The prevalence, predominance, and metabolic potentials of *Candidatus* Prochlorobium terpiosii in the coral-killing sponge, *Terpios hoshinota*

Running title: Dominant cyanobiont in *Terpios hoshinota*

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Abstract

Terpios hoshinota is a ferocious, space-competing sponge that kills a variety of stony corals by overgrowth. Outbreaks of this species have led to intense coral reef damage and declines in living corals on the square kilometer scale in many geographical locations. Our large-scale 16S rRNA metagenome study across three oceans revealed that the core microbiome of *T. hoshinota* included OTUs related to *Prochloron*, *Endozoicomonas*, *Pseudospirillum*, SAR116, *Magnetospira*, and *Ruegeria*. A *Prochloron*-like OTU was the most dominant cyanobacterium in *T. hoshinota* in the western Pacific Ocean, South China Sea, and Indian Ocean. The high-quality draft genome of the *Prochloron*-like cyanobacterium and our pigment analysis revealed that, unlike the previously described *Prochloron* species and their genomes, this bacterium had phycobiliproteins and phycobilins and lacked chlorophyll *b*. Furthermore, the phylogenetic analyses demonstrated that the bacterium was phylogenetically distinct to *Prochloron*, strongly suggesting that it should be a sister taxon to *Prochloron*; we therefore proposed this symbiotic cyanobacterium as a novel species under a new genus: *Candidatus* Prochlorobium terpiosii. In addition, we also characterize the metabolic potentials of the cyanobacterium in carbon and nitrogen cycling and propose a model for the interaction between *Ca*. Pb. terpiosii LD05 and *T. hoshinota*. This study builds the foundation for *T.*

hoshinota's microbiome and paves a way for understanding the ecosystem, invasion mechanism, and causes of outbreak of this coral-killing sponge.

Introduction

The coral-killing sponge, *Terpios hoshinota*, has received attention since its outbreaks were detected in several coral reefs in the western Pacific Ocean, South China Sea, and Indian Ocean [1-7]. The encrusting sponge can kill various stony corals by overgrowth [8]. It grows up to 23 mm per month [9], and its fast growing and competitive nature enables it to kill scleractinian corals quickly and at a high rate (30–80%) across biogeographic regions [4, 5, 7, 10]. For instance, *T. hoshinota* overgrowth killed 30% of corals in some coral reefs of Green Island, Taiwan in just one year [2]. Similarly, the sponge jeopardizes coral reefs in numerous regions of Indonesia, Malaysia, Japan, India, and the Great Barrier Reef [3, 10-12]. The gradual spread of *T. hoshinota* makes it a serious threat to coral reefs. However, little is known about what causes its outbreaks.

Sponges are commonly known to harbor complex microbial communities, and symbiotic microorganisms play vital roles in the development, health, and nutrient acquisition of their hosts [13]. The microbe and host form an ecological unit called a holobiont. In *T. hoshinota*, the sponge associates with a bacterial community of relatively

low diversity that is dominated by cyanobacteria [14]. Ultrastructural observations have clearly shown that cyanobacteria are densely distributed in the sponge, contributing to 50% of the total cell volume [1, 14, 15]. The blackish color of *T. hoshinota* is also attributed to cyanobacteria [14]. Accordingly, the sponge is called cyanobacteriosponge. Several studies have shown that cyanobacteria play important roles in the sponge's growth and competition with corals. For instance, high numbers of cyanobacteria can be observed in the sponge's larvae [16, 17], suggesting that they are transmitted vertically during embryogenesis [18]. On the other hand, *in situ* light shading was shown to discontinue *T. hoshinota* expansion [19]. Even short-term shading can cause a long-term decrease in the biomass of symbiotic cyanobacteria and lead to irreversible damage to *T. hoshinota* [20]. Furthermore, one study found that, when *T. hoshinota* encountered a variety of corals, the sponge formed a hairy tip structure packed with dense cyanobacteria [8]. These results indicate that cyanobacteria are vital not only for *T. hoshinota* growth, but also for its overgrowth of corals.

Though important, the identity and role of symbiotic cyanobacteria are still unclear. In 2015, Yu *et al*. isolated and cultivated *Myxosarcina* sp. GI1, a baeocytous cyanobacterium, from *T. hoshinota* at Green Island [21]. However, electron microscopy has not identified vegetative cell aggregates of baeocyte in *T. hoshinota* [14-16].

Moreover, our previous study analyzed 16S ribosomal RNA gene sequences from Green Island and found that the dominant cyanobacterium in *T. hoshinota* is a novel species closely related to *Prochloron* sp. [14]. *Prochloron* is an obligate symbiont of some ascidians; it carries chlorophyll *b* and lacks phycobilin, which is unusual in cyanobacteria. However, a pigment analysis reported that the cyanobacteria in *T. hoshinota* contained phycobilins but lacked chlorophyll *b*, which is typical of cyanobacteria [15]. Hence, the identity and characteristics of the predominant cyanobacterium in *T. hoshinota* remain uncertain, as does the question of whether its predominance is universal. On the other hand, since cyanobacteria are attributed to the sponge's health and invasion capacity, the ecological relationship between *T. hoshinota* and its cyanobacteria needs to be determined.

Other bacteria in the *T. hoshinota* microbiome also contribute to holobiont functions. Microbial community in sponge can be shaped by host-related factors, such as the immune system and nutrient exchanges [22, 23]. Strict host selective pressure can stabilize the microbial community [24]. On the other hand, the community can also be determined by environmental factors, such as light availability, pH, and temperature. Nevertheless, no study has investigated the microbiome of *T. hoshinota* under different biogeographical backgrounds. Hence, this study investigated the bacterial community *T.*

hoshinota across different oceans to examine whether the bacterial community is stable and host-specific or depends on geography.

Most sponge symbionts, including the dominant cyanobacteria in *T. hoshinota*, are uncultivable. Metagenomic methods can be used to determine the microbial composition in *T. hoshinota*, and knowing this composition will help us understand the ecosystem of the *T. hoshinota* holobiont. In this study, we used 16S rRNA-based metagenomic analysis to investigate the bacterial compositions and diversities of *T. hoshinota* samples from a wide geographical range across the western Pacific Ocean, South China Sea, and Indian Ocean. Despite the geographical variation in bacterial communities, *T. hoshinota* contains a stable core community that accounts for most of the bacterial composition. Inside this core community, we found that a single species predominated the cyanobacteria in *T. hoshinota* from different locations. The draft genome of this predominant cyanobacterium was reconstructed by whole-genome shotgun sequencing. The following genomic and comparative genomic analyses elucidated the phylogenetic affiliation and taxonomy of the dominant symbiotic bacterium in *T. hoshinota* and putative symbiotic interactions between the bacterium and the host.

Materials and methods

Sample collection

Sixty-one sponge samples, summarized in Supplementary table 1, were collected from 15 coastal sites in the western Pacific Ocean, South China Sea, and Indian Ocean. The collection sites in the western Pacific Ocean included Okinoerabu (JPOKE), Bise (JPBS), Miyako (JPMYK), Ishigaki (JPISG), Lyudao (TWLD), Lanyu (TWLY), Kenting (TWKT), Guam (USGU), and the Great Barrier Reef (AUGBR). The collection sites in the South China Sea included Yongxing (CNYX), Taiping (TWTP), Redang (MYRD), and Pari (IDPR). Indian Ocean samples were collected from Faafu Atoll (MVFF).

DNA extraction

Before DNA isolation, sponge tissue was collected and placed in tubes. Samples were then washed with 1 ml 1X TE buffer. For the sponge samples from JPBS, JPISG, JPMYK, JPOKE, TWLD, TWLY, and USGU, total DNA was extracted using a DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. For the other samples, total DNA was extracted by a modified CTAB method described in

our previous study [14]. After DNA isolation, the samples were stored at -80°C until subsequent experiments.

16S rRNA gene amplification and multiplex tag sequencing

High throughput sequencing of the 16S rRNA hypervariable V6–V8 region was used to characterize bacterial community diversity and composition. V6–V8 sequences were amplified by PCR with forward primer 5´-AACGCGAAGAACCTTAC-3´and reverse primer 5´-GACGGGCGGTGWGTRCA-3´. PCR mixtures contained 33.5 µL of sterilized distilled water, 0.5 µL of 5 U TaKaRa Ex Taq (Takara Bio, Otsu, Japan), 5 µL of 10X Ex Taq buffer, 4 µL of 10 mM dNTP, 1 µL of each primer at a concentration of 10 µM, and 5 µL template DNA in a total volume of 50 µL. PCR was programmed with an initial step of 94°C for 5 min, 30 cycles of 94°C for 30 s, 52°C for 20 s, and 72°C for 30 s, followed by a final step of 72°C for 10 min. PCR was performed again to add barcodes to the amplicons. Each primer tag was designed with four extra nucleotides at the 5' end of both primers. Unique tags were used for PCR barcoding to label each sample in the study. PCR conditions were the same as that for the V6–V8 amplification, except that the number of reaction cycles was reduced to five. The products were purified by 1.5% agarose electrophoresis and QIAEX II Agarose Gel Extraction kit (QIAGEN, Hilden, Germany) according to manufacturer’s instruction. The quality of the purified products

was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Vantaa, Finland).

Illumina sequencing and community analysis

DNA concentration was measured by a Quant-iT™ assay (Thermo Fisher Scientific, USA). Equal pools of DNA were sent to Yourgene Bioscience (Taipei, Taiwan) and sequenced using the MiSeq platform (Illumina, USA). Short and low-quality reads were filtered by Mothur v.1.38.1 [25] to retain reads with an average quality score > 27 and lengths of 365–451 base pairs (bp). Reads with homopolymer > 8 bp were excluded, and chimera sequences from all samples were removed by USEARCH v8.1.1861 [26]. OTUs were determined and classified using the UPARSE pipeline [27] with reference to the Silva v128 database [28]. OTUs were defined using a 97% similarity threshold. OTUs assigned to Archaea, Eukaryota, chloroplast, and mitochondria were removed.

Alpha and beta diversity indices were determined by QIIME 2 v2020.6 [29]. Alpha diversity indices were calculated by Shannon diversity, Chao1 richness estimator, and Faith's phylogenetic diversity. Dissimilarities in microbial community compositions among samples were computed using Bray–Curtis distance dissimilarity, unweighted UniFrac, and weighted UniFrac. The results were visualized by principal component analysis (PCA) and dendrogram.

Electron microscopy

T. hoshinota samples at the sponge-coral border were collected by transmission electron microscopy (TEM), and *T. hoshinota* was observed on rubber by scanning electron microscopy.

Samples were prepared as described in our previous study [14]. They were fixed in 0.1 M phosphate buffer with 2.5% glutaraldehyde and 4% paraformaldehyde. The samples were then washed with 0.1 M phosphate buffer for 15 min three times, then immersed in 0.1 M phosphate buffer with 1% osmium tetroxide for 4 hr.

After being washed again three times, the samples for TEM were sequentially dehydrated in acetone at 30%, 50%, 70%, 85%, 95%, and 100% for 20 min each time. After being dehydrated, the samples were embedded in Spurr's resin and sectioned. The sections were stained with 5% uranyl acetate in methanone and 0.5% lead citrate. The stained samples were observed under a Philips CM-100 TEM.

Samples for SEM were sequentially dehydrated in 30% for 1 hr, 50% for 1 hr 70% for 1 hr, 85% for 2 hr, 95% for 2 hr, 100% for 2 hr, and 100% for 12 hr. The samples were dried in a Hitachi HCP-2, then gold coated with a Hitachi E-1010 and observed under an FEI Quanta 200 SEM.

Pigment analysis by UPLC-QTOF-MS

Approximately 2.5 g of wet *T. hoshinota* tissue from each sample was centrifuged at 7,000 rpm for 30 s to remove excess water. Sponge tissues were placed in mortars and ground with 4 ml of 100% acetone to homogenize the tissues. The homogeneous samples were transferred into a 5 ml centrifuge tube and centrifuged at 7000 rpm at room temperature for 30 s. The supernatant was transferred into a new 5-ml centrifuge tube. The samples were covered with aluminum foil paper for being shaded and stored at 4°C until pigment analysis.

Pigment content analysis was performed by UPLC-MS. The UPLC-MS system used the Agilent 1290 Infinity II ultra-performance liquid chromatography (UPLC) system (Agilent Technologies, Palo Alto, CA, USA) coupled online to the Dual AJS electrospray ionization (ESI) source of an Agilent 6545 quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The samples were separated using a Kinetex XB-C18 column (2.6 μm, 4.6 × 100 mm, Phenomenex, Torrance, CA, USA) at 40°C. The chromatogram was acquired; mass spectral peaks were detected and their waveform processed using Agilent Qualitative Analysis 10.0 software (Agilent, USA).

Metagenome sequencing and assembly

Bacterial DNA and cDNA were used to reconstruct the cyanobacterial genome. For DNA sequencing, *T. hoshinota* tissue was ground, and the samples were filtered through a 0.35 μm membrane. 2 x 10^3 cyanobacterial cells were purified from the samples using the MoFlo XDP cell sorter based on fluorescence and forward scatter intensity and transferring into two wells of a 96-well plate. The cells were centrifuged at 12,000 x *g* for 5 min at 4°C, and then lysed with denaturation solution containing 40 mM KOH and 90 mM EDTA in sterilized H_2O at 65°C for 3 min. The DNA was amplified with an REPLI-g Mini Kit (QIAGEN, USA). The lysates were mixed with 40 μL master mix, which contained 29 μL REPLI-g mini reaction buffer, 1 μL REPLI-g mini DNA polymerase, and 10 μL sterilized H_2O. After incubation at 30°C for 16 hr with a Thermo-cycler, the reaction was stopped by heating the samples at 65°C for 3 min. Amplification was performed twice. The amplified DNA was then purified by Microspin™ G-50 Columns. Purified DNA was sent to Yourgene Bioscience (Taipei, Taiwan) and sequenced on a MiSeq platform (Illumina, USA).

For cDNA sequencing. Total RNA was extracted from *T. hoshinota* tissues using TRI reagent (Ambion, Inc.) according to the manufacturer's instructions.

MICROBExpress kits (Ambion, Inc.) were used to remove ribosomal RNA according to the manufacturer's instructions with capture oligo. Eukaryotic mRNA was then removed by oligo-dT. After the mRNA was reverse transcribed into cDNA, cDNA sequencing was performed by NGS High Throughput Genomics Core on HiSseq platform (Illumina, USA).

DNA and cDNA reads were merged, then trimmed and filtered by trimmomatic v0.39 with the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:3: TRUE LEADING:10 TRAILING:10 SLIDINGWINDOW:5:15 MINLEN:50 [30]. The processed reads were *de novo* assembled using metaSPAdes with k-mer sizes of 21, 33, 55, 77, and 99 without scaffolding. Binning was performed using VizBin [31], and the cyanobacterial bin was confirmed by GTDB-TK taxonomy annotation [32].

Genome annotation

The genome of *Prochloron*, “Prochlorobium”, and phylogenetically close cyanobacteria were annotated using Prokka v1.13.7 with the ‘usegenus’ and ‘rfam’ options [33]. The genomes were also annotated with KEGG functional orthologs (KO numbers) and COGs [34, 35]. To annotate the KO numbers, protein sequences predicted by Prodigal were blasted against the KEGG database using BlastKoala [36, 37]. The K number annotation results were then used to reconstruct the transporter systems and metabolic pathways using KEGG mapper [38]. To annotate COGs, the predicted protein

sequences were searched against the COG database by RPSblast with an e-value threshold of 10^{-5} [39]. In addition, the transporter proteins were identified by searching the putative protein sequences against TransportDB 2.0 (August 2019) using BLASTp [40].

Phylogenetic analysis

To reconstruct a phylogenetic tree of the 16S rRNA genes, the sequences with high identities to the 16S rRNA gene in LD05 genome were retrieved by searching the NCBI nt database using BLASTn [39, 41]. The sequences were aligned by MUSCLE on MEGA7 [42, 43]. A tree was then reconstructed using IQ-TREE v2.03 using automatic model selection and 1,000 bootstraps [44]. The tree was visualized with iTOL v4 [45].

Genomes for each cyanobacteria species in GTDB database were downloaded and 120 single-copy gene protein sequences were used to reconstruct a tree [46]. Marker gene protein sequences were identified, aligned, and concatenated by GTDB-TK v1.3.0 [32]. A phylogenomic tree was then built using IQ-TREE v2.03 with automatic model selection and 1,000 Ultrafast bootstraps. The tree was visualized with iTOL v4 [44, 45].

Availability of data and materials

All the raw data and genome were submitted under BioProject ID: PRJNA665642.

Results

Bacterial diversity in *Terpios hoshinota* samples from different oceans

To characterize the bacterial communities in coral-encrusting *T. hoshinota* across different geographical backgrounds, samples were collected from 15 locations in the Pacific Ocean, South China Sea, and Indian Ocean (Table S1). 16S ribosomal RNA (rRNA) gene sequencing was used to determine the bacterial communities.

Statistical tests showed that operational taxonomic unit (OTU) Chao1 richness and Faith's phylogenetic diversity (PD) indices were not significantly associated with the oceanic regions from which the samples were collected (Chao1: $p = 0.23$, PD: $p = 0.30$) (Fig. S1a and S1b). In terms of Shannon diversity, the indices were also not significantly associated with the oceanic regions when the outliners were removed (Fig. S1c). Some samples—e.g., TWKT, MYRD, and MVFF samples—from the same locations showed high variation in Chao1 and Shannon indices. In contrast to the alpha diversity indices, the similarities in microbial composition were associated with sample origins (Fig. S2). PERMANOVA showed that samples from the same ocean were significantly more similar than those from different oceans (Bray Curtis: pseudo-$F = 5.13514$, $p = 0.001$,

Unweighted UniFrac: pseudo-F = 1.60401, p = 0.01). When relative abundances were taken into consideration, the effect of the sea on similarities increased (weighted UniFrac: pseudo-F = 6.09022, p = 0.001).

Prochloron-like bacterium dominated _T. hoshinota_ bacterial communities across different oceans

Microbial composition analysis revealed that *Cyanobacteria* and *Proteobacteria* accounted for most of the bacterial composition across the samples, ranging from 81.5–97.4% (Fig. 1, Fig. S3a, and Table S2). *Bacteroidetes* and *Spirochaetae* were present across all the samples, though their abundances were only 0.34–6.29%. Genus-level analysis showed that *Prochloron*-like OTUs were the most dominant and present in all the samples (Fig. S3b). Moreover, *Prochloron*-like OTUs accounted for most of the *Cyanobacteria* in the samples (Table S3). In total, four OTUs were assigned to *Prochloron*, but only one of them, referred to as Prochl-OTU01, was predominant across the samples.

Along with Prochl-OTU01, *Endozoicomonas*, *Pseudospirillum*, SAR116, *Magnetospira*, and *Ruegeria* OTUs were common and co-occurred across the samples, forming a putative core microbiome. This core microbiome contributed about 80% of bacterial abundance in each sample.

Structure of symbiotic *Prochloron*-like bacteria in *T. hoshinota*

To reveal the detailed structure of the *Prochloron*-like bacteria, a *T. hoshinota*-encrusting abiotic substrate was observed using transmission electron microscopy and scanning electron microscopy (Fig. 2). The *T. hoshinota* sponge, roughly 500 μm thick, were supported by the bundle of tylostyle spicules, and the spicules on the surface were interlaced (Fig. 2a-b). Inside the body, spherical bacterial cells 4–6 μm in diameter were widely distributed, and cells were observed in various stages of cell division (Fig. 2c and 2f). A study published in 2015 isolated a baeocytous cyanobacterium, *Myxosarcina* sp., from *T. hoshinota* [21]. However, no vegetative cell aggregate of baeocytes could be observed in our results.

TEM results showed that these spherical cells contained thylakoids, typical compartments inside cyanobacteria, and the arrangements were parietal (Fig. 2d–f). In addition, phosphate granules and carboxysomes were found; gas vesicles were absent in these cells, which is consistent with a previous description of *Prochloron*'s structure [47].

Genome assembly of a novel *Prochloron*-like species

The predominance of a *Prochloron*-like OTU in *T. hoshinota* from various locations indicates the intimate association between bacterium and host. To reveal the identity and characteristics of the *Prochloron*-like bacterium, a *Prochloron*-like genome, referred as to LD05, was reconstructed by *de-novo* assembly of the metagenome from the sample at Green Island, Taiwan. The metagenome-assembled genome (MAG) was 91.73% complete, with 2.16% contamination (Table 1). A *Prochloron*-like 16S rRNA gene was also successfully recovered from the genome, and the sequence was identical to that of the predominant Prochl-OTU01 identified from the community survey. The analysis of the GTDB-Tk demonstrated that the LD05 MAG was closest to *Prochloron didemni*, with 77.97% average nucleotide identity (ANI).

Recently, a putative *Prochloron* species genome called SP5CPC1 was recovered from the metagenome of a sponge microbiome [48]. The ANI analysis found that the LD05 genome shared 93.18% identity with the SP5CPC1 genome, which is below the 95% ANI cutoff for species delineation [49]. The LD05 shared a similar genome size, GC ratio, and coding density with SP5CPC1 (Table S4). On the other hand, *P. didemni* P2-P4 had a much larger genome and lower GC ratio and coding densities. There was also a discrepancy between LD05 and *P. didemni* P2-P4 based on the average amino acid identity (AAI) and percentage of conserved proteins (POCP) analyses (Fig. S4). The

LD05 shared 91% AAI with SP5CPC1, but only 70% AAI with *P. didemni* P2–P4 genomes. On the other hand, the POCP between LD05 and *Prochloron* genomes ranged from 49.8–52%, close to a proposed 50% POCP cutoff for the genera delineation [50]. Taken together, the LD05 and SP5CPC1 genomes were different species with high similarity, and both were more distantly related to *P. didemni*.

Phylogenetic and functional genomic analyses revealed distinct characteristics of LD05 and SP5CPC1 genomes compared to other *Prochloron* genomes

Although the LD05 and SP5CPC1 genomes had certain degrees of similarity to *Prochloron* genomes, the phylogenetic, genomic, and functional genomic analyses identified distinct characteristics between them and *Prochloron*, suggesting that they belong to a different genus.

A phylogenetic tree of 120 cyanobacteria was constructed based on 120 single-copy genes to determine phylogenic affiliation (Fig. 3a and Fig. S5). The tree demonstrated that the LD05 and SP5CPC1 formed a clade that was sister to the clade containing *P. didemni*. The larger clade encompassing the two clades was adjacent to the clade containing *Synechocystis*, *Myxosarcina*, and other cyanobacteria. To provide an in-depth view, homologs of the LD05 16S rRNA gene with high similarities were retrieved from

the NCBI database. A tree constructed by these 16S rRNA sequences depicted two distinct clades, referred as to Clade I and Clade II (Fig. 3b). Clade II contained 16S rRNA gene sequences from three genomes of *P. didemni* strains and other 16S rRNA sequences from various ascidians. In contrast, Clade I mainly comprised sequences from sponge holobionts, including SP5CPC1, LD05, and 16S rRNA sequences from a variety of other sponges. Although many sequences in Clade I were assigned as *Synechocystis* and *Prochloron* 16S rRNA sequences, we found that they actually shared higher identities with the members of Clade I than *Synechocystis* or *Prochloron*. Moreover, the genome phylogenomic analysis also revealed that the SP5CPC1 and LD05 are phylogenetically distant from cultivated *Synechocystis* (Fig. 3a).

To characterize the functional differences between the bacteria in the two clades, LD05, SP5CPC1, and *P. didemni* genomes were annotated with KEGG orthologs and clusters of orthologous groups (COGs). Complete-linkage clustering of COG category abundances demonstrated that the LD05 and SP5CPC1 were more functionally similar to *Microcystis* groups than to *P. didemni* (Fig. S6).

Two hallmarks of *Prochloron* are the presence of chlorophyll *b* and absence of phycobilins in the cell [51]. However, a previous study showed that the symbiotic cyanobacteria in *T. hoshinota* had phycobilins based on microspectrophotometry results

[15], which is consistent with our finding that phycobilins synthase and the phycobiliproteins genes were present in LD05 and SP5CPC1 genomes. The absence of the chlorophyll *b* synthase gene in LD05 and SP5CPC1 genomes implied that the bacteria lacked chlorophyll *b*. In addition, the failure to identify chlorophyll *b* in the ascidian *Trididemnum nubilum* holobiont, which the PCR clone AO15 (DQ357958.1) in Clade I was recovered from, implied that the bacterium also lacks chlorophyll *b*. In accordance with the genomic analyses, our pigment analysis by LC-QTOF-MS also revealed the presence of chlorophyll *a* and absence of chlorophyll *b* in *T. hoshinota* (Fig. 4). These results indicated that the bacteria in Clade I had different light-harvesting systems from *Prochloron* in Clade II.

In summary, the results indicate that the LD05 and SP5CPC1 were derived from a common ancestor, phylogenetically different from the ancestor of *Prochloron*. More importantly, the bacteria have photosynthetic machinery distinct from that of *Prochloron*. Therefore, we propose that the bacteria in Clades I and II be classified into two different genera and classify the species in Clade I as a novel genus “Prochlorobium”. Moreover, we classified LD05 as a novel species named *Candidatus* Prochlorobium terpiosii LD05 [Etymology: Gr. prep. and pref. *pro*, before, primitive; Gr. adj. *chlôros*, green; Gr. n. *bios*, life; N.L. neut. n. *Prochlorobium*, primitive green life. N.L. gen. n. *terpiosii*, of *Terpios*, a

zoological scientific genus name], and SP5CPC1 was reclassified as *Candidatus* Prochlorobium sp. SP5CPC1 (Fig. S7).

Metabolic features of the novel species *Ca.* Pb. terpiosii LD05

Ca. Pb. terpiosii LD05 possessed nearly all the genes required for photosynthesis, carbon fixation, the tricarboxylic acid cycle (TCA), and glycolysis (Fig. 5). In addition, genes related to sucrose metabolism—e.g., sucrose-6-phosphatase, sucrose synthase, and sucrose phosphorylase—were identified. These genes were also present in the SP5CPC1 genome, but not in *P*. *didemni* genomes. For light-harvesting systems, like typical cyanobacteria, the genes encoding phycobiliproteins—including phycoerythrin, phycocyanin, and allophycocyanin—were recovered, except for *cpcC*, *cpcD*, and *cpeE*. Phycobilin synthase genes were also detected in the genomes. On the other hand, the genes encoding chlorophyll *a* synthesis were present, but the gene for chlorophyll *b* synthase was absent.

Certain symbiotic cyanobacteria contain nitrogen fixation machinery and provide their host with nitrogen sources. However, the *Ca*. Pb. terpiosii LD05 and SP5CPC1 lacked nitrogen fixation genes. The two genomes had genes for nitrate uptake and assimilatory nitrate reduction pathways to convert nitrate into ammonium. The glutamine

synthetase/glutamate synthase (GS/GOGAT) pathway was also found in the genomes, indicating that the ammonium derived from extracellular nitrate can be incorporated into amino acids by this pathway. Additionally, the genomes also carried the complete gene set for the urea transporter and urease. The bacteria may take in the urea from hosts and convert it into ammonium. More importantly, the general L-amino acid transporters can be identified in the genomes, which cannot be observed in *Prochloron* genomes. Hence, the bacteria may have multiple methods for obtaining nitrogen resources from their host and environment.

Many symbiotic bacteria in sponges have been shown to provide their hosts with cofactors and vitamins such as vitamin B_{12} (cobalamin), which can only be synthesized by prokaryotic organisms [22]. The *Ca*. Pb. terpiosii LD05 genome contained nearly complete gene sets for the synthesis pathways of vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_7 (biotin), and vitamin B_{12}. The transporter for cobalt, a key constituent of vitamin B_{12}, was also be identified. In contrast, the *Prochloron* genomes lacked a cobalt transporter and many genes for vitamin B_{12} synthesis.

Previous studies have discovered a variety of secondary metabolites from the *T. hoshinota* holobiont [52]; these metabolites may be produced by symbiotic bacteria [53]. Our analysis revealed the existence of multiple terpene synthesis clusters in *Ca*. Pb.

terpiosii LD05. Furthermore, the species also contains a gene encoding squalene cyclase, suggesting that the bacterium can produce cyclic triterpenes.

Many symbiotic bacteria in sponge carry eukaryotic-like proteins (ELPs), which may originate from eukaryotes [54]. The ELPs—e.g., leucine-rich repeat proteins (LRRs), ankyrin repeat proteins (ARPs), WD-40 containing proteins (WD-40), and tetratricopeptide repeat proteins (TPRs)—may be used to interact with hosts during various biological processes. The genomic analysis demonstrated that *Ca*. Pb. terpiosii LD05 carried these ELPs. However, some closely related free-living cyanobacteria also possess ELPs. On the other hand, the ELPs were highly enriched across *Prochloron* genomes.

Discussion

T. hoshinota is one of the most important biological threats to coral. Its associated cyanobacteria and other symbiotic bacteria are essential for maintaining the sponge's function. Nevertheless, the present study is the first to explore the composition and role of *T. hoshinota*'s microbiome. Our unprecedented large-scale survey of the bacterial community in *T. hoshinota* based on 16S amplicon sequencing of samples from various

regions across the western Pacific Ocean, Indian Ocean, and South China Sea enabled us to characterize the sponge's core microbiome.

We discovered a bacterium, closely related to *Prochloron*, that was dominant across our sponge samples. The prevalence and predominance of this bacterium in *T. hoshinota* highlight the strong relationship between bacteria and their hosts. Recovery of the bacterial genome, followed by phylogenetic and functional analyses, demonstrated that this cyanobacterium has a distinct phylogenetic affiliation and genomic and metabolic characteristics from *Prochloron*, indicating that it belongs to a novel genus. We proposed a new genus, "Prochlorobium", and refined the membership of *Prochloron*. Lastly, a functional analysis was performed to understand the lifestyle of this novel bacterium in the *T. hoshinota* holobiont. This study provides insights into the role of the cyanobacterium in *T. hoshinota*, which will facilitate future studies to investigate the ecosystem inside *T. hoshinota*, the causes of its outbreaks, and its invasion mechanism.

Biogeographical variation in the *T. hoshinota* microbiome and its core microbial members

The microbial community in sponges can be shaped by several environmental factors—e.g., light, nutrients, temperature, and pH—and may change in response to stress [23, 24, 55, 56]. Environmental changes may force a sponge holobiont to alter its

microbial community to acclimatize [57]. On the other hand, host-related factors can also be important for determining community composition. For example, previous studies have shown that sponges have several immune receptors, such as NOD-like and Toll-like receptors [13]. These receptors enable sponges to discriminate between symbionts and prey. In order to live inside a sponge, microbes have to evade host immunity and phagocytosis [58, 59]. Furthermore, metabolites and nutrients provided by hosts also contribute to the community structure [60]. Some sponges show high stability in their microbiome due to strict selective pressures from their host. For instance, *Ircinia* and *Hexadella* sponges exhibit host-specific and stability in their associated bacterial communities, despite large geographic distances between sampling sites [24, 61]. In contrast, certain sponges, such as *Petrosia ficiformis*, harbor biogeographical-dependent bacterial compositions [62].

Our survey of three oceans enabled us to determine whether bacterial communities vary across different biogeographical backgrounds and experience strong selective pressure from their host. Our dissimilarity analysis of the microbial composition of *T. hoshinota* showed a weak correlation between sample location and bacterial composition. The differences in microbial composition among *T. hoshinota* samples from different locations may result from local acclimatization. Samples from the same oceanic regions

that share communities may equip their holobionts with similar metabolic functions to cope with stress or increase fitness in certain environments.

However, despite the biogeographical variation, we still identified core bacterial members that were present in all samples (Fig. S3). This core bacterial community comprised only a few genera, but accounted for ~80% of the community across all samples, indicating that it is vital to the holobiont. Host-related factors may help keep this core microbiome stable because its members carry out core functions of the holobiont [23]. The core members may contain the metabolic capability to utilize nutrients from the sponge host's environment and play important roles in nutrient exchanges, such as sulfur, carbon, and nitrogen cycling. This core group may be also responsible for defending against predators and protecting the sponge symbionts from toxins and pathogens [23].

The most dominant OTU in the core microbiome was closely related to *Prochloron*, a genus of symbiotic cyanobacteria found in various ascidians. The biogeographically-independent prevalence and predominance of this *Prochloron*-like bacterium in *T. hoshinota* suggest that it has a crucial role in the holobiont.

Candidatus Prochlorobium terpiosii gen. nov., sp. nov., a _Prochloron_-like bacterium prevalent in _T. hoshinota_

Although the genome of the *Prochloron*-like bacterium in *T. hoshinota* is similar to that of *Prochloron*, deeper genomic, phylogenetic, and functional analyses, combined with the recent recovery of another symbiont *Prochloron*-like bacterial genome in the sponge, revealed that the two cyanobacteria are distinct, especially in terms of their pigment contents and phylogenetic divergence. Hence, a new genus, “Prochlorobium”, was formally erected to distinguish these two groups.

Our 16S rRNA metagenomic analysis revealed that *Candidatus* Prochlorobium terpiosii LD05 is the most dominant cyanobacterium within *T. hoshinota* specimens collected across three different oceans. In order to determine whether *Ca*. Pb. terpiosii LD05 is adapted to different locations, a small-scale microbial composition survey was conducted in Taiwan and Japan using 16S rRNA PCR cloning. The results suggested that the variations among the symbiotic *Ca*. Pb. terpiosii LD05 are small, and the sequences from the same location could not be grouped in the resulting phylogenetic tree (Fig. S8), indicating that there is no significant local adaptation.

Why does the dominant cyanobacterium species remain identical in *T. hoshinota* samples across different oceans? The reason remains unclear. Recent studies suggest that *T. hoshinota* larvae, which carry vertically transmitted cyanobacteria, may have a short dispersal distances because they are more dense than the water, leading them to settle

rapidly after leaving their mother sponge [16-18]. In this circumstance, the symbiotic *Ca*. Pb. terpiosii LD05 from various locations might accumulate genetic differences to adapt to local environments. The absence of evident genetic alterations in our study may be the result of the tight and stable symbiotic interactions between *Ca*. Pb. terpiosii LD05 and *T. hoshinota* restricting genetic changes in the bacterium. Another possibility is that the species becomes broadly dispersed through some other mechanism, such as via ocean currents or transportation via vessels; this would enable *T. hoshinota* larvae to spread quickly across the ocean with few genetic alterations. Evidence of recent *T. hoshinota* outbreaks support this latter scenario [2].

Comparison between "Prochlorobium" and *Prochloron*

Comparative genomics between "Prochlorobium" and *Prochloron* enables us to infer their evolutionary histories and respective relationship to their host. Previous studies have shown that symbiotic bacteria usually have reduced genomes because certain genes erode as symbiosis develops [63, 64]. Furthermore, a model of symbiont evolution has proposed that during the evolutionary history of symbiosis large-scale pseudogenization can happen during transitional events, such as a strict host association or vertical

transmission, leading to a sudden drop in coding density [64]. Eventually, the coding density gradually bounces back due to deletion bias.

Comparing *Prochloron* and “Prochlorobium”, we found that “Prochlorobium” had a smaller genome but similar coding density to other phylogenetically closely related cyanobacteria. *Prochloron*, on the other hand, had a similar genome size but lower coding density. This difference may indicate that the transition toward the host-restricted lifestyles occurred more recently in *Prochloron* than “Prochlorobium”. The hypothesis can be supported by our observation that *Prochloron* harbored highly-enriched transposase genes as the elevation of mobile genetic element quantities, such as transposons and insertion sequences, is thought to be associated with the recent transition to a host-restricted symbiotic lifestyle [65].

Along with its unusual pigments, another hallmark of *Prochloron* is that is produces patellamides, cytotoxic cyclic peptides. However, the synthesis gene cluster cannot be found in *Ca*. Pb. terpiosii LD05 or SP5CPC1. On the contrary, the latter two genomes have many terpene synthesis gene clusters and contain genes that encode the protein that circulates terpene. Hence, “Prochlorobium” and *Prochloron* may produce distinct secondary metabolites to increase the fitness of themselves or their entire holobiont.

Certain cyanobacteria contain genes for sucrose metabolism. Though the role of sucrose in cyanobacteria remains understudied, several articles have shown that sucrose can be utilized as a compatible solute, serve as signal molecule, or be used for glycogen synthesis [66-69]. Interestingly, our genomic analysis found that the genes involved in sucrose metabolism were present in *Ca*. Pb. terpiosii LD05 and SP5CPC1 but absent in *Prochloron*. We hypothesize that "Prochlorobium" can use sucrose as an osmolyte to cope with osmotic stress; this is supported by a previous finding that the genes related to osmotic stress are enriched in sponge-associated bacterial genomes [22]. On the other hand, we also found that *Ca*. Pb. terpiosii LD05 and SP5CPC1 contained osmoprotectant transporter genes, which were not identified in *Prochloron* genomes. These results indicate that the "Prochlorobium" species may live in environments with higher osmotic stress, like places with high osmolarity or fluctuations in osmolarity, compared to *Prochloron*. Alternatively, "Prochlorobium" and *Prochloron* may use different strategies to deal with osmotic stress.

Another observation that drew our attention is that eukaryotic-like proteins (ELPs) are highly-enriched in *Prochloron* but not "Prochlorobium". ELPs can be used to perform protein-protein interactions, phagocytosis evasion, host cell binding, and interference with the host ubiquitin system [70]. ELPs are also thought to be necessary for sponge

symbionts [8]. The differences in ELP quantities between *Prochloron* and "Prochlorobium" may indicate that "Prochlorobium" uses an unidentified mechanism to interact with their hosts. Another possibility is that the long-term symbiotic history of "Prochlorobium" propelled bacteria to retain their essential ELPs and reduce unnecessary ones.

Symbiotic interactions between *Ca*. Pb. terpiosii and *T. hoshinota*

Sponges harbor complex bacterial communities [13], and the symbiotic bacteria play vital and diverse roles in the physiology and ecology of sponges. For instance, bacteria can provide their hosts with various nutrients and recycle waste. Moreover, symbiotic microorganisms can produce diverse secondary metabolites to compete with other holobionts or defend from invasion by predators. The predominance of *Ca*. Pb. terpiosii LD05 highlights its role in the *T. hoshinota* and the genome of *Ca*. Pb. terpiosii LD05 enables us to understand its metabolic potential, the symbiotic relationship, and even how the holobiont overgrows corals.

Many sponges are mixotrophs and certain sponges can obtain carbon resources from cyanobacteria [71]. Some sponges even acquire more than 50% of their energy demand from the symbiotic cyanobacteria in the form of photosynthates [72]. Photosynthesis was

observed in the *T. hoshinota* holobiont, and its efficiency increased when the coral-killing holobiont came into contact with coral; this mechanism may help sponges overgrow the coral [73]. *Ca*. Pb. terpiosii LD05 is the only cyanobacterium in the core microbiome, and it has been found to have the genes needed for photosynthesis. Hence, the bacterium may be an important carbon source for *T. hoshinota* and facilitate competition with coral.

Ammonia secreted by sponges may serve as a nitrogen source for symbiotic bacteria. Our analysis identified ammonium transporters and the GS-GOGAT pathway in *Ca*. Pb. terpiosii LD05. Hence, the bacterium may recycle the ammonium from host. On the other hand, the bacterium harbors the urea transporter and urease. Therefore, urea can be used as an alternative nitrogen source. A previous study showed that the level of free amino acids in *T. hoshinota*-living cyanobacteria increased when the holobiont encountered coral. Our finding of an amino acid transporter in *Ca*. Pb. Terpiosii LD05 suggests that the bacterium may benefit from “coral killing” by leaching amino acids or ammonium that are released as the coral disintegrates.

Some microbes that live in sponges can store polyphosphate granules, which can account for 25–40% of the total phosphate content in sponge tissue [74, 75]. These polyphosphate granules can be used for energy storage and may enable the holobiont to withstand periods of phosphate starvation. Our electron microscopy revealed

polyphosphate granules in *Ca*. Pb. terpiosii LD05. We also found genes encoding polyphosphate kinase. Hence, *Ca*. Pb. terpiosii LD05 may play a pivotal role in phosphate storage and cycling in the *T. hoshinota* holobiont. A previous study demonstrated that the phosphate concentration in seawater was highly correlated with *T. hoshinota* overgrowth [76], highlighting the importance of phosphate in the spread of the *T. hoshinota* holobiont. Another study also proposed that *T. hoshinota* accumulates nutrients to compete with corals [8]. We propose that *Ca*. Pb. terpiosii LD05 helps *T. hoshinota* outcompete corals by serving as a carbon and phosphate storage. However, how the phosphate concentration is correlated with *T. hoshinota* cover is unclear, as are details about phosphate cycling among the host, *Ca*. Pb. terpiosii LD05, and other symbiotic microbes.

Animals cannot synthesize essential vitamins, so symbiotic microorganisms are thought to be important sources of essential vitamins for sponges. Our analysis of the *Ca*. Pb. terpiosii LD05 genome identified the biosynthesis pathway for vitamin B_1, vitamin B_7, and vitamin B_{12}. Thus, *Ca*. Pb. terpiosii LD05 may help maintain *T. hoshinota*'s health by providing the holobiont with vitamins.

One of the leading questions in the study of *T. hoshinota* is how it kills corals. Several mechanisms have been proposed. One argues that the sponge produces cytotoxic

allelochemicals to damage coral cells [77]. Another suggests that the sponge overgrows corals and competes with them for nutrients [8]. Finally, the sponge may cause the coral's microbial composition to change, causing it to malfunction [78]. These hypotheses are not mutually exclusive. For the first, several cytotoxic compounds, including nakiterpiosin, nakiterpiosinon, and terpiodiene, have been isolated from *T. hoshinota* holobionts [79, 80]. Nakiterpiosin, and nakiterpiosinon are C-nor-D-homosteroids. Previous studies showed that cyanobacteria can produce sterols by the cyclization of squalene, a triterpene [81]. In the *Ca*. Pb. terpiosii LD05 genome, the synthesis pathway of squalene and squalene cyclases were identified. Hence, the bacterium may be responsible for the production of the toxins. These toxins may facilitate overgrowing corals by damaging coral tissue directly or weakening the coral's defenses.

Conclusions

This study makes several fundamental discoveries about the bacterial community associated with *T. hoshinota*. The core microbiome of *T. hoshinota* includes a *Prochloron*-like bacterium, as well as *Endozoicomonas*, *Pseudospirillum*, SAR116, *Magnetospira*, and *Ruegeria*. Our finding that the dominant cyanobacteria in *T. hoshinota* is the same throughout the western Pacific Ocean, South China Sea, and Indian Ocean

suggests that this particular cyanobacterium is the obligate symbiont of the sponge. The high-quality draft genome of this cyanobacterium was recovered, and the phylogenetic and genomic analyses revealed that the genome should be classified as a novel species under a new genus “Prochlorobium”, close to *Prochloron*. The draft genome enables us to understand the carbon and nitrogen metabolism of the bacterium, and the putative symbiotic interaction between the bacterium and *T. hoshinota*. By identifying the core microbiome and revealing the metabolic potential of the dominant cyanobacterium in *T. hoshinota*, our conclusions will help future studies explore the detailed ecosystem inside the holobiont, unveil how *Ca*. Pb. terpiosii LD05 contributes to the invasion and coral-killing nature of *T. hoshinota*, and determine the cause of the outbreaks in the future.

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YHC, HJC, and SLT designed the study and prepared the manuscript. YHC and HJC analyzed and interpreted the data. HJC, CYY, JHS, DZH, PWC, MHL, and WSC collected and sequenced the samples. HJC, CYY, and JHS observed the electron microscopy. HJC, THS, SHL, and CMY examined the pigments. CAC, JDR, EH, BHI,

HH, PJS, CHJT, and HY assisted with the sampling.

All authors read and approved the manuscript.

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940

Figure Legends

FIG 1. Proportions of *T. hoshinota*-associated bacterial communities across oceans. *Proteobacteria* (blue) and *Cyanobacteria* (green) were the dominant groups, ranging from 81.5–97.4% of community. (a) Bacterial compositions across the western Pacific Ocean, South China Sea, and Indian Ocean. Maps on the right show results in Okinawa, Japan (b), and Taiwan (c). Different colors represent different bacterial phyla. Percentage composition of phyla less than 0.05% are grouped as "Others."

FIG 2. Electron micrographs of *T. hoshinota* and associated cyanobacteria. (a-c, SEM; d-f, TEM). (a) Sponge covering an abiotic substratum. The sponge's skeleton was arranged in a tangential style, and the mesophyll was supported by a tract of spicules. (b, c) Close view of the mesophyll. Numerous spheres composing the inside of the mesophyll, and cross-matched with (d); their thylakoids identified the spheres as cyanobacteria. The detailed structures of the cyanobacteria were observed from a single cyanobacterial cell (e) and a dividing cell (f); they included the cell wall, thylakoid, carboxysome (ca), phosphate granule (p), and nucleoid (n).

FIG 3. Molecular phylogenetic analyses of *Prochloron* and *Prochloron*-like bacteria.

(a) Pruned phylogenetic tree based on the concatenation of 120 single-copy gene protein sequences. The complete phylogenetic tree including other cyanobacterial genera can be found in Supplementary figure 5. The branches with Ultrafast bootstrap (UFBoot) value >95% are highlighted with the red. The *Prochloron* genomes are labeled with yellow, and LD05 and SP5CPC1 are labeled with pink. *Vampirovibrio chlorellavorus* C was used as the outgroup. (b) A phylogenetic tree was constructed using the 16S rRNA gene by the maximum-likelihood method with 1,000 bootstraps. The tree included 16S rRNA gene sequences from SP5CPC1, LD05, *Prochloron* genomes—including *P. didemni* P2, *P. didemni* P3, and *P. didemni* P4—and other related 16S rRNA gene sequences identified by PCR cloning from environmental samples including. The 16S rRNA gene sequence of *Stanieria* sp. NIES-3757 genome was used as an outgroup. The scare bar represents the number of changes per nucleotide. Asterisk represents the genomes are available.

FIG 4. Extracted ion-chromatograms (EICs) of UPLC-QTOF-MS of the extracts of *T. hoshinota*. Chlorophyll *a* analytical standard (a) and chlorophyll *b* analytical standard (b) were used in the analysis. The analysis included two *T. hoshinota* samples. EIC of MS spectra within the m/z value of 893.543 (a, c, e) and 907.522 (b, d, f). *a*: chlorophyll *a* peak. *b*: chlorophyll *b* peak.

FIG 5. Metabolic potentials and putative nutrient cycling between *T. hoshinota* and *Ca.* Pb. terpiosii LD05.

The bacterium may recycle nitrogen waste from host cells and provide them with vitamins. The bacterium may also produce secondary metabolites as toxins to help the sponge weaken coral tissues and facilitate overgrowth. On the other hand, the bacterium may also store phosphate as an energy reservoir for the holobiont to prepare for the competition with coral. Abbreviation: amt, ammonia transporter; NRT, nitrate transporter; NR, nitrate reductase; NiR, ferredoxin--nitrite reductase; CA, carbonic anhydrase; PPK, polyphosphate kinase; GS, glutamine synthetase; GOGAT, glutamine oxoglutarate aminotransferase; RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase; pcyA, phycocyanobilin:ferredoxin oxidoreductase; HO, heme oxygenase; pebA, dihydrobiliverdin:ferredoxin oxidoreductase; pebB, phycoerythrobilin:ferredoxin oxidoreductase. *The structures of nakiterpiosin and nakiterpiosinone are depicted based on Shuanhu Gao et al (2012).

Figure 1

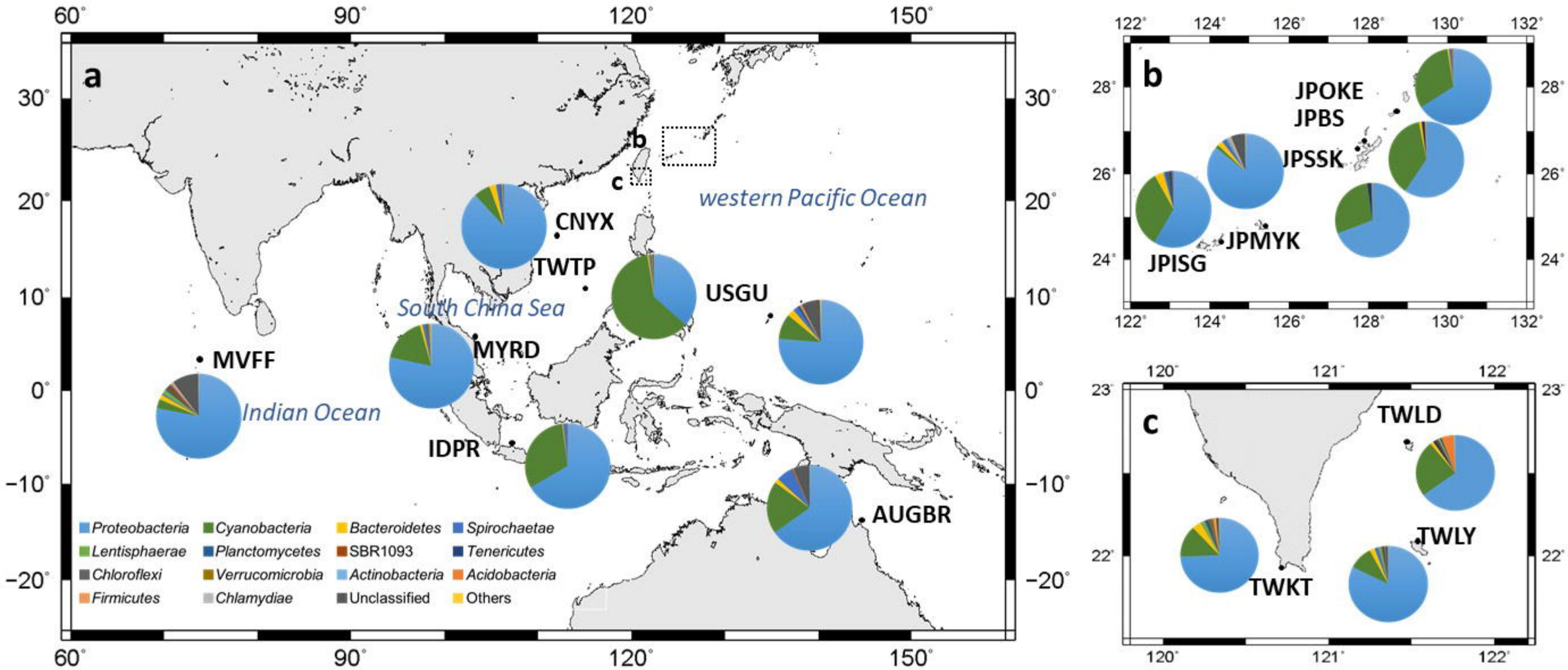

a
b
c
western Pacific Ocean
South China Sea
Indian Ocean
CNYX
TWTP
USGU
MYRD
MVFF
IDPR
AUGBR
Proteobacteria
Cyanobacteria
Bacteroidetes
Spirochaetae
Lentisphaerae
Planctomycetes
SBR1093
Tenericutes
Chloroflexi
Verrucomicrobia
Actinobacteria
Acidobacteria
Firmicutes
Chlamydiae
Unclassified
Others
JPOKE
JPBS
JPSSK
JPMYK
JPISG
TWLD
TWLY
TWKT

Figure 2

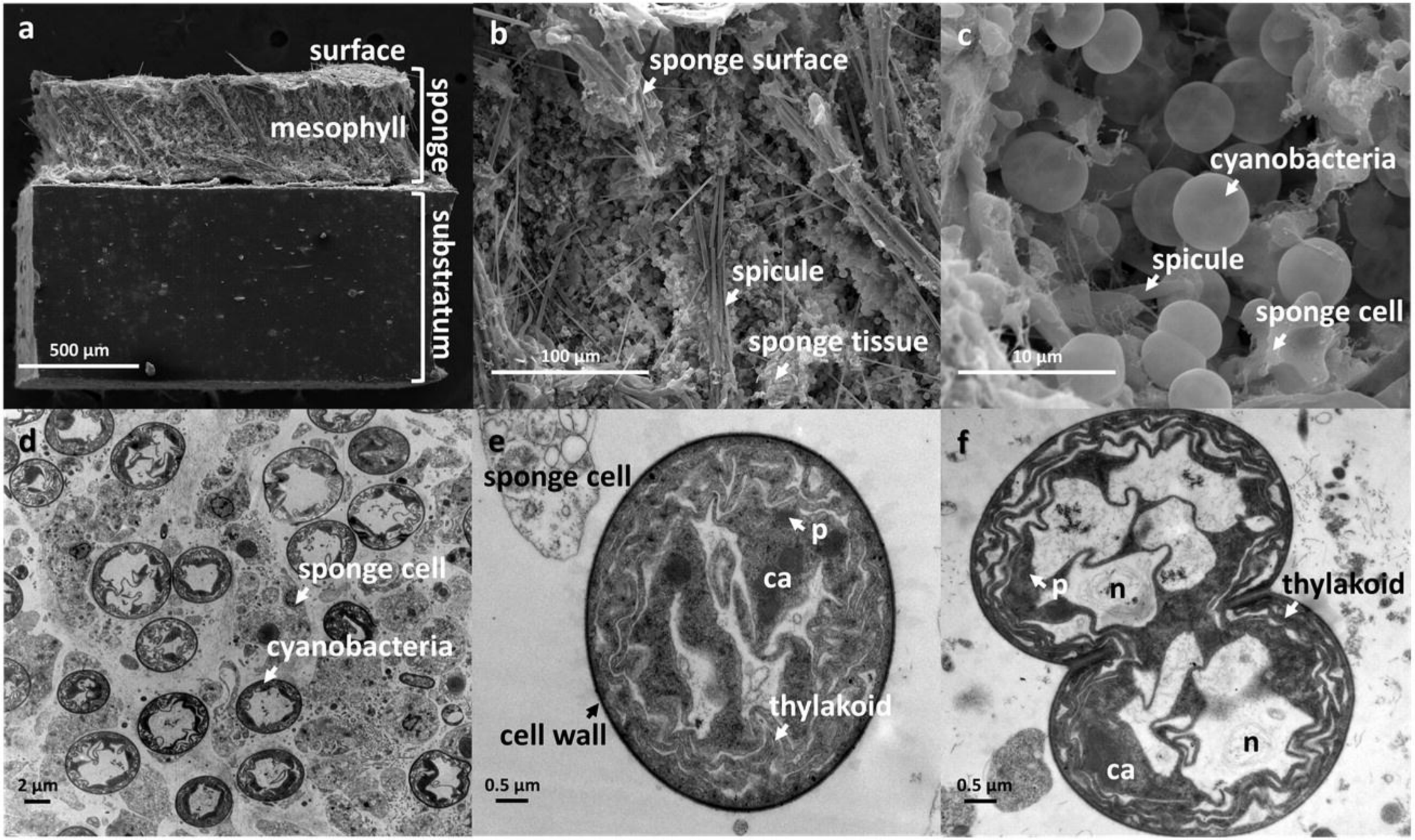
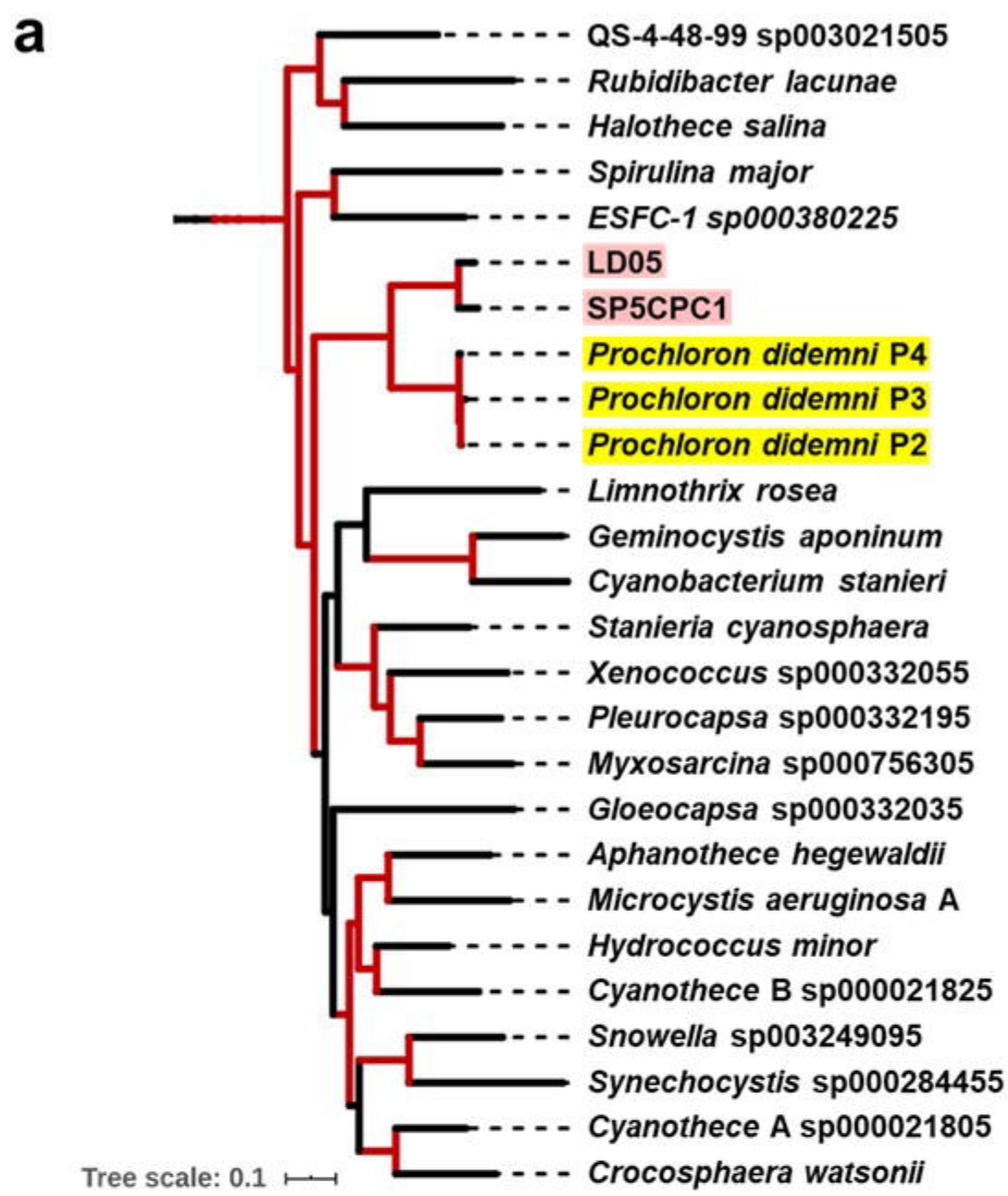

a
surface
mesophyll
sponge
substratum
500 µm
b
sponge surface
spicule
sponge tissue
100 µm
c
cyanobacteria
spicule
sponge cell
10 µm
d
sponge cell
cyanobacteria
2 µm
e
sponge cell
p
ca
thylakoid
cell wall
0.5 µm
f
p
n
thylakoid
n
ca
0.5 µm

Figure 3


a
QS-4-48-99 sp003021505
Rubidibacter lacunae
Halothece salina
Spirulina major
ESFC-1 sp000380225
LD05
SP5CPC1
Prochloron didemni P4
Prochloron didemni P3
Prochloron didemni P2
Limnothrix rosea
Geminocystis aponinum
Cyanobacterium stanieri
Stanieria cyanosphaera
Xenococcus sp000332055
Pleurocapsa sp000332195
Myxosarcina sp000756305
Gloeocapsa sp000332035
Aphanothece hegewaldii
Microcystis aeruginosa A
Hydrococcus minor
Cyanothece B sp000021825
Snowella sp003249095
Synechocystis sp000284455
Cyanothece A sp000021805
Crocosphaera watsonii
Tree scale: 0.1

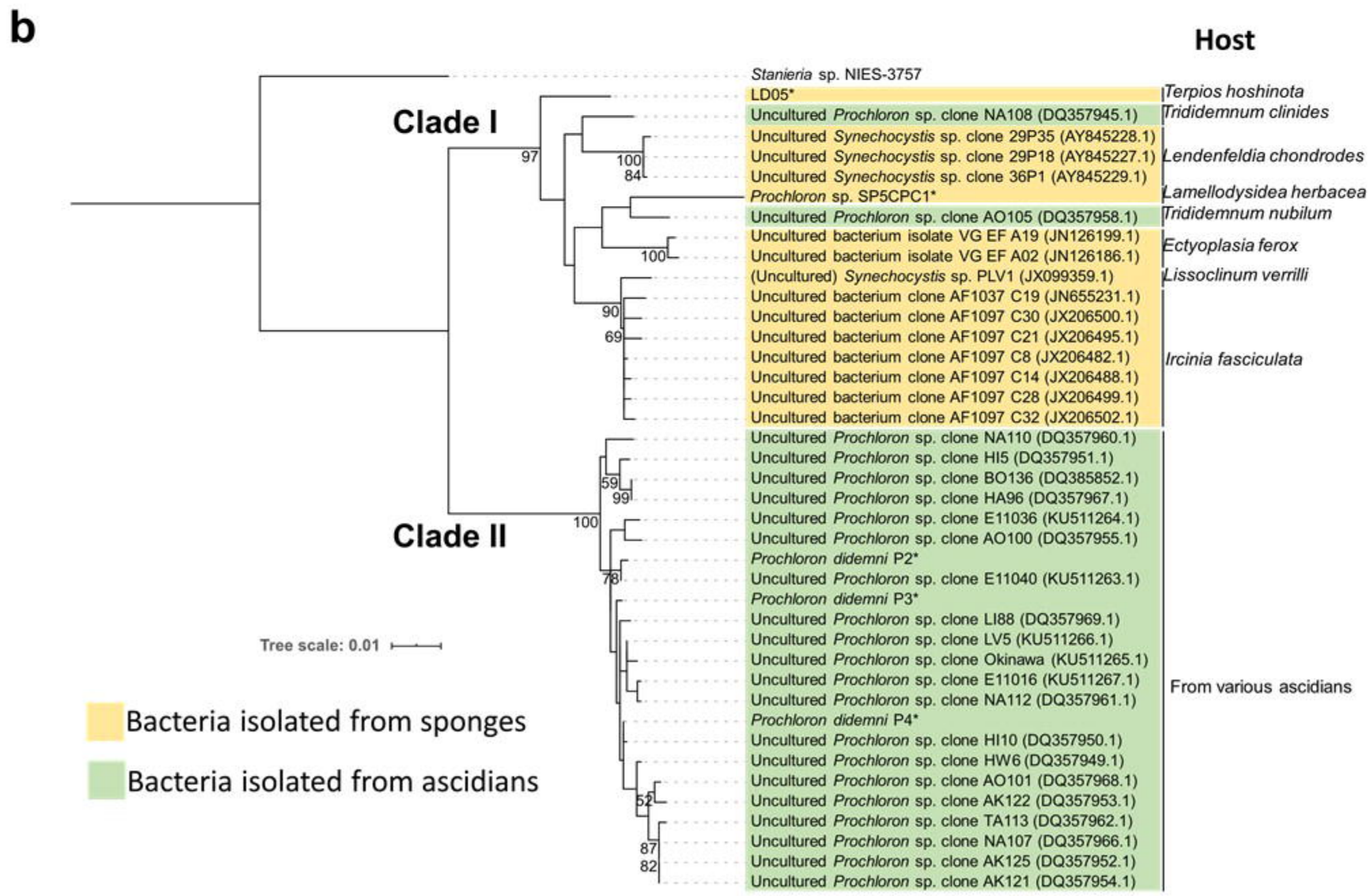

b
Host
Clade I
Clade II
Stanieria sp. NIES-3757
LD05*
Uncultured Prochloron sp. clone NA108 (DQ357945.1)
Uncultured Synechocystis sp. clone 29P35 (AY845228.1)
Uncultured Synechocystis sp. clone 29P18 (AY845227.1)
Uncultured Synechocystis sp. clone 36P1 (AY845229.1)
Prochloron sp. SP5CPC1*
Uncultured Prochloron sp. clone AO105 (DQ357958.1)
Uncultured bacterium isolate VG EF A19 (JN126199.1)
Uncultured bacterium isolate VG EF A02 (JN126186.1)
(Uncultured) Synechocystis sp. PLV1 (JX099359.1)
Uncultured bacterium clone AF1037 C19 (JN655231.1)
Uncultured bacterium clone AF1097 C30 (JX206500.1)
Uncultured bacterium clone AF1097 C21 (JX206495.1)
Uncultured bacterium clone AF1097 C8 (JX206482.1)
Uncultured bacterium clone AF1097 C14 (JX206488.1)
Uncultured bacterium clone AF1097 C28 (JX206499.1)
Uncultured bacterium clone AF1097 C32 (JX206502.1)
Uncultured Prochloron sp. clone NA110 (DQ357960.1)
Uncultured Prochloron sp. clone HI5 (DQ357951.1)
Uncultured Prochloron sp. clone BO136 (DQ385852.1)
Uncultured Prochloron sp. clone HA96 (DQ357967.1)
Uncultured Prochloron sp. clone E11036 (KU511264.1)
Uncultured Prochloron sp. clone AO100 (DQ357955.1)
Prochloron didemni P2*
Uncultured Prochloron sp. clone E11040 (KU511263.1)
Prochloron didemni P3*
Uncultured Prochloron sp. clone LI88 (DQ357969.1)
Uncultured Prochloron sp. clone LV5 (KU511266.1)
Uncultured Prochloron sp. clone Okinawa (KU511265.1)
Uncultured Prochloron sp. clone E11016 (KU511267.1)
Uncultured Prochloron sp. clone NA112 (DQ357961.1)
Prochloron didemni P4*
Uncultured Prochloron sp. clone HI10 (DQ357950.1)
Uncultured Prochloron sp. clone HW6 (DQ357949.1)
Uncultured Prochloron sp. clone AO101 (DQ357968.1)
Uncultured Prochloron sp. clone AK122 (DQ357953.1)
Uncultured Prochloron sp. clone TA113 (DQ357962.1)
Uncultured Prochloron sp. clone NA107 (DQ357966.1)
Uncultured Prochloron sp. clone AK125 (DQ357952.1)
Uncultured Prochloron sp. clone AK121 (DQ357954.1)
Terpios hoshinota
Trididemnum clinides
Lendenfeldia chondrodes
Lamellodysidea herbacea
Trididemnum nubilum
Ectyoplasia ferox
Lissoclinum verrilli
Ircinia fasciculata
From various ascidians
97
100
84
100
90
69
100
59
99
78
52
87
82
Tree scale: 0.01
Bacteria isolated from sponges
Bacteria isolated from ascidians

Figure 4

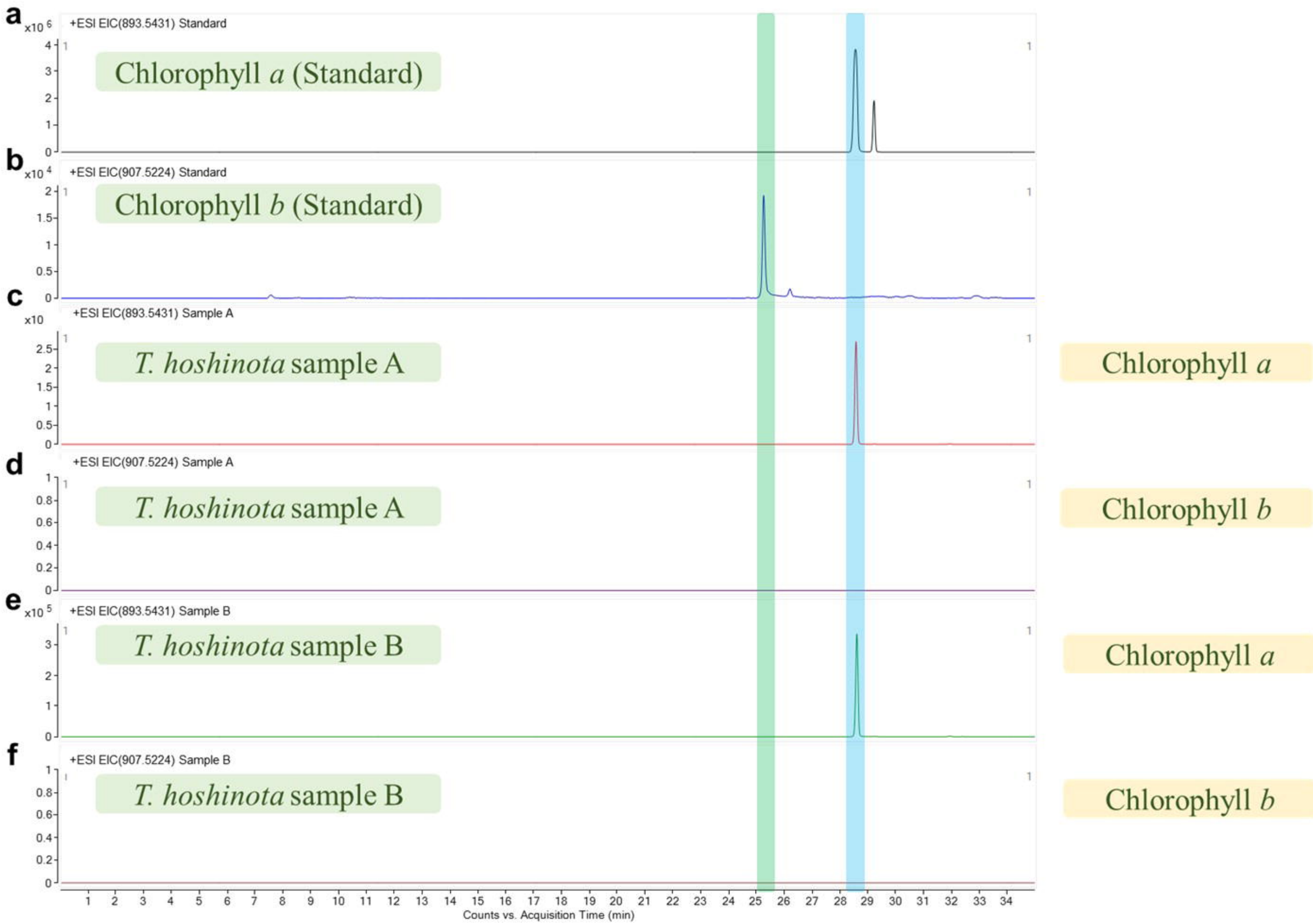
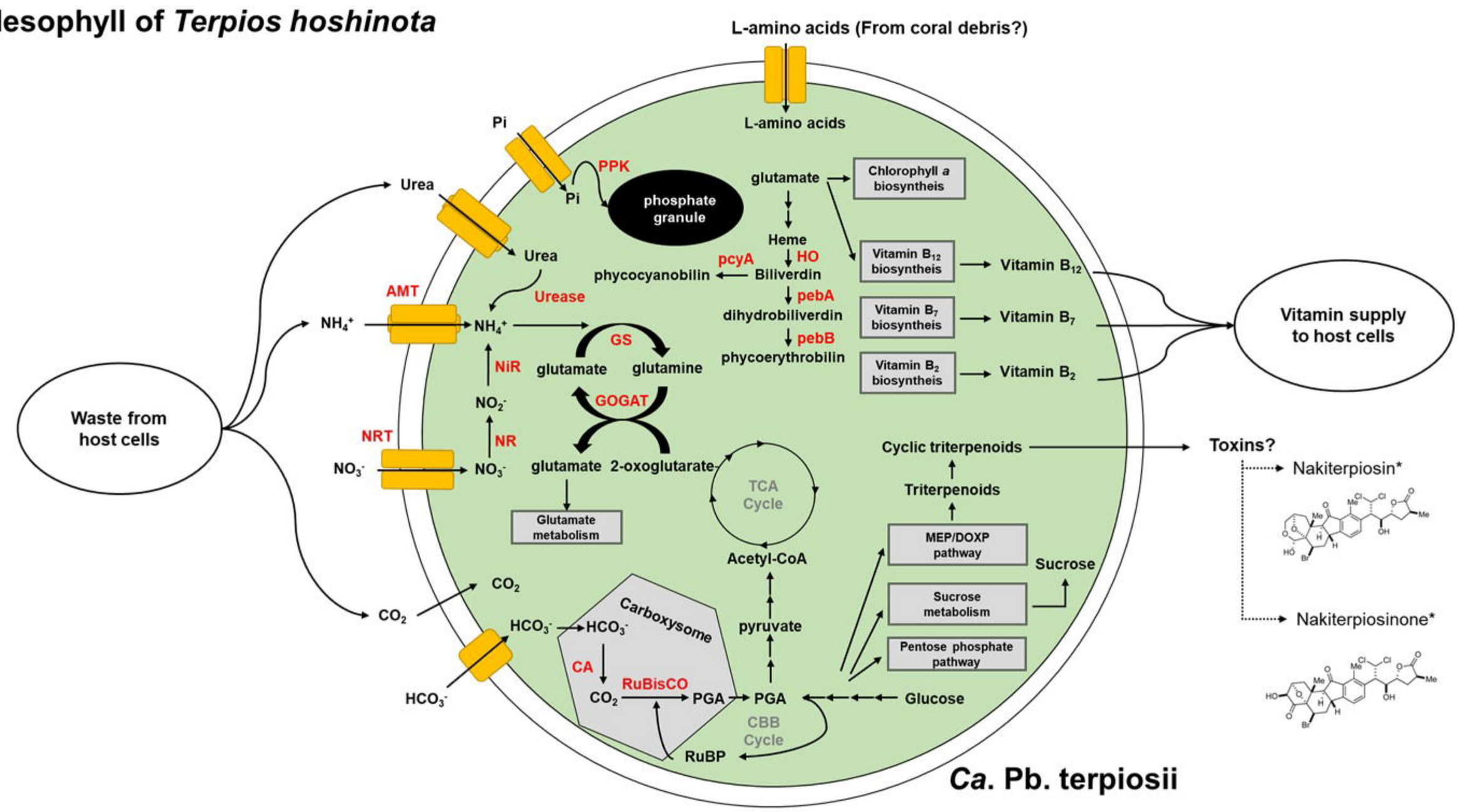

a
+ESI EIC(893.5431) Standard
Chlorophyll a (Standard)
b
+ESI EIC(907.5224) Standard
Chlorophyll b (Standard)
c
+ESI EIC(893.5431) Sample A
T. hoshinota sample A
Chlorophyll a
d
+ESI EIC(907.5224) Sample A
T. hoshinota sample A
Chlorophyll b
e
+ESI EIC(893.5431) Sample B
T. hoshinota sample B
Chlorophyll a
f
+ESI EIC(907.5224) Sample B
T. hoshinota sample B
Chlorophyll b
Counts vs. Acquisition Time (min)

Figure 5


Mesophyll of *Terpios hoshinota*
L-amino acids (From coral debris?)
L-amino acids
Pi
PPK
Pi
phosphate granule
Urea
Urea
Urease
AMT
NH_4^+
NH_4^+
NiR
NO_2^-
NR
NRT
NO_3^-
NO_3^-
GS
glutamate
glutamine
GOGAT
glutamate
2-oxoglutarate
Glutamate metabolism
TCA Cycle
Acetyl-CoA
pyruvate
PGA
PGA
CBB Cycle
RuBP
Glucose
CO_2
CO_2
HCO_3^-
HCO_3^-
HCO_3^-
Carboxysome
CA
CO_2
RuBisCO
PGA
glutamate
Chlorophyll *a* biosyntheis
Heme
HO
pcyA
phycocyanobilin
Biliverdin
pebA
dihydrobiliverdin
pebB
phycoerythrobilin
Vitamin B_{12} biosyntheis
Vitamin B_{12}
Vitamin B_7 biosyntheis
Vitamin B_7
Vitamin B_2 biosyntheis
Vitamin B_2
Vitamin supply to host cells
Waste from host cells
Cyclic triterpenoids
Toxins?
Triterpenoids
MEP/DOXP pathway
Sucrose
Sucrose metabolism
Pentose phosphate pathway
Nakiterpiosin*
Nakiterpiosinone*
Ca. Pb. terpiosii