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ORIGINAL ARTICLE

Down under the tunic: bacterial biodiversity hotspots and widespread ammonia-oxidizing archaea in coral reef ascidians

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Ascidians are ecologically important components of marine ecosystems yet the ascidian microbiota remains largely unexplored beyond a few model species. We used 16S rRNA gene tag pyrosequencing to provide a comprehensive characterization of microbial symbionts in the tunic of 42 Great Barrier Reef ascidian samples representing 25 species. Results revealed high bacterial biodiversity (3 217 unique operational taxonomic units ($OTU_{0.03}$) from 19 described and 14 candidate phyla) and the widespread occurrence of ammonia-oxidizing *Thaumarchaeota* in coral reef ascidians (24 of 25 host species). The ascidian microbiota was clearly differentiated from seawater microbial communities and included symbiont lineages shared with other invertebrate hosts as well as unique, ascidian-specific phylotypes. Several rare seawater microbes were markedly enriched (200–700 fold) in the ascidian tunic, suggesting that the rare biosphere of seawater may act as a conduit for horizontal symbiont transfer. However, most OTUs (71%) were rare and specific to single hosts and a significant correlation between host relatedness and symbiont community similarity was detected, indicating a high degree of host-specificity and potential role of vertical transmission in structuring these communities. We hypothesize that the complex ascidian microbiota revealed herein is maintained by the dynamic microenvironments within the ascidian tunic, offering optimal conditions for different metabolic pathways such as ample chemical substrate (ammonia-rich host waste) and physical habitat (high oxygen, low irradiance) for nitrification. Thus, ascidian hosts provide unique and fertile niches for diverse microorganisms and may represent an important and previously unrecognized habitat for nitrite/nitrate regeneration in coral reef ecosystems.
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Introduction

Symbiotic microbial communities are a common feature of marine invertebrates and include diverse lineages of bacteria, archaea, fungi, microalgae and viruses (Rowan, 1998; Taylor *et al.*, 2007). Prokaryotic symbionts are a particularly rich component of invertebrate microbiota and encompass nearly all major branches of bacterial and archaeal life. Many of these symbiont lineages are primarily host-associated (i.e., obligate symbionts) and represent novel microbial taxa from species level (e.g., *Synechococcus spongiarum* in sponges, Usher *et al.*, 2004) to phylum level, (e.g., *Poribacteria*, Fieseler *et al.*, 2004) while others exist in both free-living and host-associated states, (i.e., facultative symbionts) though generally enriched in the invertebrate microhabitat and rare in seawater communities (Sunagawa *et al.*, 2010). The phylogenetic diversity of symbiotic microbes is associated with a diversity of metabolic pathways in the carbon, (Wilkinson, 1983) nitrogen (Hoffmann *et al.*, 2009) and sulfur cycles (Hoffmann *et al.*, 2005), spurred by the utilization of host waste products (e.g., ammonia), the presence of dimethylsulfoniopropionate (DMSP, Raina *et al.*, 2010) and physico-chemical conditions of the host microenvironment (e.g., oxygen gradients; Hoffmann *et al.*, 2008; Kühl *et al.*, 2012). The structural and functional diversity of symbiotic microbial communities indicate that invertebrate hosts provide fertile microbial niches

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that contribute to prokaryotic biodiversity and nutrient cycling in coastal marine ecosystems.

Invertebrate-microbe symbioses also play critical roles in host ecological success through the provision of supplemental nutrition and production of defensive secondary metabolites. For example, sponges, corals and ascidians are able to supplement their heterotrophic filter-feeding activities with fixed carbon sourced from photosynthetic symbionts (Muscatine and Porter, 1977; Pardy and Lewin, 1981; Freeman and Thacker, 2011), utilizing autotrophic symbiont metabolism to enhance their growth rates in nutrient-limited environments. Sponge symbionts are also responsible for the synthesis of vitamin B1, which animals need to obtain from their diet (Fan *et al.*, 2012), while the cyanobacteria in the genus *Prochloron* appear to provide UV-absorbing molecules to their ascidian hosts (Hirose *et al.*, 2004). Further, symbiont biosynthesis of secondary metabolites contributes to the chemical defenses of marine invertebrates (Schmidt *et al.*, 2005; Freeman *et al.*, 2012), a key strategy for sessile organisms to deter predation, avoid surface fouling and compete for substrate (Armstrong *et al.*, 2001; Pawlik, 2011). In addition to their roles in host biology and ecology, many of these unique and structurally diverse secondary metabolites have pharmaceutical applications and substantial importance for biotechnology and drug discovery (Paul and Ritson-Williams, 2008; Erwin *et al.*, 2010).

Ascidians (Class Ascidiacea) are sessile, filter-feeding invertebrates that inhabit diverse benthic ecosystems in tropical, temperate and polar marine environments. As a basal lineage in the phylum Chordata, ascidians occupy a key stage in deuterostome evolution (Delsuc *et al.*, 2006). Ascidians are also a prolific source of novel marine natural products (Erwin *et al.*, 2010) and the involvement of microbial symbionts in bioactive compound production (Schmidt and Donia, 2010) has prompted recent studies of the ascidian microbiota (Donia *et al.*, 2011; Kwan *et al.*, 2012). Historically, most studies of microbial symbionts in ascidians have focused on cyanobacteria, in particular the genera *Prochloron* and *Synechocystis.* These symbionts associate with colonial ascidians on the colony surface, inside the common cloacal cavities or as endosymbionts in the tunic, a polysaccharide envelope surrounding the zooids (Cox *et al.*, 1985; Cox, 1986; Hernández-Mariné *et al.*, 1990; Hirose *et al.*, 1996, 2006a, b, 2012; Turon *et al.*, 2005; Martínez-García *et al.*, 2007). Even when inhabiting the colonial tunic, the symbionts are mostly extracellular, with only a few instances of intracellular associations (Hirose *et al.*, 1996; Moss *et al.*, 2003; Kojima and Hirose, 2010). However, few studies to date have employed the molecular approaches required to accurately assess microbial biodiversity in ascidians (Martínez-García *et al.*, 2007, 2008, 2011; Münchhoff *et al.*, 2007; Tait *et al.*, 2007; López-Legentil *et al.*, 2011; Behrendt *et al.*, 2012; Erwin *et al.*, 2013). For example, DNA sequence analysis and fluorescence *in situ* hybridization techniques only recently revealed the first archaeal symbionts in the ascidian tunic, indicating that *Thaumarchaeota* may be involved in nitrification inside host tissues (Martínez-García *et al.*, 2008).

A growing body of literature suggests that ascidian-associated microbes may play a critical role in the metabolic needs of their host, (Hirose and Maruyama, 2004; Martínez-García *et al.*, 2008; Kühl *et al.*, 2012), yet the microbial communities inhabiting most ascidian species remain unknown. The advent of high-throughput, next-generation DNA sequencing platforms offers new opportunities for in-depth microbial diversity evaluation across large sample sets. Deep sequencing of microbial communities from soils, seawater and sponges has revealed diversity estimates over an order of magnitude higher than that recovered by traditional sequencing techniques (Huber *et al.*, 2007; Roesch *et al.*, 2007; Webster *et al.*, 2010), including the detection of bacterial phyla not represented in first-generation sequencing datasets (e.g., Webster and Taylor, 2012). Similarly, the recent application of next generation sequencing to the ascidian microbiota has revealed a high diversity of symbiotic microbes and uncovered new ascidian-associated microbial lineages in the colonial host *Lissoclinum patella* (Behrendt *et al.*, 2012) and solitary host *Styela plicata* (Erwin *et al.*, 2013), highlighting the depth of microbial biodiversity and unknown facultative and obligate symbiotic microbes awaiting discovery within ascidian hosts.

In this study, we used 16S rRNA gene tag pyrosequencing to investigate the diversity, structure and specificity of microbial communities inhabiting the tunic of 42 samples of Great Barrier Reef (GBR) ascidians (representing 25 species, 7 families and 3 orders) in order to provide the most comprehensive characterization of the ascidian microbiome to date. The diversity and composition of ascidian-associated microbial communities were compared to free-living communities in ambient seawater and among ascidian host species, including intraspecific variability among replicates for 10 ascidian species. In addition, the spatial localization of symbionts within the ascidian tunic was visualized by electron microscopy, and the genetic identity of ascidian hosts was established by analysis of mitochondrial (cytochrome oxidase subunit I) and ribosomal (18S rRNA) gene sequences. This comprehensive assessment of microbial diversity in GBR ascidians will provide the basis for future research within the fields of symbiosis, drug discovery and ascidian holobiont resilience to environmental change or anthropogenic disturbance. Exploration of ascidian microbiomes may also highlight a hidden reservoir for primary productivity and nitrogen metabolism and enable

more reliable predictions of biogeochemical cycling in coral reef environments.

Material and methods

Sample collection

Ascidian ($n = 42$) and seawater ($n = 3$) samples were collected by SCUBA between 2–14 m depth from several localities within the Great Barrier Reef, North Queensland, Australia (Supplementary Table S1). Ascidian samples were processed for: (1) taxonomic analyses, by preservation in 4% formaldehyde, (2) molecular analyses, by immediate submersion in liquid nitrogen and storage at −80 °C and (3) electron microscopy analyses, by preservation in 2.5% glutaraldehyde using filtered seawater as buffer. Seawater samples (2 l) were transported to the laboratory, concentrated on 0.2 μm sterivex filters (Durapore; Millipore, North Ryde, New South Wales, Australia) with a peristaltic pump and aseptically frozen at −80 °C.

DNA extraction

Frozen ascidian tissues (approximately 0.5 g per sample) were thawed, dissected under a stereomicroscope into inner tunic and zooid fractions and aseptically transferred to 1.5 ml tubes using sterile scalpels and tweezers. Inner tunic (i.e., beneath the surface layer) was chosen to avoid epibionts and ambient seawater microbes. These tunic samples were processed for microbial analysis, while zooids were processed for barcoding each ascidian specimen. DNA extraction was conducted separately for inner tunic and zooid tissue fractions with the Power Plant DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. DNA extraction from concentrated seawater samples (filters) was performed by the addition of 1.8 ml lysis buffer (40 mM EDTA, 50 mM Tris and 0.75 M sucrose) and 200 μl of Lysozyme (10 mg/ml), incubation at 37 °C for 45 min, the addition of 40 μl of Proteinase K (10 μg of Proteinase K in 1 ml of 10% SDS) and incubation at 55 °C for 1 h. Lysates were transferred to sterile tubes and DNA was extracted using standard phenol:chloroform procedures and resuspended in 20 μl of distilled water. All PCR products were visualized on 1% agarose gels to assess amplification specificity and initial product quantity.

Identification and barcoding of host ascidians

Ascidian samples were assigned to the lowest taxonomic group possible based on morphological examination (Supplementary Text S1). Genetic identification was also performed using the mitochondrial gene cytochrome oxidase subunit I (COI) and 18S rRNA gene sequences. Both gene regions are commonly used to determine species boundaries and diversity among ascidian taxa (Tarjuelo *et al.*, 2004; López-Legentil and Turon, 2005; Yokobori *et al.*, 2006; Pérez-Portela *et al.*, 2009) and COI is the metazoan standard for the Barcode of Life Project (www.barcodeoflife.org).

DNA extractions from zooid tissue were used as templates for PCR amplification of a 519 – 621 bp fragment of the COI gene. Total PCR reaction volume was 50 μl, including 10 μl of 5 × Buffer, 0.4 μl of bovine serum albumin (BSA; 10 mg/ml), 0.25 μl of My *Taq* DNA Polymerase (Bioline, London, United Kingdom), 2 μl of each primer (10 μM) and 1 μl of template DNA. Two sets of primer pairs were used for COI amplification, the 'universal' primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and the ascidian-specific primers Tun_forward and Tun_Reverse2 (Stefaniak *et al.*, 2009). PCR conditions for amplification with universal primers were: an initial denaturing step of 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 50 s; and a final elongation step at 72 °C for 5 min. PCR conditions for amplification with ascidian-specific primers were: an initial denaturing step of 94 °C for 1 min; 60 cycles of 94 °C for 10 s, 50 °C for 30 s and 72 °C for 50 s; and a final elongation step at 72 °C for 10 min. PCR products were purified and bi-directionally sequenced at Macrogen, Inc. (Seoul, South Korea). Quality-checked sequences are archived in GenBank under accession numbers KC017426 to KC017444. Additional genetic identification and phylogenetic analyses of host ascidians were performed with 18S rRNA gene sequences recovered from the non-target, eukaryotic data component of the pyrosequencing run (Supplementary Text S2, Figure S4).

16S rRNA gene tag pyrosequencing

DNA extractions from inner tunic tissue and seawater samples were used as templates for PCR amplification of a ca. 466 bp fragment of the 16S rRNA gene encompassing the V6 – V8 regions using the primer set pyro926F (5′-AAACTYAAAKGAA TTGRCGG-3′) and pyro1392R (5′-ACGGGCGGTG TGTRC-3′) complemented with adaptors B and A, respectively (Roche, Basel, Switzerland), as detailed previously (Erwin *et al.*, 2013). Multiplex identifier (MID) barcodes unique to each sample were attached to reverse primers (Supplementary Table S2). PCR products were sent to Macrogen, Inc. for purification and further processing. Amplicon library was constructed using 5 μg of DNA from each sample (ascidian and sweater), resulting in a final concentration of 700 513 297 molecules/μl. Massively parallel 16S rRNA gene tag pyrosequencing was performed using the Roche 454 GS-FLX Titanium system, and the resulting data were deposited as flowgrams (sff file) in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information under the accession number SRA056317.

Sequence data were processed with stringent filtering and screening criteria to minimize the occurrence of spurious sequences and overestimation of microbial diversity (Huse *et al.*, 2010; Schloss *et al.*, 2011), using the mothur software package (Schloss *et al.*, 2009), as detailed previously (Erwin *et al.*, 2013). Briefly, adaptor, MID and primer sequences were removed from raw sequences and the dataset de-noised (removal of reads with ambiguous base calls, long homopolymers and barcode or primer mismatches) and quality filtered (removal of short sequences and low quality reads). Non-target sequences (e.g. eukaryotic 18S rRNA, mitochondria, chloroplast) were removed using Metaxa v1.1, (Bengtsson *et al.*, 2011) resulting in a dataset consisting solely of archaeal and bacterial 16S rRNA gene sequences. These sequences were aligned to the Greengenes database, trimmed to an overlapping alignment space (449 bp) and putatively chimeric sequences were removed (UChime; Edgar *et al.*, 2011).

Data analysis

High quality sequences ($n = 94\,637$) were assigned to taxonomic groups based on the improved Greengenes taxonomy template (McDonald *et al.*, 2012) with *Thaumarchaeota* elevated to the rank of phylum (Brochier-Armanet *et al.*, 2008, Spang *et al.*, 2010), grouped into $OTU_{0.03}$ based on 97% sequence similarity and the average neighbor clustering algorithm, and the taxonomic assignment of each $OTU_{0.03}$ was constructed by majority consensus (Schloss and Westcott, 2011).

Sampling coverage and expected total OTU diversity were calculated using rarefaction analysis and the bootstrap estimator (Smith and Van Belle, 1984) at six different OTU definitions corresponding approximately to the species ($OTU_{0.03}$), genus ($OTU_{0.05}$), family ($OTU_{0.10}$), order ($OTU_{0.15}$), class ($OTU_{0.20}$) and phylum ($OTU_{0.25}$) levels (97%, 95%, 90%, 85%, 80% and 75% similarity, respectively). All subsequent analyses were based on OTUs at 97% sequence identity ($OTU_{0.03}$). Sub-sampling of sequence pools from samples with greater than 2000 reads were performed in the mothur software package to standardize sampling effort and determine its effect on diversity estimates. Host-specificity of the ascidian microbiota was assessed by partitioning OTUs into core (present in $>70\%$ of host species), variable (present in at least two host species) and specific (present in a single host species) groups (*sensu* Schmitt *et al.*, 2012). To broaden the analysis of the specificity of the ascidian microbiota, abundant ascidian-associated OTUs (i.e., those represented by >100 total sequence reads) were compared to sequences in the GenBank database using a nucleotide-nucleotide BLAST search (Altschul *et al.*, 1990). To compare microbial community similarity across hosts, Bray-Curtis similarity matrices were constructed using square root transformations of relative OTU abundance per host and visualized in cluster plots using Primer v6 (Plymouth Marine Laboratory, United Kingdom). Finally, Mantel tests were conducted to test for correlations between host relatedness (18S rRNA sequence similarity) and symbiont similarity (Bray-Curtis similarity) using the ade4 package for *R* (Dray and Dufour, 2007).

Transmission electron microscopy

Bacterial cells in the tunic of the representative ascidian species *Phallusia julinea, Polycarpa aurata*, *Pycnoclavella* sp., *Clavelina meridionalis*, *Lissoclinum badium* and *Synoicum castellatum* were visualized by transmission electron microscopy. Resin blocks and semi-thin and ultra-thin sections were prepared at the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona as described in López-Legentil *et al.* (2011). Transmission electron microscopy observations were conducted on a JEOL JEM-1010 (Tokyo, Japan) electron microscope coupled with an Orius CDD camera (Gatan, Germany).

Results

Diversity and phylogeny of ascidian hosts

The 42 host ascidians examined for microbial symbionts were classified in 25 species from 7 families and all 3 recognized orders in the class Ascidiacea, with 18 species belonging to the Aplousobranchia, the largest ascidian order in terms of species and family richness (Shenkar and Swalla, 2011). Analyses of 18S rRNA gene sequences (23 of the 25 host species) and COI sequences (19 of 25 host species) confirmed morphological identifications and provide molecular datasets to facilitate additional research on the ascidian microbiota. All reference works used to identify each specimen and pertinent taxonomic remarks are provided (Supplementary Text S1), including underwater images (Supplementary Figures S1, S2 and S3) and a phylogenetic analysis using 18S rRNA sequences (Supplementary Text S2, Figure S4).

Richness and diversity of the ascidian microbiota

Collective analysis of 16S rRNA sequence reads derived from ascidian hosts ($n = 67\,826$) revealed a remarkable richness and diversity of microbial communities associated with GBR ascidians. A total of 3321 unique microbial $OTU_{0.03}$ represented the combined GBR ascidian microbiome and corresponded to 19 described bacterial phyla, 14 candidate bacterial phyla and 3 described archaeal phyla (Figure 1). This increases the taxonomic diversity known to inhabit ascidians by 14 microbial phyla. Coverage estimates of total diversity sampled were high across all taxonomic levels, ranging from 82 ($OTU_{0.03}$) to 85% ($OTU_{0.25}$). Rarefaction analysis

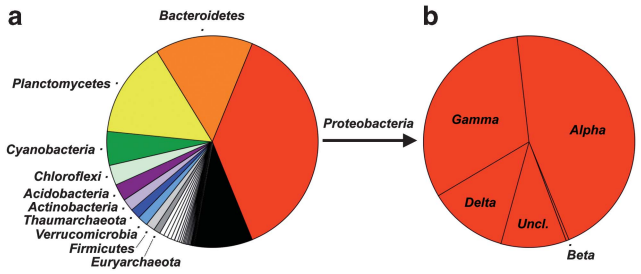

Figure 1 Taxonomic diversity of the ascidian microbiota. (**a**) Phylum level distribution of the 3321 microbial $OTU_{0.03}$ recovered from 42 GBR ascidian hosts, depicting common phyla (*in color*, >1% $OTU_{0.03}$ diversity), rare phyla (*in gray*, <1%; SBR1093, *Lentisphaerae*, *Chlamydiae*, *Tenericutes*, TM7, WS3, *Spirochaetes*, *Nitrospirae*, OP3, TM6, *Crenarchaeota*, *Chlorobi*, OP11, *Thermi*, *Armatimonadetes*, *Fusobacteria*, NKB19, *Calditrhix*, OP8, PAUC34f, BRC1, *Elusimicrobia*, GN04, KSB1 and SM2F11) and bacterial OTUs unclassified at the phylum level (*in black*). (**b**) Class level distribution of proteobacterial OTUs.

revealed that observed OTU diversity was approaching expected OTU diversity at higher level taxonomic rankings (e.g., phylum and class; Supplementary Figure S5) while additional sampling would continue to uncover new microbial OTUs at lower taxonomic levels (e.g., genus and species; Supplementary Figure S6) due to a rich rare component of the microbiota (1817 singletons).

Analyses of individual hosts and ascidian species revealed up to 486 microbial $OTU_{0.03}$ per individual and 697 unique $OTU_{0.03}$ per species (Tables 1 and 2), with many ascidians hosting more diverse microbial communities than those recovered from ambient seawater in terms of observed and expected (Chao1) OTU richness and common diversity indices (Shannon, Simpson Inverse; Supplementary Table S3). 16S rRNA sequence reads derived from seawater ($n=26\,811$) grouped into 385 unique $OTU_{0.03}$ (129–284 per replicate). While high variability in sampling effort (sequence reads per sample) can obscure direct comparisons among host species and between ascidians and seawater, over 25% ($n=11$) of the sampled ascidians exhibited higher microbial $OTU_{0.03}$ diversity than the most well-sampled seawater replicate, despite lower sampling effort (7 500–13 500 fewer sequence reads; Table 1). Further, this trend was maintained after sub-sampling of sequence pools to standardize sampling efforts across ascidian and seawater sources (Supplementary Figure S6).

Composition of the ascidian microbiota

Microbial communities in GBR ascidians were composed of diverse bacterial phyla and archaeal lineages (Figure 1, Supplementary Table S4). Bacterial OTUs dominated the ascidian microbiota, accounting for 97% ($n=3217$) of $OTU_{0.03}$ diversity and 82% of all sequence reads ($n=55\,698$). The most dominant bacterial phylum was *Proteobacteria*, representing over one-third (38%) of $OTU_{0.03}$ diversity ($n=1251$) and the only phylum detected in all examined ascidians. *Proteobacteria* accounted for over half of all sequence reads in 12 ascidian individuals and over 90% of sequences from *Aplidium protectans*, *Lissoclinum* cf. *capsulatum* and *Didemnum granulatum* (Figure 2). Within the *Proteobacteria*, the classes *Alphaproteobacteria* and *Gammaproteobacteria* were most prevalent (517 OTUs and 397 OTUs, respectively), followed by *Deltaproteobacteria* and *Betaproteobacteria* (125 OTUs and 6 OTUs, respectively). Representatives from the phyla *Bacteroidetes* and *Planctomycetes* were also common, each accounting for over 15% of $OTU_{0.03}$ diversity ($n=496$ and 486, respectively, Figure 1) and detected in the majority (>88%) of ascidian hosts (Figure 2, Supplementary Table S4).

Cyanobacteria was the fourth most diverse phyla associated with ascidians (172 OTUs, 5% of $OTU_{0.03}$ diversity) and included the genus *Procholoron*, present only in *Lissoclinum patella* (OTU0810), and 4 OTUs that were closely related (95–98% sequence identity) to the recently described *candidatus* 'Acaryochloris bahamiensis' (López-Legentil *et al.*, 2011). Most notably, two *Acaryochloris* OTUs (OTU0125, 0126) were common in all 3 individuals of the host *Eudistoma amplum* (0.7 to 8.9% relative abundance). An additional 5 described phyla were common in ascidians, including *Chloroflexi* (103 $OTU_{0.03}$), *Acidobacteria* (87), *Actinobacteria* (62), *Verrucomicrobia* (51) and *Firmicutes* (45), each accounting for 1 to 3% of $OTU_{0.03}$ diversity and detected in at least half of the ascidian hosts examined. The remaining 24 described and candidate phyla present in the ascidian microbiota were rare overall (each <1% of total $OTU_{0.03}$ diversity) and within each host ascidian (<2% of sequence reads; Figure 2, Supplementary Table S4), with the exception of *Spirochaetes* in *Polycarpa aurata* (18% relative abundance) and SBR1093 in *Eudistoma amplum* (11%).

Archaeal OTUs accounted for 18% ($n=12\,128$) of sequence reads but only 3% ($n=104$) of the $OTU_{0.03}$ richness in the ascidian microbiota. *Thaumarchaeota* were particularly abundant ($n=11\,993$; 53 OTUs) and common (present in 93% of host individuals), with most archaeal sequence reads (98%) matching to the ammonia-oxidizing genera *Nitrosopumilus* ($n=11\,630$; 36 OTUs) and *Cenarchaeum* ($n=261$; 5 OTUs). In fact, the most common $OTU_{0.03}$ in the ascidian microbiota (OTU0001, *Nitrosopumilus* sp.) was present in 37 of the 42 host individuals (22 of 25 host species) at relative abundances up to 95% (*Lissoclinum badium*), while extremely rare in ambient seawater (0–0.04%). In addition, a common archaeal symbiont in *Leptoclinides madara* (OTU0025, 17–28% relative abundance) was classified to the genus *Cenarchaeum* and closely matched (98% sequence identity) an uncultivated archaeon reported in the marine

Table 1 Taxonomic classification of ascidian hosts and sequence data summary for ascidian and seawater samples. Total values in bold refer to summed reads and unique OTUs

Species	*Order*	*Family*	*Total*		*Archaea*		*Bacteria*	
			Reads	*$OTU_{0.03}$*	*Reads*	*$OTU_{0.03}$*	*Reads*	*$OTU_{0.03}$*
Clavelina arafurensis	Aplousobranchia	Clavelinidae	490	190	57	4	433	186
Clavelina meridionalis			249	103	15	8	234	95
Clavelina meridionalis			1207	333	44	10	1163	323
Clavelina meridionalis			1023	411	38	11	985	400
Pycnoclavella sp.			1449	313	93	11	1356	302
Pycnoclavella sp.			116	47	3	3	113	44
Pycnoclavella diminuta			2040	384	294	9	1746	375
Pycnoclavella diminuta			1188	301	434	9	754	292
Pycnoclavella diminuta			347	167	66	6	281	161
Didemnum cf. *albopunctatum*		Didemnidae	3654	154	906	12	2748	142
Didemnum cf. *granulatum*			386	22	11	4	375	18
Didemnum multispirale			3035	102	10	2	3025	100
Didemnum multispirale			2799	142	21	3	2778	139
Didemnum multispirale			2979	209	25	6	2954	203
Didemnum sp.1			6905	486	255	6	6650	480
Didemnum sp.2			2684	448	762	12	1922	436
Leptoclinides madara			979	74	165	2	814	72
Leptoclinides madara			281	18	79	2	202	16
Lissoclinum badium			3224	27	3055	4	169	23
Lissoclinum badium			4670	29	4464	4	206	25
Lissoclinum cf. *capsulatum*			598	36	2	1	596	35
Lissoclinum patella			2489	86	1	1	2488	85
Eudistoma amplum		Polycitoridae	517	164	177	16	340	148
Eudistoma amplum			444	175	89	13	355	162
Eudistoma amplum			825	286	112	11	713	275
Polycitor giganteus			1602	95	6	3	1596	92
Aplidium protectans		Polyclinidae	4272	129	30	3	4242	126
Aplidium sp.			1968	176	64	7	1904	169
Synoicum castellatum			3846	382	4	2	3842	380
Synoicum castellatum			6447	344	60	3	6387	341
Synoicum castellatum			120	46	27	4	93	42
Phallusia arabica	Phlebobranchia	Ascidiidae	105	23	2	1	103	22
Phallusia arabica			338	53	39	8	299	45
Phallusia arabica			54	17	2	2	52	15
Phallusia julinea			562	97	55	4	507	93
Phallusia philippinensis			28	8	12	1	16	7
Ecteinascidia diaphanis		Perophoridae	1168	344	17	4	1151	340
Perophora aff. *modificata*			1541	189	184	9	1357	180
Polycarpa argentata	Stolidobranchia	Styelidae	561	68	446	7	115	61
Polycarpa aurata			449	18	0	0	449	18
Polycarpa aurata			159	23	2	2	157	21
Polycarpa aurata			28	8	0	0	28	8
Ascidian Microbiota Total =			**67 826**	**3321**	**12 128**	**104**	**55 698**	**3217**
Filtered Seawater	n.a.	n.a.	9 573	221	289	24	9 284	197
Filtered Seawater	n.a.	n.a.	14 441	248	134	21	14 307	227
Filtered Seawater	n.a.	n.a.	2 797	129	3	3	2 794	126
Ambient Seawater Total =			**26 811**	**385**	**426**	**26**	**26 385**	**359**
Grand Total =			**94 637**	**3604**	**12 554**	**124**	**82 083**	**3480**

Table 2 Intra-specific variation in the ascidian microbiota highlighting the shared components (i.e., present in all host individuals) of each species' microbiota

Species	*Species Cluster*	*No. Samples*	*Total Sequences*	*Total $OTU_{0.03}$*	*Shared Sequences (%)*	*Shared $OTU_{0.03}$ (%)*
Clavelina meridionalis	Y	3	2479	697	1338 (54)	26 (4)
Pycnoclavella sp.	N	2	1565	341	1077 (69)	19 (6)
Pycnoclavella diminuta	N	3	3575	673	1731 (48)	35 (5)
Didemnum multispirale	Y	3	8813	367	6192 (70)	24 (6)
Leptoclinides madara	Y	2	1260	81	1116 (89)	11 (14)
Lissoclinum badium	Y	2	7894	41	7848 (99)	15 (37)
Eudistoma amplum	Y	3	1786	491	809 (45)	31 (6)
Synoicum castellatum	N	3	10 413	620	5237 (50)	17 (3)
Phallusia arabica	N	3	497	82	104 (21)	2 (2)
Polycarpa aurata	N	3	636	39	514 (81)	3 (8)

Species cluster refers to Figure 2.

sponge *Axinella verrucosa* (GenBank accession number AF420237).

Specificity of the ascidian microbiota

Comparison of the rich ascidian microbiota with ambient seawater microbes revealed low overlap between free-living and host-associated microbial communities. A total of 283 OTUs were present in the seawater communities and absent from the ascidian microbiota, while 102 OTUs were present in both ascidian and seawater samples, representing only 3% of total $OTU_{0.03}$ diversity in the ascidian

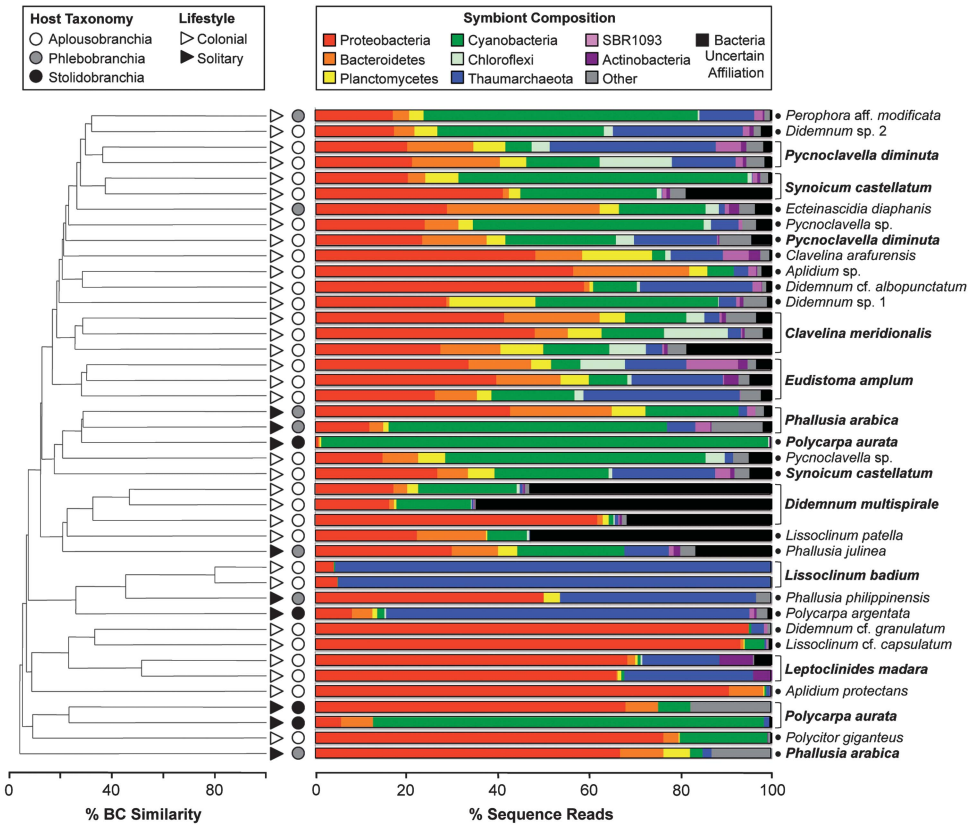

Figure 2 Microbial community similarity and composition in 42 samples of GBR ascidians. Dendrogram (*left*) based on Bray-Curtis (BC) similarity of microbial communities in ascidian hosts. Ordinal classifications of ascidians hosts are shown as circles, Aplousobranchia (*white*), Phlebobranchia (*gray*) and Stolidobranchia (*black*) and zooid organization as triangles, colonial (*white*) and solitary (*black*). Bar charts (*right*) show the relative abundance of microbial phyla in each host ascidian, with host species names listed on the right. *Bold* names indicate species with replicate samples.

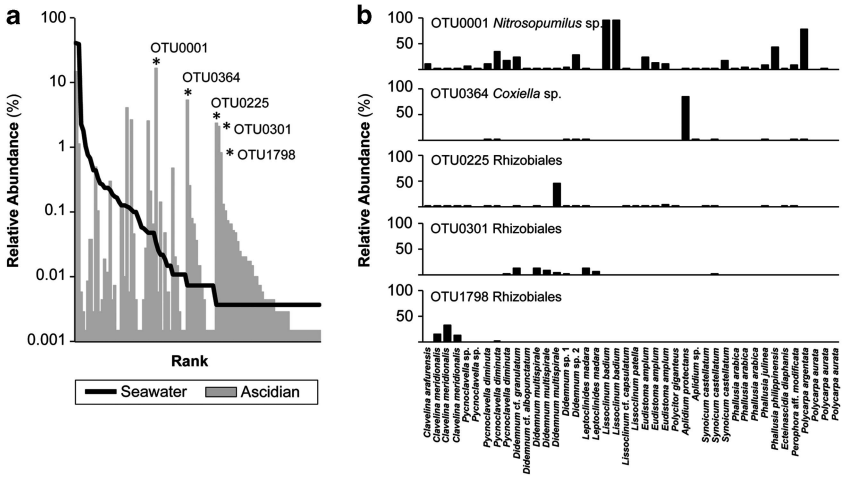

Figure 3 Relative abundance of seawater microbes in the ascidian microbiota. (**a**) Rank-abundance plots showing the relative abundance of 102 microbial OTUs present in both seawater (*black line*) and ascidian hosts (*gray bars*). Asterisks denote OTUs > 200 times more abundant in ascidian hosts than seawater. (**b**) Classification and relative abundance of 5 rare seawater biosphere OTUs among ascidian hosts.

microbiota. Further, over one-third ($n = 40$) of these shared microbial OTUs exhibited greater than an order of magnitude difference in relative abundance in seawater and ascidians assemblages, including 5 OTUs that were 200x to 700x more abundant in host ascidians (Figure 3). For example, OTU0001 (*Nitrosopumilus* sp.) accounted for 17% of sequence reads from the ascidian microbiota. The remaining 4

OTUs were specific to particular host families (e.g., OTU0301 in Didemnidae), species (e.g., OTU1798 in 3 individuals of *Clavelina meridionalis*) or individuals (e.g., OTU0225 in 1 of 3 *Didemnum multispirale* individuals) and rare or absent in most ascidian hosts (Figure 3).

Additional analysis of abundant components of the ascidian microbiota revealed symbiont overlap between ascidians and other invertebrate hosts, as well as a unique component of the ascidian microbiota (Table 3). A total of 56 microbial OTUs accounted for 78% of sequences obtained from ascidian hosts. Over two-thirds of these OTUs ($n=38$) matched closely (>97% sequence identity) to previously characterized sequences (Table 3), most commonly derived from seawater ($n=14$), corals ($n=9$), sponges ($n=6$) and sediment ($n=3$). In some cases, OTUs that were widespread among ascidians hosts and in the rare biosphere of seawater matched closely to other invertebrate-associated sequences. For example, OTU0264 (*Bacteroidetes*, *Flavobacteriaceae*) was present in 24 ascidian individuals, was rare in seawater (<0.05% relative abundance) and matched identically to coral-derived sequences from Caribbean (*Montastraea faveolata*) and Indo-Pacific (*Montipora aequituberculata*) stony corals and an Indo-Pacific soft coral (*Sinularia* sp.). The remaining 18 OTUs exhibited

Table 3 Abundant OTUs in the ascidian microbiota, showing their representation in ascidian (ASC) and seawater (SW) datasets, number of host species, closest known relative and taxonomic classification

OTU	*Reads (ASC)*	*Hosts (ASC)*	*Reads (SW)*	*BLAST Match Source (Identity, Acc. No.)*	*Phylum*	*Lowest Taxonomic Rank*
0001	11338	39	9	Sponge (98.3, AF420237)	Thaumarchaeota	G. *Nitrosopumilus*
0140	9981	42	11105	Seawater (100, GU119217)	Cyanobacteria	G. *Prochlorococcus*
0287	4964	11	0	Bivalve (92.9, EU857739)	Unclassified	K. Bacteria
0364	3669	13	13	Sponge (100, HQ241801)	γ-proteobacteria	G. *Coxiella*
0188	2836	30	33	Seawater (100, HQ338142)	α-proteobacteria	F. Rhodobacteraceae
0292	1836	34	30	Seawater (100, GU119442)	Cyanobacteria	G. *Prochlorococcus*
0189	1790	28	13	Seawater (100, JF514245)	α-proteobacteria	G. *Mesorhizobium*
0225	1633	25	1	Seawater (100, JF769651)	α-proteobacteria	O. Rhizobiales
0301	1432	10	1	Ascidian (100, DQ860066)	α-proteobacteria	O. Rhizobiales
1128	1346	3	0	Seafloor Lava (93.2, EU491218)	Proteobacteria	P. Proteobacteria
1129	985	2	0	Sediment (88.7, GU046335)	Unclassified	K. Bacteria
0851	858	5	0	Sponge (94.1, EU883386)	α-proteobacteria	O. Rhodospirillales
0310	779	32	10685	Seawater (100, JN547429)	Cyanobacteria	G. *Prochlorococcus*
1063	671	3	0	Soil (97.9, JQ059148)	α-proteobacteria	F. Rhodospirillaceae
1798	567	5	1	Seawater (95.8, HQ715140)	α-proteobacteria	O. Rhizobiales
1379	379	5	0	Soil (90.4, GQ127925)	Unclassified	K. Bacteria
3180	354	1	0	Sediment (95.4, AB374687)	Bacteroidetes	F. Flammeovirgaceae
1101	336	16	118	Seawater (100, AB540006)	Bacteroidetes	F. Flavobacteriaceae
0355	333	25	0	Coral (100, FJ809316)	SBR1093	C. VHS-B5-50
0862	329	16	0	Sponge (100, EU335078)	Chloroflexi	C. Anaerolineae
0931	327	13	0	Algae (96.7, HM474939)	Chloroflexi	C. Anaerolineae
0164	326	30	3	Seawater (100, GU119490)	Planctomycetes	O. Pirellulales
1032	326	3	0	Sediment (96.6, JQ989595)	α-proteobacteria	O. Rhizobiales
0866	324	22	0	Coral (100, DQ416621)	Bacteroidetes	F. Flavobacteriaceae
0293	261	11	0	Sponge (100, FJ625530)	Planctomycetes	O. Pirellulales
2687	260	2	0	Biofilm (94.6, FJ901434)	Cyanobacteria	F. Phormidiaceae
0296	246	2	0	Sediment (96.7, JN977252)	γ-proteobacteria	C. γ-proteobacteria
0273	245	15	0	Coral (99.6, JQ347330)	Cyanobacteria	F. Pseudanabaenaceae
0025	241	2	0	Sponge (98.3, AF420237)	Thaumarchaeota	G. *Cenarchaeum*
0875	211	3	0	Coral (97.1, FJ425620)	Bacteroidetes	F. Flammeovirgaceae
0003	208	19	0	Cyanobacteria (100, JX197041)	Thaumarchaeota	G. *Nitrosopumilus*
0335	206	26	59	Seawater (100, EU592360)	α-proteobacteria	F. Rhodobacteraceae
0300	202	4	0	Sponge (100, JN128259)	γ-proteobacteria	G. *Microbulbifer*
0344	198	9	0	Sponge (100, DQ097259)	α-proteobacteria	G. *Pseudovibrio*
2656	193	3	0	Diatom Bloom (94.4, EU734047)	β-proteobacteria	C. β-proteobacteria
0318	186	20	0	Coral (100, FJ489710)	SBR1093	C. EC214
2229	183	3	0	Seawater (98.3, HM798908)	α-proteobacteria	F. Rhodospirillaceae
0187	179	17	2	Seawater (100, HM103531)	α-proteobacteria	F. Rhodobacteraceae
0161	165	19	0	Sediment (100, GQ249478)	γ-proteobacteria	F. Chromatiaceae
0306	157	16	0	Coral (100, FJ203575)	α-proteobacteria	F. Hyphomicrobiaceae
0850	153	3	0	Biofilm (98.7, DQ167245)	α-proteobacteria	G. *Kiloniella*
2389	152	3	0	Coral (95.8, EF206859)	γ-proteobacteria	C. γ-proteobacteria
0133	147	20	0	Coral (100, GU118991)	Bacteroidetes	F. Flammeovirgaceae
0264	147	24	13	Coral (100, FJ809398)	Bacteroidetes	F. Flavobacteriaceae
0294	145	3	0	Algae (99.6, GU451475)	α-proteobacteria	G. *Pseudovibrio*
1065	143	4	0	Coral (93.2, GU118840)	α-proteobacteria	O. Rhodospirillales
0186	138	17	0	Sediment (99.6, FJ358900)	Bacteroidetes	F. Flammeovirgaceae
0307	137	13	0	Algae (99.6, HM474882)	α-proteobacteria	F. Rhodospirillaceae
2811	137	2	0	Seawater (94.5, EF572701)	Bacteroidetes	F. Flavobacteriaceae
0297	132	12	0	Coral (99.6, FJ203345)	Planctomycetes	O. Pirellulales
0939	130	13	0	Sediment (100, DQ256661)	Cyanobacteria	G. *Leptolyngbya*
2749	124	1	0	Sediment (96.2, EU287328)	α-proteobacteria	O. Rhizobiales
0686	121	7	0	Bivalve (92.5, EU857738)	Unclassified	K. Bacteria
2875	117	1	0	Seawater (98.3, JN216763)	α-proteobacteria	C. α-proteobacteria
1132	107	1	0	Mammal Gut (89.2, EU459272)	Unclassified	K. Bacteria
0172	101	4	0	Seawater (99.2, GQ349494)	δ-proteobacteria	G. *Nitrospina*

greater divergence from both free-living and host-associated microbes, including 11 OTUs that exhibited <95% sequence identity to known microbial sequences (Table 3).

Core, variable and specific microbial OTUs

Comparison of the microbial communities among ascidian hosts revealed a high degree of host specificity in the ascidian microbiota and the presence of a small number of very abundant and widespread microbial OTUs. No universal symbiont OTUs (i.e., present in all host species) were detected and core OTUs (present in >70% of host species) were represented by 7 OTUs at high relative abundance, accounting for 40% of all sequence reads. These OTUs corresponded to 2 *Prochlorococcus* sp. (*Cyanobacteria*; OTU0140, OTU0310) that were also common in seawater communities (41 and 40% relative abundance, respectively), as well as *Nitrosopumilus* sp. (*Thaumarchaeota*; OTU0001), *Prochlorococcus* sp. (*Cyanobacteria*; OTU0292), Rhodobacteraceae sp. (*Alphaproteobacteria*; OTU0188), Pirellulales sp. (*Planctomycetes*; OTU0164) and an OTU from the candidate phylum SBR1093 (OTU0355) that were rare (0.01–0.12% relative abundance) or absent in seawater samples. Variable OTUs (present in at least 2 host species) were represented by 950 OTUs and accounted for 49% of sequence reads, while specific OTUs (present in a single host species) were represented by 2364 OTUs and accounted for 11% of sequence reads.

Community-level analysis of tunic-associated microbes among ascidian species revealed a significant correlation between host relatedness (18S rRNA sequence similarity) and symbiont community similarity (Mantel test, $r=0.37$, $P<0.001$). This relationship was maintained when replicate samples were removed ($r=0.28$, $P<0.001$) and when using sub-sampled sequence pools to standardize sampling effort ($r=0.50$, $P<0.001$), indicating that high symbiont similarity among individuals of the same species and sampling artifacts were not the sole drivers of the observed correlation. Indeed, while symbiont communities were consistent across replicate individuals for 5 colonial ascidian species, other host species exhibited high intra-specific variability among replicates, including two solitary and three colonial species (Table 2). The lowest intra-specific diversity in symbiont structure was seen in *Lissoclinum badium*, where shared symbionts accounted for 37% of $OTU_{0.03}$ diversity and 99% of sequence reads. The highest intra-specific diversity was seen in *Phallusia arabica*, where shared symbionts only accounted for 2% of $OTU_{0.03}$ diversity and 21% of sequence reads (Table 2). Symbiont communities did not strictly cluster by higher-level host taxonomy (order to genus-level) or lifestyle (solitary or colonial; Figure 2), likely obscured by the observed variability in symbiont specificity among hosts.

Bacterial ultrastructure in the ascidian tunic

Transmission electron microscopy examination of the solitary ascidians *Phallusia julinea* and *Polycarpa aurata* revealed randomly distributed and extremely rare bacterial cells in the inner tunic of these two species. All bacterial morphotypes observed in *P. julinea* were ovoid to rod-shaped cells (ca. 0.4 μm × 2 μm; Supplementary Figure S7A), while ovoid cells (ca. 0.12 μm), cyanobacteria (ca. 0.15 μm, with ca. 5 thylakoids evenly spaced along the periphery of the cell), and a spiral bacterium (Supplementary Figure S7B) were observed in *P. aurata*. Colonial ascidians were characterized by a higher number of bacteria in their tunic. *Pycnoclavella* sp. featured groups of 2 to 5 cyanobacteria encased in a network of fibers (Supplementary Figure S7C). Both clavelinids (*Pycnoclavella* sp. and *C. meridionalis*) contained ovoid-shaped bacteria often surrounded by irregular inclusions spread throughout the tunic (Supplementary Figure S7D). In *Lissoclinum badium* and *Synoicum castellatum*, all bacterial cells were ovoid or rod-shaped (ca. 0.5 μm × 2 μm, and ca. 0.3 μm × 1 μm, respectively) and observed either in isolation or forming small groups of 2–6 bacteria in close proximity to ascidian cells (Supplementary Figure S7E and S7F, respectively).

Discussion

Bacterial biodiversity hotspots in the ascidian tunic

In this study, we provide the most comprehensive characterization of the ascidian microbiota to date and reveal exceptional bacterial biodiversity inhabiting the tunic of GBR ascidians. Encompassing 3321 unique $OTU_{0.03}$ from 19 described bacterial phyla, 14 candidate bacterial phyla and 3 described archaeal phyla, the ascidian microbiota exhibited comparable diversity to the rich microbiota associated with marine sponges (Schmitt *et al.*, 2012) and corals (Sunagawa *et al.*, 2010) and indicates that the ascidian tunic represents a previously unrecognized hotspot for marine microbial diversity. Visualization of microbial cells by transmission electron microscopy confirmed the presence of microbes in the ascidian tunic and was consistent with results from 16S rRNA gene tag pyrosequencing, for example, the prevalence of cyanobacterial OTUs (>50% of sequence reads) and cyanobacterial cells encased in a fiber network in *Pycnoclavella* sp. and the detection of a *Spirochaetes* OTU (18% relative abundance) and a bacterium with spiral morphology in *Polycarpa aurata*.

Phylum-level composition of the ascidian microbiota retrieved herein was similar to what has been described for other ascidian species and was comprised of mostly *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* (Martínez-García *et al.*, 2007; Tait *et al.*, 2007; Behrendt *et al.*, 2012). Moreover, as found for other tropical ascidians (e.g., Behrendt

et al., 2012), *Cyanobacteria* were particularly abundant in most of our ascidian samples. In addition, the ascidian microbiota demonstrated some overlap with other host-associated microbial communities yet clear distinction from ambient planktonic communities in coral reef seawater, except for the widespread presence of *Cyanobacteria* from the *Prochlorococcus* genus. Consistently, previous studies have noted multiple shared symbiont lineages among microbiota of sponges and corals (Taylor *et al.*, 2007; Simister *et al.*, 2012), indicating microbial lineages adapted to host-associated lifestyles may disperse among disparate host organisms. However, the ascidian microbiota also maintained distinguishing characteristics in comparison to other host-associated communities. For example, the phylum *Planctomycetes* exhibited high diversity in ascidian hosts, whereas members of this phylum are typically rare in microbiota of sponge (Schmitt *et al.*, 2012; Webster and Taylor, 2012) and coral hosts (Sunagawa *et al.*, 2010; Barott *et al.*, 2011). Further, 11 of the 56 most common OTUs in the ascidian microbiota exhibited high sequence divergence (>5%) from any previously described marine microbe. The unique niches inside invertebrate tissues are becoming recognized hotspots for microbial biodiversity and our results suggest that ascidian tunics offer a similarly fertile habitat for marine microorganisms.

Rare seawater microbes enriched in the ascidian tunic

The vast majority of OTUs in the ascidian microbiota were not present in planktonic communities. However, a cautionary note is necessary here as seawater samples were collected in only one of our sampling sites and at one given time (October 2011), while ascidian samples were collected from different locations (separated by less than 120 km) and times (May through November 2011; Supplementary Table S1). Microbes in seawater are known to vary seasonally, occur in patches or be stratified according to their microenvironmental requirements or to microscale turbulences (e.g., Giovannoni and Stingl, 2005). Accordingly, the low number of shared OTUs (3%) between the seawater and the ascidian samples may be partly due to an insufficient sampling of the surrounding seawater.

Nevertheless, we found that several microbes from the rare biosphere of seawater exhibited high relative abundance in ascidian-associated communities. Five microbial OTUs exhibited 200 to 700 times higher relative abundance in the ascidian tunic than in the plankton, suggesting the selective enrichment of rare seawater microbes in ascidian hosts as observed for the microbiota in marine sponges (Webster *et al.*, 2010; Taylor *et al.*, 2013) and reef-building corals (Sunagawa *et al.*, 2010). Notably, 3 of the 5 OTUs enriched in the ascidian microbiota were classified to the order Rhizobiales, a lineage of *Alphaproteobacteria* well known for their nitrogen-fixation capacity and mutualistic relationships with terrestrial plants (Lodwig *et al.*, 2003) and more recently documented as dominant nitrogen-fixing symbionts in the coral microbiome (Lema *et al.*, 2012). In this study, a total of 176 OTUs affiliated with Rhizobiales were present in the ascidian microbiota and detected in all 25 ascidian host species prompting further study of nitrogen-fixing bacteria in the ascidian microbiota and their potential contribution to nitrogen cycles in the ascidian holobiont. These results also indicate the potential for horizontal symbiont transfer among hosts with the rare biosphere of seawater acting as a conduit among host habitats.

Host specificity of the ascidian microbiota

The vast majority of symbiont OTUs (71%) were present in a single host species and absent in seawater, indicating a high degree of host specificity in the microbiota of coral reef ascidians. Indeed, no universal symbionts (i.e., present in all ascidian hosts) occurred and only 7 core OTUs (of 3321 total OTUs) were detected. While few 16S rRNA gene sequence datasets from ascidians are available for comparative analyses, several OTUs exhibited specific associations with particular host taxa across a broad geographic range. For example, OTU3073 from *Ecteinascidia diaphanis* matched to the candidate genus *Endoecteinascidia*, a distinct lineage of *Gammaproteobacteria* described solely from ascidians in the genus *Ecteinascidia*, including *E. turbinata* from the Mediterranean (Moss *et al.*, 2003) and Caribbean (Pérez-Matos *et al.*, 2007). The detection of this candidate genus from a GBR ascidian expands the known geographic range of this symbiont taxon and further supports its specificity to the host genus *Ecteinascidia*. In addition, this symbiont lineage is particularly notable for its putative role in secondary metabolite synthesis within the animal cell, including the production of the anticancer agent ET-743 (Rath *et al.*, 2011), which may constitute a key functional aspect of ascidian-bacterial symbioses (Kwan *et al.*, 2012).

Even among replicate individuals of the same ascidian species, some intra-specific variability was observed. Consistent microbial community structure was observed in 5 of the 10 ascidian species where multiple individuals were analyzed, while the remaining half exhibited greater similarity to the microbiota of unrelated species than to conspecific hosts, suggesting a non-obligate symbiosis. These results suggest different factors structuring the symbiont communities in different ascidian species, with more homogenous communities potentially maintained in some hosts by vertical symbiont transmission or specific functional requirements and more heterogeneous communities in other hosts determined by more stochastic or dynamic factors. This observation is in agreement with mounting evidence suggesting that colonial ascidians, such as

the Didemnidae, establish stable symbiotic microbial associations that are vertically transmitted (Kott, 1980, 1982, 2001; Hirose, 2000; Schuett *et al.*, 2005; Hirose *et al.*, 2006a, b; Hirose and Hirose, 2007; Bright and Bulgheresi, 2010; López-Legentil *et al.*, 2011; Kojima and Hirose, 2012), while others, such as solitary ascidians may selectively acquire symbionts from the surrounding seawater (Erwin *et al.*, 2013).

Widespread ammonia-oxidizing archaea (AOA) in the ascidian microbiota

Nitrification is a key process in the global nitrogen cycle that results in the conversion of ammonia to nitrite (ammonia-oxidation) and nitrite to nitrate (nitrite-oxidation), a two-step process mediated solely by prokaryotic organisms (Ward *et al.*, 2007). The archaeal component of the ascidian microbiota was notably comprised of lineages with known ammonia-oxidization capabilities. In particular, sequences affiliated with the genus *Nitrosopumilus* dominated the archaeal communities in GBR ascidians and several *Nitrosopumilus* OTUs exhibited a widespread distribution among hosts and high relative abundance within hosts. In coral reef waters, observations of high nitrite/nitrate concentrations compared to adjacent, open water habitats have long suggested active nitrification among reef-associated microbes (Webb *et al.*, 1975). More recent studies have reported that host-associated microbes in sponges and corals contributed to nitrification in these reef habitats to a larger extent than reported for free-living communities in sediments and seawater (Diaz and Ward, 1997; Southwell *et al.*, 2008). The finding herein of widespread ammonia-oxidizing archaea in coral reef ascidians suggests an additional and potentially important source of nitrification in reef habitats.

In fact, the most dominant of all OTUs in the ascidian microbiota (17% of total reads) was classified in the genus *Nitrosopumilus* and matched nearly identically (>99% sequence identity) to a symbiotic ammonia-oxidizing archaea (AOA) previously described in the Mediterranean ascidian *Cystodytes dellechiajei*, where active nitrification was detected in the tunic layer (Martínez-García *et al.*, 2008). Another OTU recovered from two individuals of the ascidian *Leptoclinides madara* at high relative abundance (17–28%) was classified in the genus *Cenarchaeum*, a candidate taxon erected for the sponge-associated symbiont *Cenarchaeum symbiosum* (Preston *et al.*, 1996) whose genome includes homologues of genes associated with chemolithotrophic ammonia oxidation (Hallam *et al.*, 2006). Finally, some ascidians (e.g., *Lissoclinum badium*) hosted *Nitrospina* symbionts, a genus of *Deltaproteobacteria* whose members are capable of nitrite-oxidation, in addition to dominant AOA lineages, suggesting that the complete nitrification process may occur in the ascidian tunic of at least some species.

Ammonia is the primary form of nitrogenous waste produced by ascidians (Goodbody, 1974) and may be recycled *via* uptake or oxidation by resident microbes. For example, the widespread AOA reported herein may utilize the ammonia-rich waste products of their host ascidians as substrate for nitrification reactions. Indeed, nitrifying microbes require not only a reduced form of inorganic nitrogen, but also high oxygen and low irradiance levels, as marine AOA are particularly susceptible to photoinhibition at higher irradiance levels (Merbt *et al.*, 2012). Thus, the ascidian tunic habitat not only satisfies the ammonia and oxygen requirements of AOA (Kühl *et al.*, 2012), but may also shelter these populations from the high irradiance levels characteristic of shallow water reefs (e.g., Vermeij and Bak, 2002) and represent important habitats for nitrite/nitrate regeneration in coral reef environments. Further, the dynamic chemical landscapes in and around ascidians (Behrendt *et al.*, 2012, Kühl *et al.*, 2012) may offer periodic windows of optimal conditions for additional metabolic pathways and maintain the complex microbiota observed in ascidian tunics.

While the taxonomic scope of the ascidian species examined herein was broad, the geographic scope was restricted to shallow water habitats of the GBR. Yet even within this single biome, our results show a remarkably rich and diverse microbial community associated with coral reef ascidians. Given the broad distribution of ascidians in the marine environment, (Lambert, 2005) expanded efforts to document the diversity of the ascidian microbiota will continue to clarify the role of ascidians as habitats for novel microbial communities and their importance for microbial-mediated processes in marine biogeochemical cycles.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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