Cyanobacterial Diversity and a New Acaryochloris-Like Symbiont from Bahamian Sea-Squirts

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Abstract
Symbiotic interactions between ascidians (sea-squirts) and microbes are poorly understood. Here we characterized the cyanobacteria in the tissues of 8 distinct didemnid taxa from shallow-water marine habitats in the Bahamas Islands by sequencing a fragment of the cyanobacterial 16S rRNA gene and the entire 16S–23S rRNA internal transcribed spacer region (ITS) and by examining symbiont morphology with transmission electron (TEM) and confocal microscopy (CM). As described previously for other species, Trididemnum spp. mostly contained symbionts associated with the Prochloron-Synechocystis group. However, sequence analysis of the symbionts in Lissoclinum revealed two unique clades. The first contained a novel cyanobacterial clade, while the second clade was closely associated with Acaryochloris marina. CM revealed the presence of chlorophyll d (chl d) and phycobiliproteins (PBPs) within these symbiont cells, as is characteristic of Acaryochloris species. The presence of symbionts was also observed by TEM inside the tunic of both the adult and larvae of L. fragile, indicating vertical transmission to progeny. Based on molecular phylogenetic and microscopic analyses, Candidatus Acaryochloris bahamiensis nov. sp. is proposed for this symbiotic cyanobacterium. Our results support the hypothesis that photosymbiont communities in ascidians are structured by host phylogeny, but in some cases, also by sampling location.

Introduction
Symbioses between cyanobacteria and marine invertebrates are common, especially in sponges (reviewed in [1]) and ascidians (reviewed in [2]). However, little is known about the nature of these symbioses and much remains to be described. In particular, few studies have employed molecular approaches to more accurately assess bacterial diversity and host-specificity [3,4,5,6,7,8]. The majority of ascidian-microbe studies have focused on species within the ascidian family Didemnidae (Aplosoubranchia), which often establish symbiotic relationships with unicellular cyanobacteria from the genera Prochloron (Prochlorales) and Synechocystis (Chroococcales). The type species of these genera are Prochloron didemni, first found in Didemnum spp. from Baja California [9,10], and Synechocystis trididemni, found in the Caribbean ascidian Trididemnum solidum [11]. The cell morphology of both cyanobacterial species is very similar [11], and molecular phylogenetic analyses revealed that they had evolved from a common cyanobacterial ancestor [12,13].

More recently, a new oxygenic photosynthetic pigment was found in the tropical didemnid Lissoclinum patella [14]. This photosymbiont was tentatively named Acaryochloris marina and presented a set of unique characteristics, the most remarkable being that it uses chlorophyll d (chl d) as a predominant photosynthetic pigment [14,15]. Chl d is a minor photosynthetic pigment that was found in association with red macroalgae and though to be an artifact [16]. Since then, the presence of Acaryochloris has also been reported in other ascidian species. Kuhl et al. [17] reported Acaryochloris-like cells growing on biofilms beneath the didemnid ascidians L. patella, Diplosoma similis and D. irens. Martinez-Garcia et al. [8] also observed small patches of Acaryochloris-like cells on the basal tunic layer of the Mediterranean ascidian Cystodytes dellechiajei (Polycitoridae).

Although the role of photosymbionts in most symbiotic relationships is unknown, the few studies that have investigated ascidian-cyanobacterial symbioses proposed a mutualistic relationship (reviewed in [18] for Prochloron symbiosis), with direct transmission of symbionts between adult generations through the larva [19,20,21,22,23]. Vertical transmission allows the maintenance of the symbiotic relationship and ensures that offspring have immediate access to the microbes necessary for their survival [18,24]. This strategy is believed to give hosts a competitive edge from an early stage, and it is normally associated with obligate symbioses.

In the Caribbean, the colonial ascidian Trididemnum solidum Van Name 1902 is known to overgrow and kill corals [25]. This species is distributed in patches and releases larvae throughout the year, the majority settling within 15 min [26]. Both the larvae and the adult of T. solidum are associated with cyanobacteria of the genus Synechocystis [12,27,28]. Concentrations of chl a are much higher in the larvae than in the adults, suggesting that the ascidian is highly
dependent on its photosymbionts [28,29]. *Trididemnum cyanophorum* Lafargue & Duchaux 1979 was first described together with its symbiont *Synechocystis trididemni* Lafargue & Duclaux 1979 was first described together with its *Trididemnum cyanophorum* dependent on its photosymbionts [28,29].

The cosmopolitan didemnid *Lissoclinum fragile* (Van Name, 1902) is also found in the Caribbean and is known to carry symbiotic cyanobacteria [30,31]. Monniot [30] described the symbiont of *L. fragile* as an alga located in the cloacal cavities of the colonies, in the tunic pouches around the abdomen of each zooid, in the mantle surrounding the gonads, and in the surface layer of the larvae [30]. In contrast, Kott et al. [32] and Cox [33] reported that the symbiont of *L. fragile* was a species of *Prochloron*, usually found in patches on the surface of the colonies.

The aim of this study was to assess the diversity of the cyanobacterial community inhabiting didemnid ascidians from the Bahamas Islands. We established the genetic identity and diversity of both the ascidian hosts and their photosymbionts in order to better understand the degree of host-specificity. To achieve this goal, we determined host phylogeny by sequencing a fragment of the mitochondrial gene cytochrome oxidase I (COI) that is commonly used to determine species boundaries and diversity among ascidian taxa [34,35,36,37,38]. In order to identify and describe the photosymbiont diversity from within ascidian tissues, we sequenced a fragment of the 16S rRNA gene and the entire 16S–23S rRNA internal transcribed spacer region (16S–23S ITS).

We also examined the morphology of the photosymbionts by transmission electron microscopy (TEM). Finally, we used confocal microscopy (CM) to investigate the presence of chlorophyll *d* (chl *d*) and phycobiliproteins (PBPs) in some of our ascidian samples.

### Materials and Methods

**Ascidian samples and identification**

Ascidian samples were collected from mangroves of Sweeting’s Cay, and coral reefs of Little San Salvador and Plana Cay (Bahamas) by SCUBA diving in 2008 and 2010 (Table 1). Collection of samples was performed with the permission of the Government of the Bahamas (to JRP). Pictures of each sampled colony were taken *in situ* before fixation in absolute ethanol (Figure 1). A piece of each colony was anaesthetized by cold exposure as described elsewhere [39], and fixed in formaldehyde for examination of zooids in a relaxed state. Spicules were obtained from small pieces of the tunic (≈5 mm²) previously boiled in commercial bleach until complete oxidation of the tissue, Spicules were then washed several times in water, dehydrated in absolute ethanol, and sputter-coated with gold. All spicule samples were obtained from small pieces of the tunic (5 mm²) previously boiled in commercial bleach until complete oxidation of the tissue, and then washed several times in water, dehydrated in absolute ethanol, and sputter-coated with gold. All spicule samples were processed. To maximize ascidian DNA yield, several zooids were carefully separated from the tunic under a stereomicroscope. DNA extraction and PCR amplification

Samples fixed in absolute ethanol were kept at −20°C until processed. To maximize ascidian DNA yield, several zooids were carefully separated from the tunic under a stereomicroscope. DNA extraction and PCR amplification

### Table 1. Ascidian species analyzed in this study.

| Species              | Date      | Code | Location          | GPS position | Acc. Num. COI |
|----------------------|-----------|------|-------------------|--------------|---------------|
| *Trididemnum cyanophorum* | 30-May-08 | SC 2-1 | Sweeting’s Cay     | 26 38° 35’N; 77 57° 44’W | JF506187 |
| *Lissoclinum aff. fragile A* | 4-Jun-08 | LSS 1-7 | Little San Salvador | 24 35° 9’N; 75 58° 26’W | JF506183 |
| *Lissoclinum fragile A* | 7-Jun-08 | WPC 1-1 | West Plana Cay    | 22 36° 15’N; 73 37° 39’W | JF506185 |
| *Lissoclinum fragile B* | 7-Jun-08 | WPC 3-6 | West Plana Cay    | 22 35° 50’N; 73 37° 45’W | JF506184 |
| *Lissoclinum fragile C* | 8-Jun-08 | EPC 1-2 | East Plana Cay    | 22 36° 23’N; 73 33° 37’W | JF506181 |
| *Lissoclinum fragile D* | 8-Jun-08 | EPC 1-5 | East Plana Cay    | 22 36° 23’N; 73 33° 37’W | JF506180 |
| *Lissoclinum aff. fragile B* | 9-Jun-08 | LSS 1-2 | Little San Salvador | 24 35° 7’N; 75 58° 25’W | JF506182 |
| *Trididemnum solidum* | 9-Jun-08 | LSS 2-1 | Little San Salvador | 24 35° 6’N; 75 58° 20’W | JF506186 |

Species name, sampling date, code, location within Bahamas, GPS position, and GenBank accession numbers for the cytochrome oxidase I (COI) gene.

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Figure 1. Images of Bahamian ascidians and their spicule types. (A) *Trididemnum cyanophorum* from Sweeting’s Cay (SC 2-1); (B) *Lissoclinum* aff. *fragile* from Little San Salvador (LSS 1-7); (C) *Lissoclinum fragile* from West Plana Cay (WPC 1-1); (D) *Lissoclinum fragile* from West Plana Cay (WPC 3-6); (E) *Lissoclinum fragile* from East Plana Cay (EPC 1-2); (F) *Lissoclinum fragile* from East Plana Cay (EPC 1-5); (G) *Lissoclinum* aff. *fragile* from Little San Salvador (LSS 1-2); and (H) *Trididemnum solidum* from Little San Salvador (LSS 2-1). Scale bar on ascidian photos = 2 cm. Scale bar on spicule photos = 50 μm.

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Cyanobacterial Symbionts of Bahamian Ascidians

Several ascidian colonies per species and sampling site were ground together in absolute ethanol using a sterile pestle. The resulting greenish liquid was decanted into two 20 mL glass scintillation vials and evaporated under vacuum to leave a powdery organic residue. To re-suspend the extract, 1 mL of lysis solution from the Puregene kit (Qiagen) was added to each scintillation vial, vortexed, and incubated at 55°C for 65 min. The remaining solution was placed in two 2 mL sterile tubes and incubated for an additional hour at 55°C. After allowing the tubes to cool down, we continued with the DNA extraction protocol following manufacturer's instructions. The primer set CYA781F [44] and CYA2381R or Primer 340 [45] was used to amplify the 3’ end of the 16S rRNA gene, the complete 16S–23S ITS region, and a small fragment of the 23S rRNA gene. Total reaction volume was 25 μL with: 12.5 μL of GoTaq mastermix (Promega), 1 μL of each primer (10 μM), 10.5 μL of PCR grade water, and 1 μL of DNA. PCR program was set as follows: A single soak at 95°C for 5 min, 38 amplification cycles (denaturation at 94°C for 1.5 min; annealing at 50°C for 2 min; and extension at 72°C for 2 min), and a final extension at 72°C for 10 min, in a Peltier PTC-200 gradient PCR. PCR products were run in a low-melting-point agarose gel (1%) to confirm insert size before sequencing using BigDye TM terminator v. 3.1 on an ABI Prism 3100 automated sequencer. Because of the length of some of our sequences (up to 1445 bp), direct sequencing with primers T7 and M13R did not always result in a complete sequence, so we used the primer U1098F [46] to close any remaining gap. All our sequences (up to 1445 bp), direct sequencing with primers T7 and M13R did not always result in a complete sequence, so we used the primer U1098F [46] to close any remaining gap. All sequences have been deposited in GenBank (accession numbers and codes in Figures 2, 3, and 4). Neighbor-joining (NJ) analyses were performed on the deconvolved image stacks after 3D surface detection ranges for chl d (chl d) and phycoïl-proteins (PBPs) were set to 700–750 nm and PBPs were set to 700–750 nm and 640–670 nm, respectively. Laser power and PMT gain and offset were equally set up for both signals. Confocal images were acquired at the maximum resolution of the objective and the stacks were deconvolved with Huygens Essential (Scientific Volume Imaging, B.V.). Deconvolution was calculated with the optimized iteration mode of this software and all image parameters were read from the Leica. lif format. Object counting and measurements were performed on the deconvolved image stacks after 3D surface rendering using Imaris (Bitplane A.G.) and the Imaris MeasurementPro module. Length and width of at least 80 cells per sample was calculated using the software ImageJ 1.41o. Each stack was
analyzed setting the fluorescence threshold to the value that resulted in the maximum number of individual objects identified. Quantification was performed on those objects that showed both signals (PBPs and chl\textsubscript{d}). Final artwork was done with Photoshop CS (Adobe). Chl\textsubscript{d} and PBPs images were colored in red and green, respectively.

Results and Discussion

Host phylogeny

Partial COI gene sequences were obtained for all the samples, with a final alignment length of 605 bp. As expected, phylogenetic analyses grouped the two \textit{Trididemnum} sequences studied in a well-supported clade (bootstrap values $>$99\% in all analysis; Figure 2I). The taxonomic status of \textit{T. cyanophorum} has been argued in the past, with some authors suggesting that it may be a synonym of \textit{T. solidum} \cite{30}. Here, we have found 12\% sequence variability between \textit{T. solidum} and \textit{T. cyanophorum} and some morphological differences in terms of color and colony shape (Figure 1), suggesting that both species names are valid despite the lack of morphological differences among zooids. All the sequences of \textit{Lissoclinum} obtained in this study formed a well-supported clade (bootstrap values = 100\% in all analysis; Figure 2II). Moreover, within the \textit{Lissoclinum} clade, two subgroups with 3\% sequence variability were further distinguished. The \textit{Didemnum vexillum} cluster includes GenBank sequences: EU419439, -57, EU742661, -68, -69, -73. Scale bar represents 0.05 substitutions per site.

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**Support Values**

| Clade | NJ | MP | ML | PP |
|-------|----|----|----|----|
| 0     | 100| 100| 99 | 1  |
| 1     | 75 | 76 | 55 | 0.94|
| 2     | 100| 100| 100| 1  |
| 3     | 97 | 98 | 94 | 0.99|
| 4     | 100| 100| 100| 1  |
| 5     | 90 | 89 | 69 | 0.81|
| 6     | 99 | 100| 98 | 1  |
| 7     | 95 | 99 | 97 | 1  |
| 8     | 100| 100| 85 | 0.98|

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Figure 2. Host phylogeny with partial COI gene sequences. Sequences obtained in this study are highlighted (bold lettering). The Stolidobranchia species \textit{Botryllus schlosseri} was used as an outgroup taxa. Labels on terminal nodes of reference sequences indicate the ascidian species and GenBank accession number or code in the Barcode of Life database. Labels on terminal nodes of sequences from this study include species name and sample code as in Table 1 (underlined: SC: Sweeting’s Cay; LSS: Little San Salvador; WPC: West Plana Cay, and EPC: East Plana Cay). Bars and labels highlight the two clades of interest (I and II). Samples corresponding to \textit{Lissoclinum aff. fragile} and \textit{L. fragile} are further highlighted with a grey bar (IIA and IIB, respectively). Tree topology was obtained from neighbor-joining (NJ) analysis. Individual bootstrap values from NJ, maximum parsimony (MP) 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 bootstrap support greater than 50\% from at least 2 of the 4 phylogenetic criteria, and dashed lines indicate weakly supported branches. The \textit{Didemnum vexillum} cluster includes GenBank sequences: EU419439, -57, EU742661, -68, -69, -73. Scale bar represents 0.05 substitutions per site. doi:10.1371/journal.pone.0023938.g002
## Support Values

| Clade | NJ | MP | ML | PP |
|-------|----|----|----|----|
| 1     | 52 | -- | -- | -- |
| 2     | 65 | -- | -- | -- |
| 3     | 95 | 92 | 86 | 0.99 |
| 4     | 99 | 99 | 99 | 1   |
| 5     | 86 | 64 | 83 | 1   |
| 6     | 69 | -- | 83 | 0.86 |
| 7     | 99 | 98 | 96 | 1   |
| 8     | 99 | 98 | 79 | 1   |
| 9     | 50 | -- | -- | -- |
| 10    | 99 | 98 | 99 | 1   |
| 11    | 72 | -- | -- | 0.86 |
| 12    | 50 | -- | 61 | -- |
| 13    | 56 | -- | 70 | 0.98 |
| 14    | 92 | 66 | 95 | 0.99 |
| 15    | 87 | 75 | 58 | 1   |
| 16    | 82 | 79 | 83 | 1   |
| 17    | 100| 99 | 100| 1   |
| 18    | 63 | -- | 64 | 0.75 |
| 19    | 99 | 84 | 81 | -- |
| 20    | 99 | 93 | 96 | 0.99 |
| 21    | 97 | 89 | 67 | -- |
| 22    | 96 | 93 | 89 | 1   |
| 23    | 97 | 75 | 53 | 0.99 |
| 24    | 88 | 69 | 71 | 0.93 |
| 25    | 71 | 75 | 82 | 0.89 |
| 26    | 65 | 56 | -- | 0.52 |
| 27    | 74 | 67 | 52 | 0.98 |
| 28    | 60 | -- | 53 | -- |
| 29    | 100| 98 | 80 | 0.90 |
| 30    | 100| 99 | 100| 1   |
| 31    | 74 | 53 | -- | 0.51 |
| 32    | 92 | -- | -- | -- |
| 33    | 56 | -- | -- | -- |
| 34    | 92 | 86 | 74 | 0.83 |
| 35    | 99 | 86 | 64 | 0.97 |
| 36    | 83 | 60 | 55 | 0.56 |
| 37    | 97 | 87 | 56 | 0.68 |

### Additional Information

- **Prochloron spp.**
  - **Trididemnum cyanophorum A (3,4,7)**
  - **Trididemnum cyanophorum B (1)**
    - Prochloron sp. DQ357944
  - **Trididemnum cyanophorum A (1,2,5,6,8)**
  - **Trididemnum solidum (1 to 8)**
    - Synechocystis trididiemni AB011380
    - Prochloron sp. DQ357958
    - Synechocystis sp. YW845228
    - Sponge symbiont YW701294
  - **Trididemnum cyanophorum B (4)**
    - Trichodesmium sp. X70767
    - Blepharitix sp. EU253968
    - Phormidium animale EF654087
    - Phormidium autumnale DQ493074
    - Microcoleus vaginatus EF654078
    - Lissoclinum fragile A (3) WPC 1-1
      - Oscillatoria corallinae X84812
      - Oscillatoria spongiae AF534686
      - Oscillatoria spongiae AF534693
    - Lissoclinum fragile D (5 to 8) EPC 1-5
    - Lissoclinum fragile C (3,4,5,7) EPC 1-2
    - Acaryochloris marinaAY512879
  - **Trididemnum cyanophorum B (6)**
    - Oscillatoria neglecta AB003166
    - Lissoclinum fragile A (5) WPC 1-1
      - Sponge symbiont YW190174
      - Synechococcus sp. AF448073
    - Lissoclinum fragile B (3) WPC 3-6
    - Lissoclinum fragile A (6,8) WPC 1-1
    - Lissoclinum fragile D (1 to 4) EPC 1-5
    - Lissoclinum fragile C (1,2,6,8) EPC 1-2
    - Cyanobium sp. AM710363
      - Synechococcus sp. EF638720
    - Cyanodictyon sp. AM710382
      - Merismopedia tenuissima AJ639891
    - Trididemnum cyanophorum B (5)
      - Sponge symbionts
      - Escherichia coli U18997

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**Note:** The diagram depicts the phylogenetic relationships among various cyanobacterial species associated with Bahamian ascidians, with support values (NJ, MP, ML, PP) indicated for each clade. The bar at the bottom indicates a branch length of 0.02.
Lissoclinum aff. fragile (Figure 2 IIA). The second subgroup was formed by identical sequences for L. fragilis from Plana Cay (Figure 2 IIB). All in all, phylogenetic analyses confirmed morphological observations and determined the taxonomic status of species and groups of samples presenting small morphological differences.

Cyanobacteria phylogeny

After alignment, 68 partial 16S rRNA gene sequences ranging between 740 and 756 bp were obtained, 19 of which were unique. All sequences from Lissoclinum aff. fragile were identical and differed from the ones obtained for L. aff. fragile B by one mutation. Unique sequences were retrieved from L. fragile, 3 of which were only encountered in one clone. All sequences obtained from Trididemnum solidum were identical, while 6 unique sequences were retrieved from T. cyanophorum, 4 of which were only sequenced once. The same sequence was never found in both Lissoclinum and Trididemnum species. Analyses conducted using the 16S rRNA fragment described above plus the complete ITS region (269–647 bp) plus a small fragment of the 23S rRNA gene (44 bp), hereafter called the16S–23S ITS region, yielded 56 unique sequences. Identical sequences were observed among specimens of L. aff. fragile A and B (6 clones total), and within clones of L. aff. fragile B (2 clones), L. fragile B (2 clones) and T. cyanophorum A (2 clones).

Blast searches in GenBank with the 16S rRNA fragment showed that the best match for Trididemnum solidum and Trididemnum cyanophorum symbionts were uncultured Prochloron and Synechocystis sequences (≥97% max. identity; >93% coverage in all cases). Three other sequences obtained for T. cyanophorum B (clones 4, 5, 6) shared similarities with a range of uncultured cyanobacteria, including Oscillatoriales and Chroococcales. All the sequences from Lissoclinum aff. fragile and most of the sequences from L. fragile (19) shared up to 95% identity with Avenacorialis sp. (100% coverage). The remaining sequences from L. fragile (11 in total) moderately resembled Leptolyngbya australica (93% max. identity; 100% coverage). Finally, two sequences of L. fragile from West Plana Cay (A, clones 3 and 5) shared 94% and 97% identities with Phormidium sp. (100% coverage) and Synecococcus sp. sequences (100% coverage), respectively.

Phylogenetic analyses based on a fragment of the 16S rRNA gene (Figure 3), and the 16S–23S ITS region (Figure 4), revealed a total of 11 groups, 10 of which were equivalent among analyses. The first one (named A in Figure 3 and 4) included 4 sequences obtained from T. cyanophorum and had strong bootstrap support (>99%). Phylogenetic analyses conducted with the 16S rRNA fragment showed that these sequences from T. cyanophorum grouped with 9 sequences of Prochloron spp. retrieved from GenBank. Five sequences from T. cyanophorum and all sequences from T. solidum were grouped with two species of Synechocystis and one sequence of Prochloron in clade B (Figure 3 and 4). High similarity of the cyanobacterial sequences obtained here for T. solidum and T. cyanophorum with Synechocystis trididemni is not surprising (clade B), as this cyanobacterium was first described in T. cyanophorum [11], and has also been found in other Trididemnum species [3]. Along with Synechocystis, some Trididemnum species can also establish symbiotic relationships with Prochloron (e.g. T. manusutaum [58], T. paracyclus [3]). For T. cyanophorum, symbionts of both Prochloron and Synechocystis appeared to coexist. This phenomenon has also been reported for other ascidians, and is especially common in Trididemnum species [32,59,60].

The third clade (named C) from the 16S rRNA analyses was formed by different cyanobacterial species belonging to the Chroococcales, together with one sequence obtained here for T. cyanophorum B (clone 4). The position of the sequence from T. cyanophorum B (4) within this group was not resolved. Further analyses, including those of the 16S–23S ITS region, did not yield clearer results, probably due to the limited number of available cyanobacterial ITS sequences in GenBank (Figure 4). Other sequences of Chroococcales were found in the 16S rRNA clade I, which grouped clone 5 from L. fragile A, an uncultured cyanobacterium sequence obtained from the Australian sponge Cymbastela sp., and a cultured strain of Synechococcus sp. This clade had a bootstrap support >80% in all analyses, however, it did not form a monophyletic group with other Synechococcus sequences retrieved from GenBank (located in clade K). Clade K was entirely formed by Chroococcales, including many sponge symbionts, some free-living species, and clone sequence 5 from T. cyanophorum B (Figure 3 and 4). Cyanobacteria are common members of sponge-associated microbial communities. In particular, Synechococcus species have been widely reported as the major photosymbionts inhabiting sponges [48,61,62,63]. Some of these symbionts were reported to be host-specific, others were found in different sponge species, and some varied according to location [48]. The relative integration of these symbionts varies depending on the sponge species, ranging from obligate symbiosis to a commensal existence, with cyanobacterial cells interspersed among sponge cells [64,65]. In this study, only one sequence related to the sponge symbiont Synechococcus was found, indicating that this cyanobacterial genus may only form facultative associations in ascidians. Moreover, the sequence obtained herein appeared more closely related to free-living Synechococcus, and may just have been captured from the water column by the ascidian.

In the 16S rRNA analyses, one sequence (clone 5) obtained from L. fragile A formed clade D with cyanobacterial species belonging to the Oscillatoriales (Figure 3). In contrast, the 16S–23S ITS sequence obtained for L. fragile A (5) (Figure 4) held a basal position within the Prochloron–Synechocystis group (clades A and B). Other sequences of Oscillatoriales appeared in clade H, which, for the 16S rRNA analysis, only included 2 sequences: one obtained from T. cyanophorum B (clone 6) and a sequence from Oscillatoria neglecta. Although the existence of this clade appeared to be supported by bootstrap analysis, its connection with the other clades remained undetermined, especially considering that other Oscillatoriales were grouped in clade D (Figure 3). The 16S–23S
ITS analysis (Figure 4) showed that the *T. cyanophorum* B (clone 6) sequence appeared basal to all clades except clade K (bootstrap support 84% in all analyses). Thus, based on 16S rRNA and 16S–23S ITS results, the identity and phylogenetic position of *L. fragile* A (clone 5) and *T. cyanophorum* B (clone 6) within the Oscillatoriales could not be resolved.

The last clade formed by sequences of Oscillatoriales was found only in the 16S rRNA analysis and grouped three sequences of *Leptolyngbya* spp. retrieved from GenBank (clade E, Figure 3). Although Blast searches returned *Leptolyngbya* species as best match for 11 of our 16S rRNA sequences from *L. fragile*, none of them were included in this clade based on phylogenetic analyses. Several studies have reported the occurrence of Oscillatoriales associated with ascidians [9,33,66,67,68,69,70,71]. However, most of these studies include only short references to these symbionts [9,67,68], a few are accompanied by electron microscopical observations [33,66,69,70,71], and even fewer provided a name or a sequence to identify the symbiont [66,70]. Thus, to date, the degree of host-specificity and strength of association between ascidians and Oscillatoriales remains unresolved.

**Figure 4. Phylogeny of 16S–23S ITS gene sequences from cyanobacteria isolated from Bahamian ascidians.** Sequences obtained in this study are highlighted (bold lettering). Labels on terminal nodes of sequences include sample name and clone sequenced (in parenthesis). Labels on terminal nodes of sequences from this study include sample name, clone sequenced (in parenthesis), and sample location (underlined: SC: Sweeting’s Cay, LSS: Little San Salvador; WPC: West Plana Cay, and EPC: East Plana Cay). Bars as in Figure 3. Tree topology corresponds to the consensus obtained from neighbor-joining (NJ) analysis. Individual bootstrap values from NJ, maximum parsimony (MP) 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 bootstrap support greater than 50% from at least 2 of the 4 phylogenetic criteria, and dashed lines indicate weakly supported branches. Scale bar represents 0.05 substitutions per site.

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ITS analysis (Figure 4) showed that the *T. cyanophorum* B (clone 6) sequence appeared basal to all clades except clade K (bootstrap support >84% in all analyses). Thus, based on 16S rRNA and 16S–23S ITS results, the identity and phylogenetic position of *L. fragile* A (clone 5) and *T. cyanophorum* B (clone 6) within the Oscillatoriales could not be resolved.

The last clade formed by sequences of Oscillatoriales was found only in the 16S rRNA analysis and grouped three sequences of *Leptolyngbya* spp. retrieved from GenBank (clade E, Figure 3). Although Blast searches returned *Leptolyngbya* species as best match for 11 of our 16S rRNA sequences from *L. fragile*, none of them were included in this clade based on phylogenetic analyses. Several studies have reported the occurrence of Oscillatoriales associated with ascidians [9,33,66,67,68,69,70,71]. However, most of these studies include only short references to these symbionts [9,67,68], a few are accompanied by electron microscopical observations [33,66,69,70,71], and even fewer provided a name or a sequence to identify the symbiont [66,70]. Thus, to date, the degree of host-specificity and strength of association between ascidians and Oscillatoriales remains unresolved.
The 16S rRNA clade F (Figure 3) grouped most of the sequences obtained from *L. fragile* A, B, C, and D, all the sequences from *L. aff. fragile*, and a single sequence obtained from the Bahamian sponge *Pseudoaxinella flava* by [63]. The best matches of this group according to blast searches were *Acaryochloris* species. Based on 16S rRNA analyses, *Acaryochloris marina* (clade G in Figure 3) appeared at the base of clades A, B, C, D, E, and F, while the 16S–23S ITS analyses showed that *A. marina* was related only to clade F (Figure 4). *Acaryochloris marina* is a recently discovered oxygenic photoautotroph that uses chl *d* as the predominant photosynthetic pigment [17,72,73]. A closer analysis of the *Acaryochloris*-like sequences (clade F) compared to *A. marina* (clade G) reported a sequence divergence >5%, while sequence variation within the *Acaryochloris*-like clade obtained here was <1%.

Within clade F, sequences were grouped according to host and sampling location. The first sub-clade contained all sequences of *L. aff. fragile* from Little San Salvador (Figures 3 and 4, IIA). Sequences for *L. fragile* formed two groups according to sampling site: West Plana Cay (samples A and B), and East Plana Cay (samples C and D, 7 kilometers away from West Plana Cay, and 330 km away from Little San Salvador). Thus, even adjacent populations had distinct cyanobacterial symbionts. All the remaining sequences of *L. fragile* A, B, C, and D formed a well-supported clade (J) by themselves, without any clear correlation with location (bootstrap support >97% in all analyses except ML for 16S–23S ITS; Figure 3 and 4). Therefore, as previously reported for sponges [48], some ascidian photosymbionts are host-specific, while a few may depend on environmental parameters associated with a given location.

The phylogenetic position of clade J within the cyanobacteria could not be resolved with analyses of the 16S rRNA fragment or the 16S–23S ITS region. Moreover, although the closest blast match with an identified cyanobacterium was *Leptolyngbia antarctica*, none of the phylogenetic analyses related clade J with other Oscillatoriales (clades D, E, and H; Figure 3 and 4). Based on currently available information, we are unable to identify or provide a taxonomic position for sequences in clade J.

**Cyanobacteria morphology**

Photosymbionts of *Trididemnum solidum* and *T. cyanophorum* were embedded in the adult tunic, but were never observed to be in direct contact with ascidian cells (Figure 5A). The symbionts observed in *T. solidum* and *T. cyanophorum* symbionts were morphologically identical to those reported from previous studies of *Synechocystis trididemni* [11]. Specifically, *S. trididemni* was reported to be round, with a diameter of 8 to 11 µm, and 5 to 7 thylakoids around the periphery of the cell. The diameter of *Synechocystis* observed herein fell within the range indicated above, however they had a few less thylakoids per cell (3 to 5 instead of 5 to 7). As described for *S. trididemni*, some of these thylakoids were also irregular and developed bladders or vesicles close to the nucleoplasma [11]. Polyedric bodies were also observed in close contact with the thylakoid membranes (Figure 5B) [11]. Thus, morphological observations of the ultrastructure of photosymbionts of *T. solidum* and *T. cyanophorum* confirmed our phylogenetic results, indicating that *Synechocystis* was the major symbiont in these ascidian species.

A very different type of cyanobacterium was observed in *L. fragile* and *L. aff. fragile* samples (Figure 5). As hinted by the phylogenetic analyses, the overall structure of the photosymbionts (i.e. cell shape, size, and major features) was consistent with previous research describing the ultrastructure of *Acaryochloris* species [14,74,75,76]. A consensus on the number and arrangement of the thylakoids within *Acaryochloris* cells does not exist in the literature. The first studies on *A. marina* reported more than 7 thylakoids surrounding the cytoplasm [14,74,75], while subsequent studies reported a lower number of thylakoids evenly spaced along the periphery of the cell [76,77]. The *Acaryochloris*-like cells observed herein better fit this last description, and presented 5 to 7 thylakoids evenly spaced along the periphery of the cell (Figure 5C). Several symbiont cells were also observed undergoing division, and followed the major division steps described by Marquart et al. [75]. Notably, the photosymbiont cells observed herein also remained connected by a common peptidoglycan layer after cell division (Figure 5D). Another cyanobacterial type containing abundant glycogen granules was observed in the tunic around the zooid abdomen (Figure 5E). Cyanobacteria were consistently found outside the sheaths that surround the calcareous spicules of the tunic (Figure 5F).

*A. marina*-like cells were also found inside the tunic of the larva isolated from *L. fragile*, indicating that the symbionts were
transmitted to the progeny (Figure 5E inset). Vertical transmission of photosymbionts to larvae has often been observed in ascidians and is assumed to be essential for host survival [10,78]. To date, three transmission modes have been described. The first mode involves the formation of a tunic extension at the posterior end of the larval trunk, just above the tail insertion called rastum [40]. The rastum has been described in most Diplosoma species with photosymbionts in the cloacal cavities [22,40,68,78,79,80,81,82,83,84]. The second mode has been observed in some Didemnum, Trididemnum and Lissoclinum species and is associated with the adhesion of the photosymbionts to either the posterior end of the larval trunk or around the entire larva except for the sensory and adhesive organs [19,21,40,78,85,86]. These ascidian species also harbor their photosymbionts in the cloacal cavities and, as in the first mode, symbionts are acquired when larvae pass through these cloacal cavities.

A more recently described transmission mode applies to ascidian species that harbor photosymbionts within their tunic. This process was described for Trididemnum minutum and is thought to involve host cells acting as a vehicle for transporting symbionts from the tunic of the adult to that of the larvae [20]. Although no larvae were obtained from T. solidum and T. cyanophorum, the process described for T. minutum or a similar mechanism may apply to the Trididemnum species analyzed in the present study. Our observations for L. fragile suggest that this species may also acquire their symbionts by an active transport mechanism during the formation of the deeper layer of the tunic, or inner tunic. In support of this hypothesis, the cyanobacteria were observed in the inner tunic separated by a folded cuticle from the outer tunic (Figure 5E inset). Photosymbionts are sufficiently abundant in the inner tunic to confer obvious pigment to the tadpole larve, except for the regions around the adhesive and sensory organs. Further research is needed to assess the exact process involved in the transfer of photosymbionts to the larvae and to determine whether ascidian cells are implicated in this process.

Chlorophyll d in Lissoclinum aff. fragile

Confocal microscopic examination revealed the presence of numerous cells containing both chlorophyll d (chl d) and phycobiliproteins (PBPs) in the tunic of Lissoclinum aff. fragile (Figure 6). Chl d is a predominant photosynthetic pigment in Acaryochloris species, a genus discovered to live in close association with ascidians [8,17,72,73,87]. Measurements performed on the deconvoluted image stacks of these Acaryochloris-like cells revealed an average length of 1.75 μm (±0.38; SD), width of 1.13 μm (±0.24; SD), and volume of 2.32 μm³ (±1.07; SD). The diameter and length of the observed cells were within the range reported in the literature for Acaryochloris spp. (1.5–1.7 μm diameter and 1.8–2.1 μm in length [75], 1–1.5 μm in diameter and 1.5–3 μm in length [15]).

To date, Acaryochloris spp. have only been found growing on biofilms beneath didemnid ascidians [17,72,73] or forming small aggregations at the base of the ascidian tunic [8]. However, in this study, Acaryochloris-like cells were abundant (Figure 6) and appeared scattered throughout the tunic. Three-dimensional reconstruction of these cells also revealed that chl d and PBPs co-localized in the center of each Acaryochloris-like cell, however, they were compartmentalized, with chl d mainly present on one side and PBPs on the other (Figure 6d). A particular orientation of chl d and PBPs within the photosymbiont cell and the host tunic may be necessary to ensure optimal sunlight absorption.

Taken together with phylogenetic analyses and TEM observations, our results indicate that L. fragile harbors photosymbionts from the Acaryochloris group within its tunic. Based on unique 16S rRNA and 16S–23S ITS sequences, we propose here to name Candidatus Acaryochloris bahamiensis nov. sp. for this photosymbiont. The importance of A. bahamiensis nov. sp. to the survival of its host remains to be assessed. However, vertical transfer of A. bahamiensis nov. sp. to the larvae suggests a close association between host and symbiont, and that this relationship is important to host survival. This is the first report of Acaryochloris forming a symbiotic relationship with an ascidian and living within its tunic.

Conclusion

In conclusion, using molecular and electron microscopic techniques, we have shown that the ascidians examined in this study harbor a considerable diversity of photosymbionts. The primary taxa of symbionts found were Synechocystis in the tunic of Trididemnum solidum and T. cyanophorum, and Acaryochloris-like symbionts in Lissoclinum fragile and L. aff. fragile. Host identity strongly correlated with the identity of the photosymbionts found in the tunic, although in some cases (e.g. Lissoclinum fragile) differences could be related to sampling location. Analyses of 16S
rRNA and 16S–23S ITS sequences from the symbionts in two varieties of *Lissoclinum fragile* revealed two major clades (*F* and *J*). Clade *F* could not be associated with any known cyanobacterium, while clade *F* was related to *Acaryochloris*. Ultrastructural examination confirmed the similarity of clade *F* symbionts with other varieties of *Acaryochloris*. These two varieties were also observed in the inner tunic of the larvae, suggesting that specialized mechanisms of vertical transmission exist. Analyses using CM revealed the presence of chl *d* and PBP, further reinforcing the classification of these photosymbionts as *Acaryochloris* spp. Based on these results and unique 16S rRNA and 16S–23S ITS sequences, we propose the name *Candidatus Acaryochloris bahamensis* nov. sp. for this photosymbiont. Substantial research is still required to determine the diversity, host-specificity, and function of microbial symbionts in ascidians.

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**Author Contributions**

Conceived and designed the experiments: SL-L, BS XT. Performed the experiments: SL-L, BS XT. Analyzed the data: SL-L MB. Contributed reagents/materials/analysis tools: SL-L BS MB JRP. Wrote the paper: SL-L. Revised the manuscript: BS MB JRP XT.

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