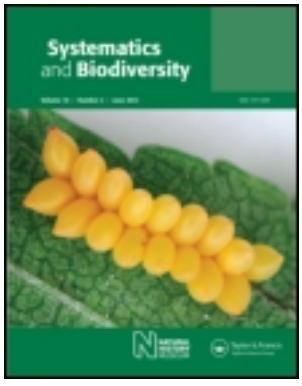

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First records of didemnid ascidians harbouring Prochloron from Caribbean Panama: genetic relationships between Caribbean and Pacific photosymbionts and host ascidians

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Research Article

First records of didemnid ascidians harbouring *Prochloron* from Caribbean Panama: genetic relationships between Caribbean and Pacific photosymbionts and host ascidians

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Two didemnid ascidians associating with cyanobacterial symbionts (*Prochloron* spp.) were firstly recorded from Caribbean Panama: *Lissoclinum verrilli*, which facultatively harboured *Prochloron* cells on the colony surface, and *Diplosoma simile*, which obligately harboured algal cells in the peribranchial and common cloacal cavities within the colonies. While *L. verrilli sensu stricto* has been exclusively recorded from the Bermudas and Caribbean islands, *D. simile* is widely distributed in tropical Indo-Pacific regions including oceanic islands such as Hawaii. Partial COI sequences of *D. simile* from the Caribbean were identical to those from the West Pacific, suggesting a high larval-dispersal ability and broad range of environmental tolerance. Molecular phylogenetics of the symbionts, based on 16S rRNA gene sequences, revealed that both ascidian species were associated with *Prochloron*, while a *Synechocystis* sp. sequence was also obtained for *L. verrilli.* In addition, *L. verrilli* and *D. simile* harboured different phylotypes within the *Prochloron* lineage that included symbionts from various hosts and various Pacific sites. Our results indicate that multiple phylotypes of *Prochloron* exist in Caribbean Panama and that considering the abundance and the number of host species in the Pacific, *Prochloron* and *D. simile* may have come from tropical Indo-West Pacific.

Key words: coral reefs, cyanobacteria, *Diplosoma*, *Lissoclinum*, molecular phylogeny, symbiosis

Introduction

Ascidians (Chordata; Tunicata), or sea squirts, are sessile marine organisms encountered all over the world. This group is informally classified in two main types: solitary and colonial. Within colonial ascidians, several genera are known to form symbiotic relationships with cyanobacteria (Kott, 1982; Kott *et al*., 1984), while few instances have been described for solitary species (De Leo & Patricolo, 1980; Lambert *et al*., 1996). In fact, the majority of ascidian-cyanobacterial studies have focused on species within the family Didemnidae that are known to establish symbiotic associations with a unicellular cyanobacterium from the genus *Prochloron* (Prochlorales). *Prochloron* is found in both tropical and subtropical waters, and is characterized by having both chlorophyll *a* and *b* pigments but lacking phycobilins (reviewed in Lewin & Cheng, 1989; Hirose *et al.*, 2009). The type species of this genus is *Prochloron didemni*, which was first described in *Didemnum candidum* from Baja California (Lewin, 1975; Lewin & Cheng, 1977), and this is the only species of the genus *Prochloron* so far described. However, it is uncertain whether *Prochloron* consists of several species or a single species (i.e. *P. didemni*). To date, a stable *in vitro* culture of *Prochloron* cells has never been established, and *Prochloron* associated with host ascidians are generally referred to as *Prochloron* sp. or *Prochloron* spp. Some host species occasionally harbour these photosymbionts on the colony surface (i.e. facultative association), while others always harbour the *Prochloron* cells in the tunic or in the peribranchial–common cloacal cavities (i.e. obligate symbiosis) (Hirose *et al.*, 2009). At present, about 30 species from four didemnid genera have been described as host species (e.g. Kott, 2001; Monniot & Monniot, 2001), mostly from the tropical Indo-Pacific region. In contrast, there have been only few reports of *Prochloron* in the Atlantic region; *Trididemnum* cf. *solidum* from Curaçao and Virgin Islands, *Didemnum* cf. *candidum* and *Diplosoma* cf.

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virens from Puerto Rico, *Diplosoma virens* from Belize, and *Trididemnum cyanophorum* from Bahamas (Lewin *et al.*, 1980; Goodbody, 2000; López-Legentil *et al.*, 2011). However, details of the photosymbionts were not documented therein although this symbiotic system forms the only lifelong photosymbiosis known in the phylum Chordata.

On the other hand, there are many reports of the association between didemnids and cyanobacteria lacking chlorophyll *b* in the Atlantic Ocean (Lafargue & Duclaux, 1979; Monniot, 1983, 1984; Rocha *et al.*, 2005; López-Legentil *et al.*, 2011) and in the Mediterranean Sea (Hernández-Mariné *et al.*, 1990; Martínez-García *et al.*, 2011). The most common of such associations is with the reddish-brown cyanobacterium *Synechocystis* (Chroococcales: Lafargue & Duclaux, 1979; Cox, 1986). *Synechocystis* was first reported in the Caribbean ascidian *Trididemnum cyanophorum* (Lafargue & Duclaux, 1979) and was accordingly named *Synechocystis trididemni.* Since then, *S. trididemni* has also been reported in *T. clinides*, *T. nubilum*, *T. solidum*, *T. tegulum* and *Didemnum viride* (Bak *et al.*, 1981; Cox, 1986; Münchhoff *et al.*, 2007; López-Legentil *et al.*, 2011). To date, no *Synechocystis* species isolated from an ascidian has been cultured. Thus, new sequences obtained for *Synechocystis* symbionts in ascidians are referred to as *Synechocystis* sp. or *Synechocystis* spp. Molecular phylogenetics based on 16S rRNA gene sequences indicated that all sequences obtained for *Synechocystis* species associated with ascidians, including *S. trididemni*, were closely related and formed a sister taxa to the *Prochloron* lineage (Münchhoff *et al.*, 2007).

In this study, we described two didemnid ascidians associated with *Prochloron* from the vicinity of Bocas del Toro, Caribbean Panama. *Lissoclinum verrilli* (Van Name, 1902) was first described in the Bermudas Islands, while *Diplosoma simile* (Sluiter, 1909) is commonly found in the Indo-Pacific region. Although intense surveys of ascidian biodiversity were previously conducted in this region (Rocha *et al.*, 2005), this is the first record of the association between cyanobacteria and *L. verrilli* and the first record of *D. simile* in the Atlantic Ocean. In addition to *Prochloron*, 16S ribosomal RNA gene sequences corresponding to *Synechocystis* sp. were also obtained from *L. verrilli.* Besides describing these species and their association with cyanobacteria, we also obtained partial sequences of the cytochrome *c* oxidase subunit I (COI) gene from both ascidians and the cyanobacterial 16S rRNA gene to establish host–symbiont and Caribbean–Pacific relationships.

Materials and methods

Animals

Surveys were carried out by snorkelling in shallow subtidal zones (<3 m) in the vicinity of Bocas del Toro, Panama in 2011 (Supplementary Fig. 1, see supplementary material, which is available on the Supplementary tab of the article's Taylor & Francis Online page at http://dx.doi/10.1080/14772000.2012.735716). Ascidian colonies were photographed *in situ*, and then collected together with their substrate. One colony of *Lissoclinum verrilli* was collected at Isla Cristobal (9°16′56.4″N, 82°15′25.7″W) on 27 June. *Diplosoma simile* colonies were collected from Crawl Key (9°15′47.78″N, 82°07′51.64″W) on 22 June and from Isla Cristobal on 26 and 27 June. At least one specimen of each species was anaesthetized with menthol and 0.37 M $MgCl_2$ for approximately 2 h, and then fixed with 10% formalin-seawater. Other specimens were fixed in 2.5% glutaraldehyde-seawater without anaesthesia to perform electron microscopy analyses. Finally, pieces from colonies from both species were also preserved in 99% ethanol for DNA extraction.

Light and electron microscopy

Fixed specimens were dissected under a binocular stereomicroscope for species identification. Zooids and cyanobacterial cells isolated from the fixed materials were observed under a light microscope equipped with differential interference contrast (DIC) optics.

The glutaraldehyde-fixed colonies stored at 4 °C were rinsed with 0.1 M cacodylate buffer with 0.45 M sucrose and post-fixed for 1.5 h in 1% osmium tetroxide in 0.1 M cacodylate buffer. The specimens were then dehydrated with ethanol, cleared with *n*-butyl glycidyl ether, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEM-1011; JEOL).

DNA extraction, amplification and sequencing

The preserved colonies were dissected under a stereomicroscope to separate the zooids from the tunic. Zooid tissue was extracted to amplify a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) from the ascidian, while tunic tissue from *D. simile* and the superficial cyanobacteria layer observed in the surface of *L. verrilli* were extracted to amplify the 16S ribosomal RNA gene from their photosymbionts. DNA was extracted using the DNeasy blood and animal tissue kit (Qiagen). For COI amplification, we used the tunicate COI specific primer pair Tun_forward/Tun_reverse2 (Table 1). PCR amplification was performed under the following conditions: 94 °C for 5 min, followed by 40–70 cycles of 94 °C for 10–30 s, 40–50 °C for 30 s, and 72 °C for 1–2 min, with a final extension at 72 °C for 10 min. Amplification was performed in a 25 μL total-reaction volume with 10 pmol of each primer, 10 nmol of each dNTP, 1X reaction buffer (Ecogen)

Table 1. PCR primers used for amplification and sequencing of COI and 16S rRNA genes.

Primer	Sequence (5′– 3′)	Region	Specificity	Citation
Tun_forward	TCGACTAATCATAAAGATATTAG	COI	Tunicata	1
Tun_reverse2	AACTTGTATTTAAATTACGATC	COI	Tunicata	1
27F	AGAGTTTGATCCTGGCTCAG	16S	Eubacteria	2
359F	GGGGAATYTTCCGCAATGGG	16S	Cyanobacteria	3
809R	GCTTCGGCACGGCTCGGGTCGATA	16S	Cyanobacteria	2
740F	GGCYRWAWCTGACACTSAGGGA	16S	Cyanobacteria	2
1494R	TACGGCTACCTTGTTACGAC	16S	Eubacteria	2
1509R	GGTTACCTTGTTACGACTT	16S	Eubacteria	4

1 = Stefaniak *et al.*, 2009; 2 = Jungblut *et al.*, 2005; 3 = Nüebel *et al.*, 1997; 4 = Martínez-Murcia *et al.*, 1995.

and 5 units of Taq polymerase (BIOTAQ, Ecogen), or in a 20 μL total-reaction volume with 0.4 μM of each primer, 200 μM each dNTP, 1X reaction buffer (Takara), 1M betain, and 0.5 units of Taq polymerase (Takara EX *Taq* Hot-Start Version, Takara). Amplicons were directly sequenced when possible or purified and cloned into pMD20-T vector (Takara) using a DNA ligation kit (Mighty Mix, Takara). Sequences were obtained on a CEQ8800 (Beckman Coulter) automated DNA sequencing system or sent to Macrogen, Inc. (Seoul, Korea).

To obtain *Prochloron* 16S rRNA gene sequences, we used the cyanobacterial-specific primer pairs 27F/809R and 740F/1494R or 359F/1509R depending on the ascidian species (Table 1). PCR amplification was performed using the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 20–30 s, 50 °C for 30 s and 72 °C for 1.5–2 min, with a final extension at 72 °C for 7 min. PCR reaction volumes and mixes were conducted as described above. PCR products were treated with ExoSAP-IT (GE Healthcare) prior to direct sequencing reactions using a DTCS Quick Start Master Mix (Beckman Coulter) or gel-purified using the QIAquick Gel Extraction kit (Qiagen) and cloned using the pGEM-T vector system (Promega). 16S rRNA sequences were obtained on a CEQ8800 (Beckman Coulter) automated DNA sequencing system or sent for purification and sequencing to Macrogen, Inc. (Seoul, Korea). Final sequences were deposited in GenBank (Accession Nos. JX099359–JX099361 and AB723717–AB723721).

Phylogenetic analyses

In order to perform phylogenetic analyses, additional sequences were retrieved from GenBank (see accession numbers in Figs 12 and 13) and aligned using ClustalX (Thompson *et al.*, 1997). An additional colony of *D. simile* was collected in Teniya, Okinawajima Island, Japan (26 °34′78″N, 128 °9′1″E), sequenced as indicated above and added to the analyses. Neighbor-joining (NJ), minimum evolution (ME) and maximum likelihood (ML) analyses were conducted in MEGA 5 (Tamura *et al.*, 2011). For NJ and ME analyses, the Tamura-Nei model of nucleotide substitution was used considering gaps as partial deletion (95%), and data were re-sampled using 1000 bootstrap replicates (Felsenstein, 1985). The ML tree was built based on the GTR+I+G (Tavaré, 1986) model with substitution rates varying among sites according to an invariant and gamma distribution and 100 bootstrap replicates. For Bayesian inference (BI), MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to calculate the posterior probabilities of branch nodes, implementing the GTR+I+G likelihood model. The Monte Carlo Markov Chain length was set to 1 million generations for the ascidian phylogeny and 2.5 million generations for the cyanobacterial one, with sampling every 100th generation and with a burn-in value of 2500 and 6250 respectively. The average standard deviation of split frequencies between two independent chains reached values of less than 0.01 after 153 000 and 1 574 000 generations for the ascidian and cyanobacterial phylogenies, respectively.

Results

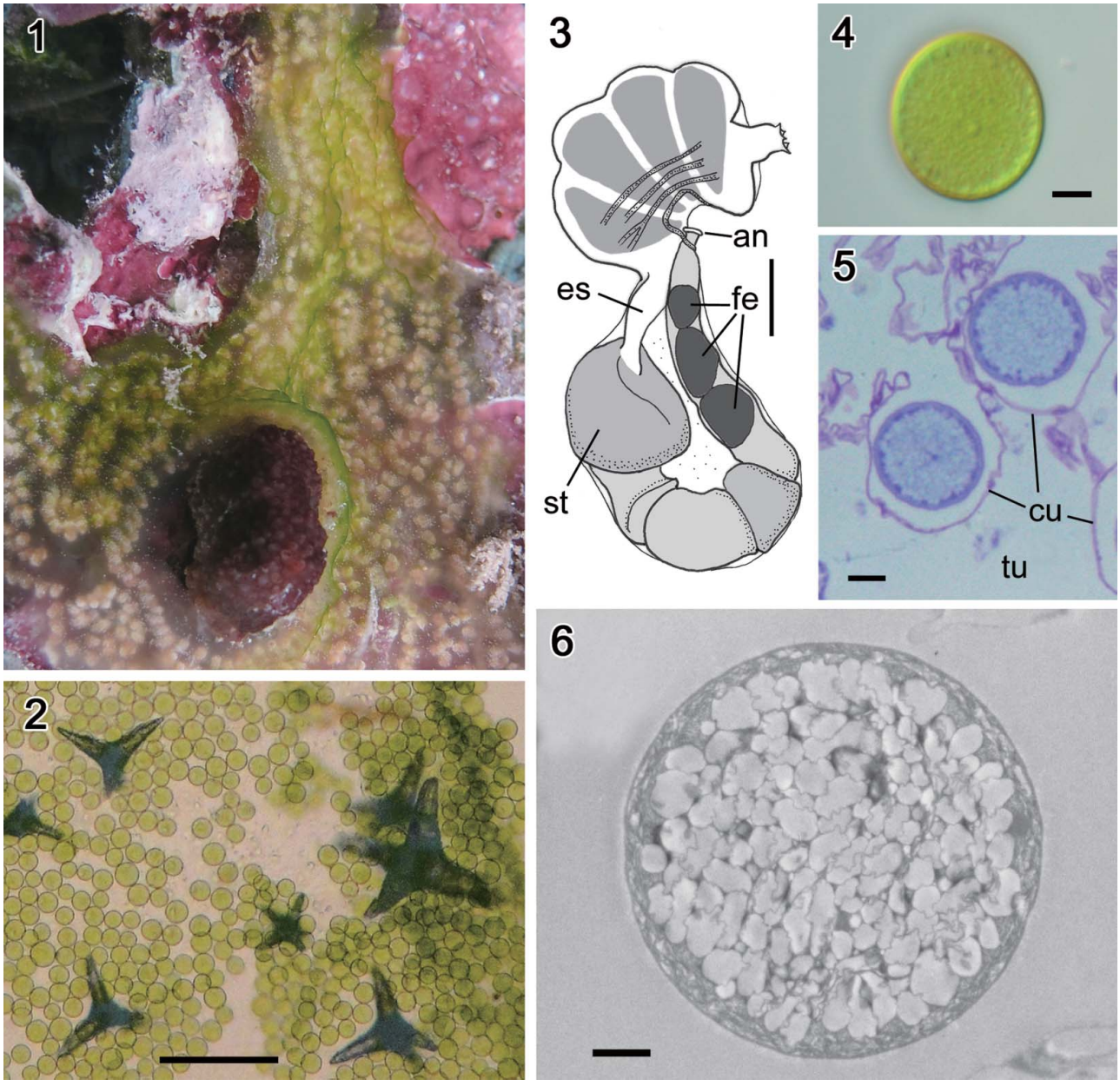
Didemnids associated with *Prochloron* in Bocas del Toro, Panama

Genus ***Lissoclinum*** Verrill, 1871

Lissoclinum verrilli (Van Name, 1902)

A transparent colony with pale yellow zooids was found on a coralline alga on the undersurface of dead coral stone (Fig. 1). The colony surface had patches of green tinge due to the presence of *Prochloron* on it (Fig. 2). The colony was a soft sheet *c*. 3 mm in thickness and the zooids were loosely arranged in double rows following elongated systems.

The tunic had consistent superficial and basal cuticles, but for the rest it was very soft. It had many granular cells (up to 10 μm) embedded throughout. The spicules were tetrahedral (sometimes with five rays), of variable sizes up to 0.15 mm between opposing rays, and without a developed central mass (Fig. 2). They were scattered in the superficial tunic layer and formed capsules around the abdomens, with three rays included in the tunic capsule and one spike



Figs 1–6. *Lissoclinum verrilli*. **1**, Colonies on the underside of a dead-coral stone. The colony surface is partly green due to the *Prochloron* cells. **2**, Tunic spicules and *Prochloron* cells isolated from the fresh colony. **3**, Line drawing of the zooid. **4**, *Prochloron* cells isolated from the fixed colony (DIC). **5**, *Prochloron* cells on the colony surface in a histological section stained with toluidine blue. **6**, Transmission electron micrograph of the *Prochloron* cell. an, anus; cu, tunic cuticle; es, esophagus; fe, feces; st, stomach; tu, tunic. Scale bars: 0.1 mm in 2 and 3, 5 μm in 4 and 5, 2 μm in 6.

protruding outwards. Spicules also surrounded the ventral sides of the thoraces. The zooids consisted of the thoraces measuring *c*. 0.3 mm and the abdomens *c*. 0.6 mm (Fig. 3). The thoraces were strongly contracted in the specimen and should be larger in the relaxed condition. The contraction prevented counting of stigmata number. There was a strong longitudinal musculature that, upon contraction, greatly narrowed the atrial aperture, consisting of one muscular band following the rim of the aperture and three bands near it (Fig. 3). No thoracic organs could be observed, likely because of the strong contraction. The abdomens' disposition ranged from not twisted (i.e. stomach on the ventral side) to 90° twisted with respect to the thorax. They had an orange-coloured stomach, followed by a colourless post-stomach. The mid-intestine was in two sections, the distal one slightly orange-pigmented, and the long posterior intestine opened at the base of the atrial aperture. No gonads or larvae were present in the colony studied.

The *Prochloron* cells were unicellular spheres of *c*. 20 μm in diameter (Fig. 4). They were often located in hollows of the ragged tunic surface and not directly adhered to the colony surface (Fig. 5). No *Prochloron* cells were distributed inside the tunic or in internal cloacal spaces. Thylakoids were stacked parallel to the cell surface in the cell periphery and the central region of the cell was filled with small vacuoles consisting of expanded portions of the thylakoids (Fig. 6).

Genus ***Diplosoma*** Macdonald, 1859

Diplosoma simile (Sluiter, 1909)

The colonies were found on the undersurfaces of dead coral rubble or on crevices in coral limestone. The colonies were irregularly shaped sheets and entirely dense green due to the abundance of *Prochloron* in the colony (Fig. 7). The colonies contained no spicules.

The zooids were *c*. 1 mm long. There were four rows of stigmata in the thorax; six stigmata in the first (top), six in the second row, six in the third row and five in the fourth (bottom) row (Fig. 8). A retractor muscle emerged from the underside of the thorax. The rectum ended at the level of the fourth row of stigmata. Bi-lobed testis was beneath the abdomen, and the vas deferens was not coiled. The present specimens did not have embryos.

The *Prochloron* cells were bright green and *c*. 15 μm in diameter and some were in the process of cell division (Figs 9–10). They were always located in the peri-branchial and common cloacal cavities. Thylakoids were stacked parallel to the cell surface in the cell periphery and the central region of the cell was occupied by a large vacuole that was an expanded portion of the thylakoids (Fig. 11).

Host and photosymbiont phylogeny

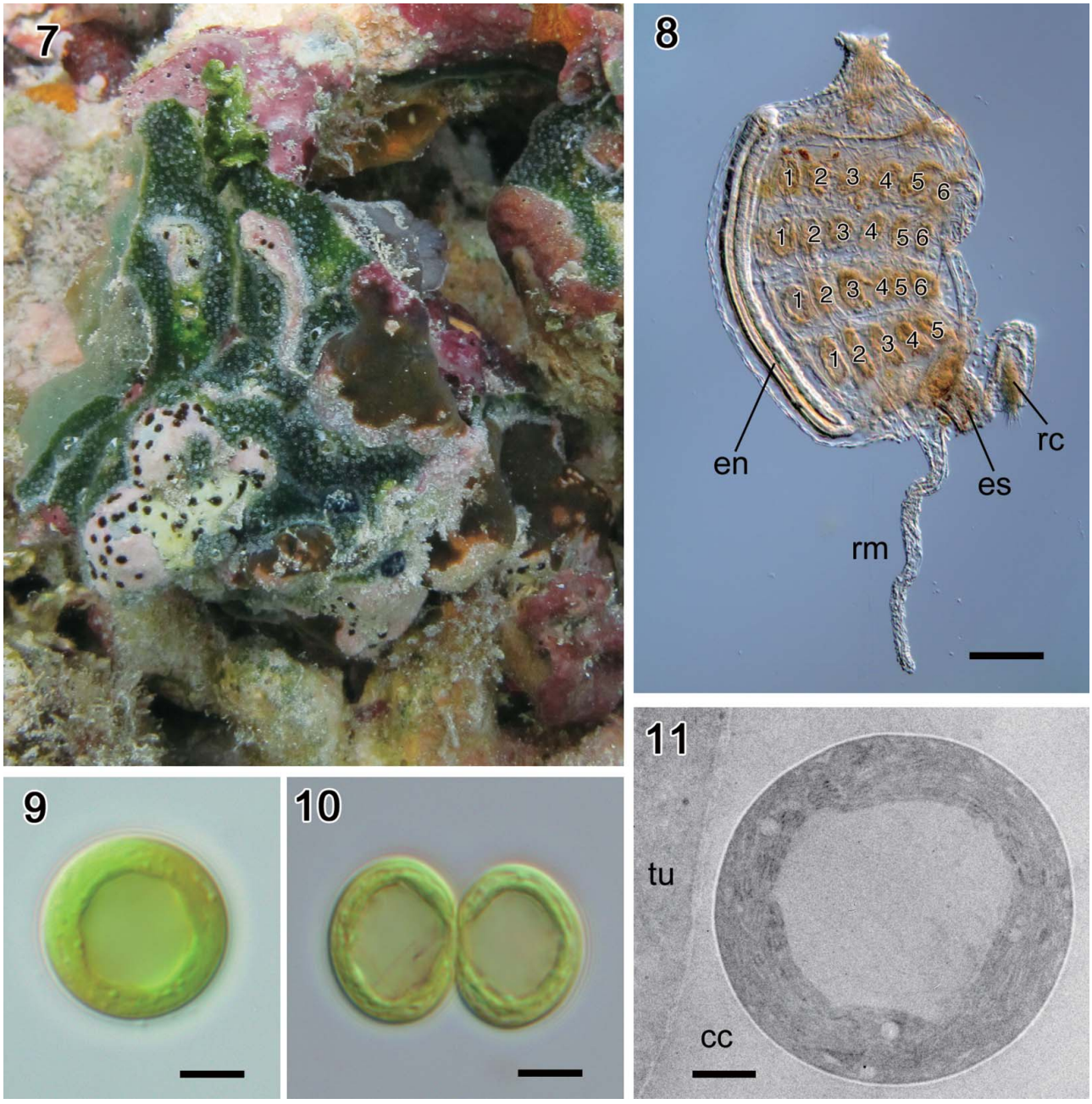
Partial COI gene sequences (552 bp) were obtained for *D. simile* and *L. verrilli* to assess host phylogeny and taxonomic relationships. Sequences for *D. simile* from the Caribbean and from the West Pacific were identical. The sequence obtained for *L. verrilli* contained a 6 bp gap (2 amino acids). The topology of the ML, ME, NJ and BI trees was nearly identical, except for the position of the *Lissoclinum fragile* and *L.* aff. *fragile* clade, which was placed either at the base of *Tridemnum*, *Diplosoma*, *Didemnun* and *Polysyncraton* or among them. Invariably, the *L. verrilli* sequence occupied a basal position within the Didemnidae while the two *D. simile* sequences formed a well-supported clade with *D. spongiforme* (Fig. 12). In all cases, phylogenetic analyses supported the monophyly of the genera *Didemnum*, *Tridemnun* and *Diplosoma*.

After alignment, partial 16S rRNA gene sequences ranging between 768 and 1408 bp were obtained for the photosymbionts associated with *D. simile* and *L. verrilli.* The three gene sequences obtained for *D. simile* and one of the sequences obtained from *L. verrilli* matched previously reported *Prochloron* sequences (>99% sequence identity). Distinct *Prochloron* phylotypes were present in *D. simile* hosts from the Caribbean and Indo-Pacific, and exhibited 1.9% sequence divergence. The other sequence obtained from *L. verrilli* was closely related to *Synechocystis tridemni* (>97% sequence identity) and other uncultured *Synechocystis* isolated from didemnid ascidians (Fig. 13). Phylogenetic analyses revealed that *D. simile* sequences from Isla Cristobal and Crawl Key in Panama formed a well-supported clade within a bigger clade grouping most of the *Prochloron* sequences obtained from Pacific host ascidians and also including the *Prochloron* sequence obtained for *L. verrilli* from Isla Cristobal (Fig. 13). A second clade grouped all *Synechocystis* sequences, most found in *Tridemnum* species from both the Pacific and the Caribbean, and now including the sequence obtained from *L. verrilli* in a basal position within this clade (Fig. 13).

Discussion

We have reported the occurrence of two didemnid ascidians harbouring *Prochloron* from the vicinity of Bocas del Toro, Panama. This is the first record of the association between *L. verrilli* and cyanobacteria (i.e. *Prochloron* and *Synechocystis*) and the first record of *D. simile* in the Atlantic Ocean. Both species inhabited shallow shaded spots under dead coral stones, which may explain why they have been overlooked in previous surveys. Host phylogeny based on partial COI sequences revealed that *L. verrilli* occupied a basal position within didemnid ascidians and did not group with other sequences of *Lissoclinum*, while the two sequences obtained for *D. simile* (one from the Caribbean and one from the Pacific) were identical. Photosymbiont phylogeny based on 16S rRNA sequences showed that the *Prochloron* sequences obtained for *L. verrilli* and *D. simile* belonged to different phylotypes. Moreover, the sequences obtained for *D. simile* symbionts from the Pacific and *D. simile* from the Caribbean were also grouped in different clades.

Since the original description as *Echinoclinum verrilli* from the Bermudas Islands (Van Name, 1902), *L. verrilli* has been widely reported in the Caribbean Sea (e.g. Monniot, 1983; Goodbody, 2000) including Bocas del Toro in Panama (Rocha *et al.*, 2005). As all species with tetrahedral spicules were initially assigned to *L. verrilli*, there were reports of this species from the Pacific waters (e.g. Tokioka, 1958; Kott, 1972; Monniot & Monniot, 1987), that are currently assigned to other species, such as *L. sente* and *L. tasmanense* (e.g. Kott, 1981, 2001), and the only record of *L. verrilli* in West Africa (Millar, 1953) may also be a different species. In fact, at present it is recognized that the so-called '*L. verrilli*-group' comprises several distinct species in the Indo-Pacific, and none corresponds to the



Figs 7–11. *Diplosoma simile.* **7**, Colonies on the underside of a dead-coral stone. **8**, Thorax of a zooid (DIC). Numbers indicate the stigmata. **9**, **10**, *Prochloron* cells isolated from the fixed colony (DIC). **11**, Transmission electron micrograph of the *Prochloron* cell in the common cloacal cavity (cc). en, endostyle; es, esophagus; rc, rectum; rm, retractor muscle; tu, tunic of the cavity wall. Scale bars: 0.1 mm in 8, 5 μm in 9 and 10, 2 μm in 11.

Atlantic *L. verrilli* (Kott, 2001). Likewise, there may be several species of this group in the Caribbean but, for the time being, we assign our specimen to the only recognized Atlantic species, *L. verrilli sensu stricto*, even if gonads and larvae were not present. The colony characteristics, the spicule shape and size, and the presence of strong longitudinal muscles (as figured also by Monniot, 1983) are in agreement with previous descriptions. The COI gene sequence obtained for *L. verrilli* presented an uncharacteristic 6 bp gap that has not been previously described for any ascidian species. Furthermore, *L. verrilli* COI sequence did not form a clade with other *Lissoclinum* species, raising doubts about the monophyly of the *Lissoclinum* genus as presently recognized. In fact, there are few (and likely plesiomorphic)

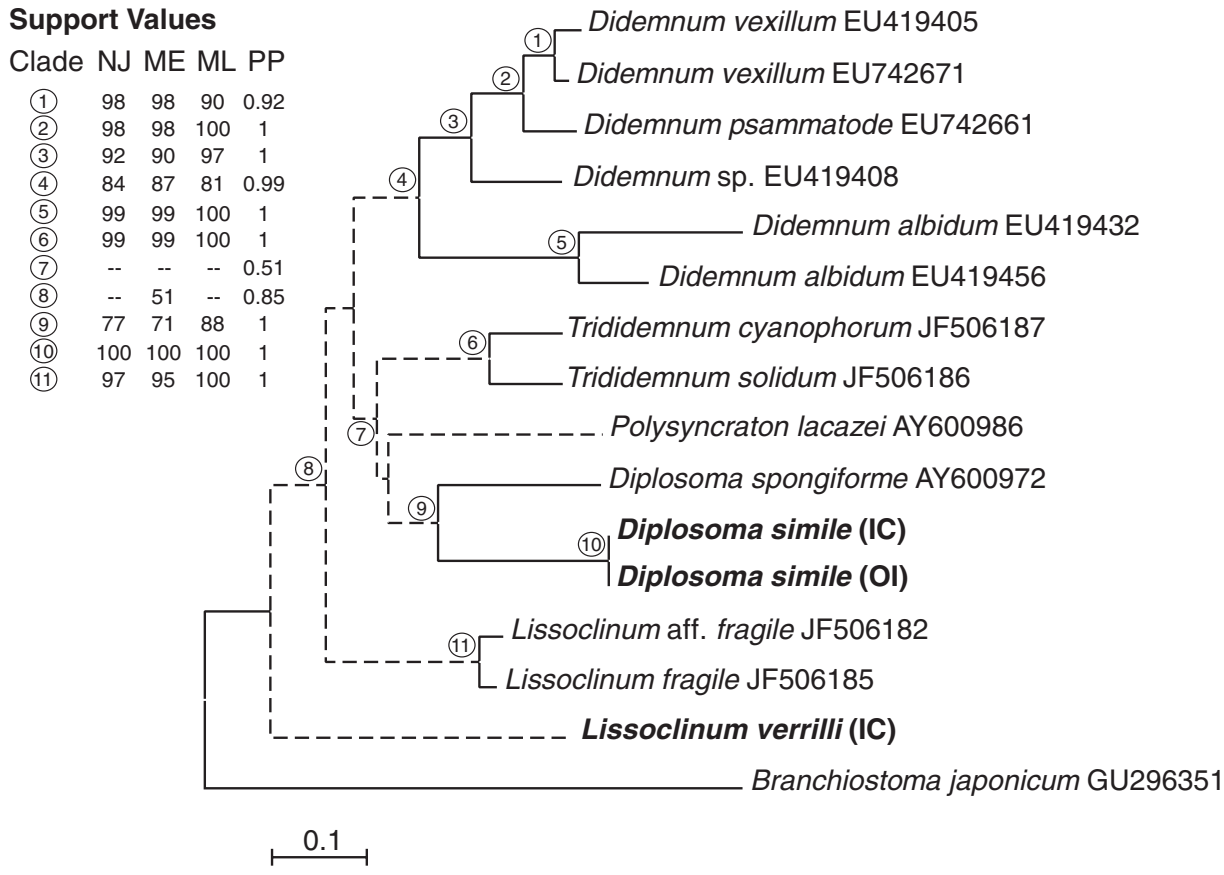
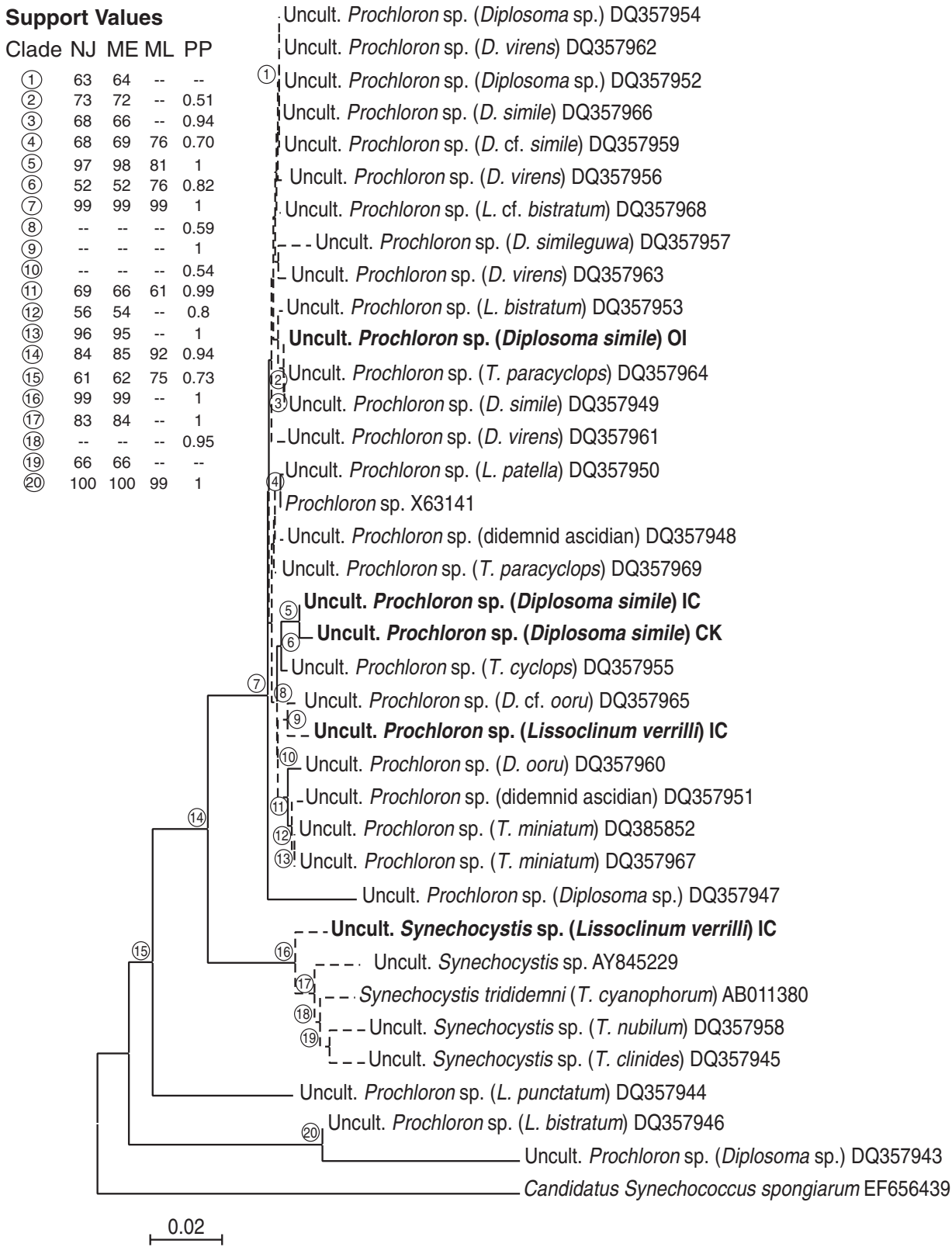


Fig. 12. Phylogeny of partial COI gene sequences from didemnid ascidians highlighting the phylogenetic position of the sequences obtained in the present study (in bold). Labels on terminal nodes of reference sequences indicate the ascidian species and GenBank accession number. Labels on terminal nodes of sequences from this study include species name and collection location (IC: Isla Cristobal, Panama; OI: Okinawajima Island, Japan). Tree topology was obtained from neighbour-joining (NJ) analysis. Individual bootstrap values from NJ, minimum evolution (ME) and maximum likelihood (ML) analyses and posterior probabilities (PP) from Bayesian inference are located in the upper-left box and correspond to circle numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 50% for all criteria) and dashed lines indicate weakly supported branches. Scale bar represents 0.1 substitutions per site.

characters, such as the spermduct shape, that define the genus *Lissoclinum* (Kott, 2001). It is clear that the observation of more specimens from the tropical Atlantic and other areas, coupled with genetic analyses, are required to ascertain the true diversity of the *L. verrilli* group in the Caribbean and elsewhere and the validity of their inclusion in the genus *Lissoclinum*. On the other hand, the association between *L. verrilli* and cyanobacterial species of *Prochloron* and *Synechocystis* has not been previously reported, indicating that it is a facultative symbiosis. This type of symbiosis is generally characterized by the formation of a photosymbiont layer on the surface of the colonies and has been often reported in didemnid ascidians including *Didemnum candidum* (Lewin, 1975), *Trididemnum banneri* and *T. cerebriforme* (Monniot, 1990), and *Lissoclinum nebulosum* (Kott, 2001). Similarly, *Prochloron* spp. have also been reported to establish facultative relationships with other invertebrates (Cheng & Lewin, 1984; Parry, 1986).

Diplosoma simile is widely distributed in the West Pacific, from the Ryukyu Archipelago, Japan to the Great Barrier Reef, Australia. Moreover, this species has been reported from oceanic islands in the Pacific Ocean, e.g. French Polynesia, Hawaii and the Bonin Islands (Monniot & Monniot, 1987; Abbott *et al.*, 1997; Hirose *et al.*, 2007). The present specimens were identified as *D. simile* based on specific features of this species (e.g. numbers of stigmata in each row and the position of retractor muscles) and molecular data showing identical COI gene sequences for specimens from the Caribbean and the West Pacific. Among the photosymbiotic *Diplosoma* species, *D. simile* has the widest distribution range to the best of our knowledge, indicating high larval-dispersal ability and broad range of environmental tolerance. Other photosymbiotic *Diplosoma* species reported in the Caribbean include *Diplosoma* cf. *virens* from Puerto Rico (Lewin *et al.*, 1980) and *D. virens* from Belize (Goodbody, 2000). Colonies of *D. virens*



always harbour *Prochloron* as found in *D. simile.* Besides the point at which the retractor muscle separates from the zooid, *D. simile* and *D. virens* are very similar in morphology, whereas the partial COI sequences clearly discriminate these species (Hirose & Hirose, 2009). Although *D. virens* is also widely distributed in the Indo-West Pacific region, the distribution range of *D. virens* is considerably smaller than that of *D. simile*; *D. virens* has not been recorded in French Polynesia, Hawaii or the Bonin Islands (Monniot & Monniot, 1987; Abbot *et al*., 1997; Hirose *et al.*, 2007). Thus, it is possible that the record of *D. virens* in Lewin *et al.* (1980) and Goodbody (2000) is a misidentification of *D. simile.* As for their photosymbionts, it is generally accepted that the association between *D. simile* and *Prochloron* is an obligate symbiosis, because the colonies of *D. simile* always harbour *Prochloron* cells in the peribranchial–cloacal cavities and the larvae always acquire *Prochloron* cells from their maternal colonies (Hirose, 2000).

The *Prochloron* cells of *L. verrilli* were very different in cytomorphology compared with the *Prochloron* cells of *D. simile* (Figs 4–6, 9–11). Cox (1986) distinguished three groups in *Prochloron* from various host ascidians: *Prochloron* cells occurring on the surface or in the tunic of the host ascidian that have small 'vacuoles' filling the central region of the cell (Group I), those in peribranchial–cloacal cavities of the host that have a large central 'vacuole' (Group II) and those in *Didemnum molle* that are characterized by little 'vacuolation' and many granular inclusions (Group III). Cox's grouping is consistent with the *Prochloron* distribution in the present specimens: the *Prochloron* cells on the colony surface of *L. verrilli* correspond to Group I, and those in cloacal cavities of *D. simile* belong to Group II.

The phylogenetic trees of *Prochloron* based on partial 16S rRNA gene sequences revealed that the Caribbean *Prochloron* harboured in *L. verrilli* and *D. simile* formed a clade with *Prochloron* from various didemnid hosts in the tropical Pacific. The two *Prochloron* sequences from Caribbean *D. simile* (*Diplosoma simile* IC and CK in Fig. 13) formed a well-supported clade within the *Prochloron* lineage. However, they did not form a clade with *Prochloron* from Pacific *D. simile* (*Diplosoma simile* OI, DQ357949 and DQ357966 in Fig. 13) and represent distinct phylotypes (1.9% sequence divergence), whereas the Caribbean host and the Pacific host exhibited identical partial sequences of the COI gene. Thus, multiple phylotypes of *Prochloron* exist in Caribbean Panama indicating low levels of both host specificity and geographic speciation, likely as a result of horizontal transmission. These results are in agreement with the observations of Münchhoff *et al.* (2007) in the West Pacific and support their hypothesis on the existence of a free *Prochloron* able to cross considerable distances prior to recolonization of a host ascidian. It is noteworthy that from all the sequences in the phylogenetic tree, the *Prochloron* sequence obtained from *L. verrilli* is the only one that has been obtained from a facultative symbiosis (photosymbiont layer on the colony surface) with a taxonomically identified ascidian host. *Prochloron* forming facultative associations occur within the clade of *Prochloron* forming obligate associations, suggesting a high degree of cytomorphological plasticity in this cyanobacterial group and horizontal transfer of photosymbionts within geographical regions and among various didemnid hosts, forming obligate or facultative associations depending on the particular ascidian host. In turn, the morphological changes of *Prochloron* cells may represent acclimation to the specific microhabitats of obligate (interior cloacal cavities) and facultative (exterior surface) symbiont locations. Periodic horizontal exchange of the photosymbionts would indeed result in the low levels of both host specificity and geographic speciation observed in *Prochloron* (Hirose *et al.*, 2009).

Additionally, a *Synechocystis trididemni*-like sequence was obtained from the colony of *L. verrilli*, although we did not observe these algal cells microscopically and it is uncertain whether this association is obligate or facultative. *Synechocystis* is known as a photosymbiont of some didemnid ascidians, including the host ascidians *Trididemnum cyanophorum* and *T. solidum* (Lafargue & Duclaux, 1979; López-Legentil *et al.*, 2011) that are also common in Bocas del Toro. In addition, *Synechocystis* species have also been sporadically reported in some sponges (Cox *et al.*, 1985; Steindler *et al.*, 2005; Erwin *et al.*, 2012), indicating broader host-specificity than *Prochloron* species. Coexistence of *Prochloron* and *Synechocystis* cyanobacteria within the same host has been previously reported in the didemnid ascidians *Trididemnum clinides* (Cox, 1986) and *Trididemnum cyanophorum* (López-Legentil *et al.*, 2011).

Fig. 13. Phylogeny of partial 16S rRNA gene sequences from photosymbionts isolated from *L. verrilli* and *D. simile*. Sequences obtained in this study are highlighted (bold lettering). Labels on terminal nodes of reference sequences indicate other cyanobacteria species, host ascidian species in parentheses, and their GenBank accession numbers (Uncult.: Uncultured; *D.*: *Diplosoma*; *L.*: *Lissoclinum*; *T.*: *Trididemnum*). All ascidian species except for *T. cyanophorum* (Caribbean) were sampled in the Indo-Pacific region. Labels on terminal nodes of sequences from this study include species name and collection location (IC: Isla Cristobal, Panama; CK: Crawl Key, Panama; OI: Okinawajima Island, Japan). Tree topology was obtained from neighbour-joining (NJ) analysis. Individual bootstrap values from NJ, minimum evolution (ME) and maximum likelihood (ML) analyses and posterior probabilities (PP) from Bayesian inference are located in the upper-left box and correspond to circle numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 50% for all criteria) and dashed lines indicate weakly supported branches. Scale bar represents 0.02 substitutions per site.

Cox *et al.* (1985) hypothesized that '*Prochloron* developed from an ancestor resembling *S. trididemni* and already living in a symbiotic relationship'. This hypothesis was further supported by the close phylogenetic relationship found in analyses of *Synechocystis* and *Prochloron* based on partial sequences obtained for the 16S rRNA gene and the large subunit of Rubisco gene (Shimada *et al*., 2003; Münchhoff *et al.*, 2007; present study).

Molecular phylogeny of didemnid ascidians inferred from 18S rRNA gene sequences supported the hypothesis that establishment of the ascidian–*Prochloron* symbiosis occurred several times within the didemnid lineage (Yokobori *et al.*, 2006). Since a facultative association is likely to precede the evolution of an obligate symbiosis, some didemnid species would have independently established a facultative association with *Prochloron* in parallel and evolved to an obligate symbiosis. Considering the abundance and the number of host species, ascidian–*Prochloron* symbioses likely originated in tropical Indo-West Pacific and were afterwards introduced to the Caribbean, possibly before the rise of the Panama isthmus. Alternatively, a more recent origin could be attributed to maritime transport through the Panama Canal. As reported by Carman *et al.* (2011), the Panama Canal may serve as a corridor between the East Pacific and Caribbean Sea for invasive ascidians. Thus, it is possible that vessels passing through the canal might also carry ascidian larvae or colonies bearing *Prochloron* in their ballast waters. The presence of multiple phylotypes of *Prochloron* within the Bocas del Toro region suggests that the photosymbionts were introduced multiple times, and that these introductions involved both facultative and obligate symbionts. The fact that the obligate symbiont of the Caribbean *D. simile* is different from that found in the Pacific, while the host species is identical (morphologically and genetically) raises doubts about whether this species is a new introduction that has switched symbionts (perhaps displaced by a strain better adapted to local conditions) or whether it has been in the Caribbean for a long time and the symbiont has differentiated there. The low host specificity and low geographic speciation described for *Prochloron* so far lends support to the former hypothesis (Münchhoff *et al.*, 2007; present study). Clearly, we need to have a comprehensive grasp of the genetic diversity in Caribbean *Prochloron* using different molecular markers and their relationship to their Pacific counterparts to disclose the histories of establishment and diversification of this unique photosymbiosis.

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