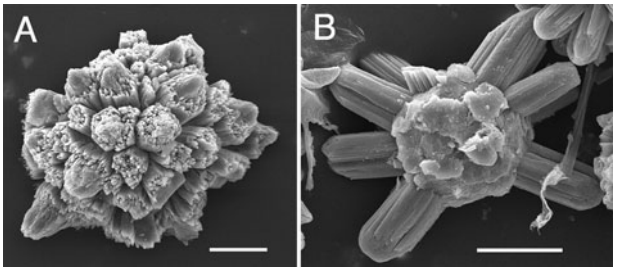

Fig. 10. Malformed tunic spicules in (A) *Lissoclinum timorense* and (B) *Tridemnum cyclops*. Scale bar: 10 μm.

should be taken into account if they are to be used as key characters for species identification.

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