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Down Under the Tunic: Bacterial Biodiversity Hotspots and Widespread Ammonia-Oxidizing Archaea in Coral Reef Ascidians

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Running Title: Structure and Function of the Ascidian Microbiota

Abstract

Ascidians are ecologically important components of marine ecosystems globally yet the ascidian microbiota remains largely unexplored beyond a few model species. In this study, 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 $OTU_{0.03}$ 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 microbiota, suggesting that the rare biosphere of seawater may act as a conduit for horizontal symbiont transfer among hosts. However, most OTUs (71.2%) 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 and steep physico-chemical gradients 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 marine microorganisms and may represent an important habitat for nitrite/nitrate regeneration in coral reef ecosystems.

Introduction

Symbiotic microbial communities are a common feature of sessile 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). Underlying the phylogenetic diversity of symbiotic microbes are 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 dimethylsulfoniopionate (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 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. The application of metagenomic approaches to studying symbiotic

function has recently revealed that sponge symbionts are also responsible for the synthesis of vitamin B1, which animals need to obtain from their diet (Fan *et al*., 2013). 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, 1998, 2006, 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 (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; Tait *et al*., 2007; Münchhoff *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 (FISH) 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 recovered by traditional sequencing techniques (Roesch *et al*., 2007; Huber *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 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 -80ºC, 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 -80ºC.

DNA Extraction

Frozen ascidian tissues (approximately 0.5 g per sample) were thawed, dissected under the binocular into inner tunic and zooid fractions and aseptically transferred to 1.5 ml Eppendorf tubes using sterile scalpels and tweezers. Inner tunic (i.e., beneath the surface

146 layer) was chosen to avoid epibionts and ambient seawater microbes. These tunic samples
147 were processed for microbial analysis, while zooids were processed for barcoding each
148 ascidian specimen. DNA extraction was conducted separately for inner tunic and zooid tissue
149 fractions with the Power Plant® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA)
150 following the manufacturer's protocol. DNA extraction from concentrated seawater samples
151 (filters) was performed by the addition of 1.8 ml lysis buffer (40 mM EDTA, 50 mM Tris and
152 0.75 M sucrose) and 200 µl of Lysozyme (10 mg/ml), incubation at 37ºC for 45 min, the
153 addition of 40 µl of Proteinase K (10 µg of Proteinase K in 1 ml of 10% SDS) and incubation
154 at 55ºC for 1 h. Lysates were transferred to sterile Eppendorf tubes and DNA was extracted
155 using standard phenol:chloroform procedures and resuspended in 20 µl of distilled water.
156
157 Identification and Barcoding of Host Ascidians
158 Ascidian samples were assigned to the lowest taxonomic group possible based on
159 morphological examination (Supplementary Text S1). Genetic identification was also
160 performed using the mitochondrial gene cytochrome oxidase subunit I (COI) and 18S rRNA
161 gene sequences. Both gene regions are commonly used to determine species boundaries and
162 diversity among ascidian taxa (Tarjuelo *et al*., 2004; López-Legentil and Turon, 2005;
163 Yokobori *et al*., 2006; Pérez-Portela *et al*., 2009) and COI is the metazoan standard for the
164 Barcode of Life Project (www.barcodeoflife.org).
165 DNA extractions from zooid tissue were used as templates for PCR amplification of a
166 519 to 621 bp fragment of COI to barcode host ascidian species. Total PCR reaction volume
167 was 50 µL, including 10 µL of 5xBuffer, 0.4 µL of bovine serum albumin (BSA; 10 mg/ml),
168 0.25 µL of My *Taq* DNA Polymerase (Bioline®, London, United Kingdom), 2 µL of each
169 primer (10µM), and 1µL of template DNA. Two sets of primer pairs were used for *COI*
170 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 94ºC for 2 min; 30 cycles of 94 ºC for 45 s, 50 ºC for 45 s and 72ºC for 50 s; and a final elongation step at 72 ºC for 5 min. PCR conditions for amplification with ascidian-specific primers were: an initial denaturing step of 94 ºC for 1 min; 60 cycles of 94 ºC for 10 s, 50 ºC for 30 s, and 72 ºC for 50 s; and a final elongation step at 72 ºC for 10 min. PCR products were purified and bi-directionally sequenced at Macrogen, Inc. (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 were used as templates for PCR amplification of a ca. 466 bp fragment of the 16S rRNA gene using the primer set pyro926F (5′-AAA CTY AAA KGA ATT GRC GG-3′) and pyro1392R (5′-ACG GGC GGT GTG TRC-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. (South Korea) for purification, amplicon library construction and massively parallel 16S rRNA gene tag pyrosequencing using the Roche 454 GS-FLX Titanium system. Pyrosequencing data were deposited as flowgrams (sff file) in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRA056317.

195 Sequence data were processed with stringent filtering and screening criteria to
196 minimize the occurrence of spurious sequences and overestimation of microbial diversity
197 (Huse *et al*., 2010; Schloss *et al*., 2011), using the mothur software package (Schloss *et al*.,
198 2009), as detailed previously (Erwin *et al*., 2013). Briefly, adaptor, MID and primer
199 sequences were removed from raw sequences and the dataset de-noised (removal of reads
200 with ambiguous base calls, long homopolymers and barcode or primer mismatches) and
201 quality filtered (removal of short sequences and low quality reads). Non-target sequences (e.g.
202 eukaryotic 18S rRNA, mitochondria, chloroplast) were removed using Metaxa v1.1
203 (Bengtsson *et al*., 2011), resulting in a dataset consisting solely of archaeal and bacterial 16S
204 rRNA gene sequences. These sequences were aligned to the Greengenes database, trimmed to
205 an overlapping alignment space (449 bp) and putatively chimeric sequences were removed
206 (UChime; Edgar *et al*., 2011).
207
208 Data Analysis
209 High quality sequences (n = 94,637) were assigned to taxonomic groups based on the
210 improved Greengenes taxonomy template (McDonald *et al*., 2012) with *Thaumarchaeota*
211 elevated to the rank of phylum (Brochier-Armanet *et al*., 2008, Spang *et al*., 2010), grouped
212 into operational taxonomic units ($OTU_{0.03}$) based on 97% sequence similarity, and the
213 taxonomic assignment of each $OTU_{0.03}$ was constructed by majority consensus (Schloss and
214 Westcott, 2011).
215 Sampling coverage and expected total OTU diversity were calculated using
216 rarefaction analysis and the bootstrap estimator (Smith and Van Belle, 1984) at six different
217 OTU definitions corresponding approximately to the species ($OTU_{0.03}$), genus ($OTU_{0.05}$),
218 family ($OTU_{0.10}$), order ($OTU_{0.15}$), class ($OTU_{0.20}$) and phylum ($OTU_{0.25}$) levels (97%, 95%,
219 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 2,000 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 hosts), variable (present in at least two hosts) and specific (present in a single host) 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 (TEM). 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). TEM observations were conducted on a JEOL JEM-1010 (Tokyo, Japan) electron microscope coupled with an Orius CDD camera (Gatan, Germany).

Results

245 Diversity and Phylogeny of Ascidian Hosts

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

256

257 Richness and Diversity of the Ascidian Microbiota

258 Collective analysis of 16S rRNA sequence reads derived from ascidian hosts (n =
259 67,826) revealed a remarkable richness and diversity of microbial communities associated
260 with Great Barrier Reef ascidians. A total of 3,321 microbial $OTU_{0.03}$ represented the
261 combined GBR ascidian microbiome and corresponded to 19 described bacterial phyla, 14
262 candidate bacterial phyla and 3 described archaeal phyla (Figure 1). This increases the
263 taxonomic diversity known to inhabit ascidians by 14 microbial phyla. Coverage estimates of
264 total diversity sampled were high across all taxonomic levels, ranging from 81.7% ($OTU_{0.03}$)
265 to 85.4% ($OTU_{0.25}$). Rarefaction analysis revealed that observed OTU diversity was
266 approaching expected OTU diversity at higher level taxonomic rankings (e.g., phylum and
267 class) while additional sampling would continue to uncover new microbial OTUs at lower
268 taxonomic levels (e.g., genus and species; Supplementary Figure S6) due to a rich rare
269 component of the microbiota (1,817 singletons).

Analyses of individual hosts and ascidian species revealed up to 486 microbial $OTU_{0.03}$ per individual and 697 $OTU_{0.03}$ per species, with many ascidians hosting more diverse microbial communities than those recovered from ambient seawater (Tables 1 and 2). 16S rRNA sequence reads derived from seawater (n = 26,811) grouped into 385 $OTU_{0.03}$ (129 to 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 to 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 Great Barrier Reef ascidians were composed of diverse bacterial phyla and archaeal lineages (Figure 1, Supplementary Table S3). Bacterial OTUs dominated the ascidian microbiota, accounting for 96.9% (n = 3,217) of $OTU_{0.03}$ diversity and 82.1% of all sequence reads (n = 55,698). The most dominant bacterial phylum was *Proteobacteria*, representing over one-third (37.7%) of $OTU_{0.03}$ diversity (n = 1,251) 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 14.6% of

295 $OTU_{0.03}$ diversity (*n* = 496 and 486, respectively, Figure 1) and detected in the majority
296 (>88%) of ascidian hosts (Figure 2, Supplementary Table S3).

297 *Cyanobacteria* was the fourth most diverse phyla associated with ascidians (172
298 OTUs, 5.2% of $OTU_{0.03}$ diversity) and included the genus *Procholoron*, present only in
299 *Lissoclinum patella* (OTU0810), and 4 OTUs that were closely related (95-98% sequence
300 identity) to the recently described *Candidatus Acaryochloris bahamiensis* (López-Legentil *et*
301 *al*., 2011). Most notably, two *Acaryochloris* OTUs (OTU0125, 0126) were common in all 3
302 individuals of the host *Eudistoma amplum* (0.7 to 8.9% relative abundance). An additional 5
303 described phyla were common in ascidians, including *Chloroflexi* (103 $OTU_{0.03}$),
304 *Acidobacteria* (87), *Actinobacteria* (62), *Verrucomicrobia* (51) and *Firmicutes* (45), each
305 accounting for 1.4 to 3.1% of $OTU_{0.03}$ diversity and detected in at least half of the ascidian
306 hosts examined. The remaining 24 described and candidate phyla present in the ascidian
307 microbiota were rare overall (each <1% of total $OTU_{0.03}$ diversity) and within each host
308 ascidian (<2% of sequence reads; Figure 2, Supplementary Table S3), with the exception of
309 *Spirochaetes* in *Polycarpa aurata* (17.9% relative abundance) and SBR1093 in *Eudistoma*
310 *amplum* (11.3%).

311 Archaeal OTUs accounted for 17.9% (*n* = 12,128) of sequence reads but only 3.1% (*n*
312 = 104) of the $OTU_{0.03}$ diversity in the ascidian microbiota. *Thaumarchaeota* were particularly
313 abundant (*n* = 11,993; 53 OTUs) and common (present in 92.8% of host individuals), with
314 most archaeal sequence reads (98.0%) matching to the ammonia-oxidizing genera
315 *Nitrosopumilus* (*n* = 11,630; 36 OTUs) and *Cenarchaeum* (*n* = 261; 5 OTUs). In fact, the
316 most common $OTU_{0.03}$ in the ascidian microbiota (OTU0001, *Nitrosopumilus* sp.) was
317 present in 37 of the 42 host individuals (22 of 25 host species) at relative abundances up to
318 95% (*Lissoclinum badium*), while extremely rare in ambient seawater (0 – 0.042%). In
319 addition, a common archaeal symbiont in *Leptoclinides madara* (OTU0025, 16.6 – 27.8%

320 relative abundance) was classified to the genus *Cenarchaeum* and closely matched (98.3%
321 sequence identity) an uncultivated archaeon reported in the marine sponge *Axinella verrucosa*
322 (GenBank accession number AF42023).
323
324 Specificity of the Ascidian Microbiota
325 Comparison of the rich ascidian microbiota with ambient seawater microbes revealed
326 low overlap between free-living and host-associated microbial communities. A total of 283
327 OTUs were present in the seawater communities and absent from the ascidian microbiota,
328 while 102 OTUs were present in both ascidian and seawater samples, representing only 3%
329 of total $OTU_{0.03}$ diversity in the ascidian microbiota. Further, over one-third (n = 40) of these
330 shared microbial OTUs exhibited greater than an order of magnitude difference in relative
331 abundance in seawater and ascidians assemblages, including 5 OTUs that were 200x to 700x
332 more abundant in host ascidians (Figure 3). For example, OTU0001 (*Nitrosopumilus* sp.)
333 accounted for 16.7% of sequence reads from the ascidian microbiota. The remaining 4 OTUs
334 were specific to particular host families (e.g., OTU0301 in Didemnidae), species (e.g.,
335 OTU1798 in 3 individuals of *Clavelina meridionalis*) or individuals (e.g., OTU0225 in 1 of 3
336 *Didemnum multispirale* individuals) and rare or absent in most ascidian hosts (Figure 3).
337 Additional analysis of abundant components of the ascidian microbiota revealed
338 symbiont overlap between ascidians and other invertebrate hosts, as well as a unique
339 component of the ascidian microbiota (Table 3). A total of 56 microbial OTUs accounted for
340 78.4% of sequences obtained from ascidian hosts. Over two-thirds of these OTUs (n = 38)
341 matched closely (>97% sequence identity) to previously characterized sequences (Table 3),
342 most commonly derived from seawater (n = 14), corals (n = 9), sponges (n = 6) and sediment
343 (n = 3). In some cases, OTUs that were widespread among ascidians hosts and in the rare
344 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 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 hosts) were detected and core OTUs (present in >70% of host species) were represented by 7 OTUs at high relative abundance, accounting for 40.4% of all sequence reads. These OTUs corresponded to 2 *Prochlorococcus* sp. (*Cyanobacteria*; OTU0140, OTU0310) that were also common in seawater communities (41.4 and 39.9% 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 49.0% of sequence reads, while specific OTUs (present in a single host individual) were represented by 2,364 OTUs and accounted for 10.6% 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 ($r = 0.28$, $P < 0.001$), indicating that high symbiont similarity among individuals of the same species was not the sole driver 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 36.6% of $OTU_{0.03}$ diversity and 99.4% of sequence reads. The highest intra-specific diversity was seen in *Phallusia arabica*, where shared symbionts only accounted for 2.4% of $OTU_{0.03}$ diversity and 20.9% 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

TEM 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 x 2 µm; Figure 4A), 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 (Figure 4B) 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 (Figure 4C). Both clavelinids (*Pycnoclavella* sp. and *C. meridionalis*) contained ovoid-shaped bacteria often surrounded by irregular inclusions spread throughout the tunic (Figure 4D). In *Lissoclinum badium* and *Synoicum castellatum*,

all bacterial cells were ovoid or rod-shaped (ca. 0.5 μm x 2 μm, and ca. 0.3 μm x 1 μm, respectively) and observed either in isolation or forming small groups of 2 to 6 bacteria in close proximity to ascidian cells (Figure 4E and 4F, 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 Great Barrier Reef ascidians. Encompassing 3,321 $OTU_{0.03}$ 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. In fact, the diversity of host-associated communities in several ascidian species exceeded that of free-living communities present in ambient seawater. Visualization of microbial cells by TEM 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 (17.9% relative abundance) and a bacterium with spiral morphology in *Polycarpa aurata*.

The composition of the ascidian microbiota demonstrated some overlap with other host-associated microbial communities yet clear distinction from ambient planktonic communities in coral reef seawater. Only 3% of ascidian-associated OTUs were present in ambient seawater samples and typically at drastically different relative abundances (see below). Several abundant OTUs in the ascidian microbiota exhibited high similarity to known

420 symbionts from other benthic invertebrates. For example, the two most common
421 *Planctomycetes* OTUs reported herein matched nearly identically (>99.5%) to sponge- and
422 coral-associated microbes (OTU0293 and OTU0297, respectively), indicating microbial
423 lineages adapted to host-associated lifestyles may disperse among disparate host organisms.
424 Consistently, previous studies have noted multiple shared symbiont lineages among
425 microbiota of sponges and corals (Taylor *et al*., 2007; Simister *et al*., 2012). However, the
426 ascidian microbiota also maintained distinguishing characteristics in comparison to other
427 host-associated communities. For example, the phylum *Planctomycetes* exhibited high
428 diversity in ascidian hosts, whereas members of this phylum are typically rare in microbiota
429 of sponge (Webster and Taylor, 2012; Schmitt *et al*., 2012) and coral hosts (Sunagawa *et al*.,
430 2010; Barott *et al*., 2011). Further, 11 of the 56 most common OTUs in the ascidian
431 microbiota exhibited high sequence divergence (>5%) from any previously described marine
432 microbe. The unique niches inside invertebrate tissues are becoming recognized hotspots for
433 microbial biodiversity and our results suggest that ascidian tunics offer a similarly fertile
434 habitat for marine microorganisms.
435
436 Rare seawater microbes enriched in the ascidian tunic
437 While the vast majority of OTUs in the ascidian microbiota were not present in
438 planktonic communities, several microbes from the rare biosphere of seawater exhibited high
439 relative abundance in ascidian-associated communities. Five microbial OTUs exhibited 200
440 to 700 times higher relative abundance in the ascidian tunic than in the plankton, suggesting
441 the selective enrichment of rare seawater microbes in ascidian hosts as observed for the
442 microbiota in marine sponges (Webster *et al*., 2010; Taylor *et al*., 2013) and reef-building
443 corals (Sunagawa *et al*., 2010). These results also indicate the potential for horizontal
444 symbiont transfer among hosts, with the rare biosphere of seawater acting as a conduit among

host habitats. In addition, vertical symbiont transmission (i.e., parent-to-offspring passage) is known to occur in several ascidians hosts (Kott, 1980, 1982, 2001; Hirose, 2000; Moss *et al*., 2003; Hirose *et al*., 2006; Hirose and Hirose, 2007; López-Legentil *et al*., 2011; Kojima and Hirose, 2012), and is assumed to be essential for host survival (Kott, 2001; Hirose and Maruyama, 2004). Together, these findings suggest that a combination of vertical and environmental symbiont acquisition establishes the microbial communities in ascidians, as hypothesized for marine sponges (Schmitt *et al*., 2008).

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.

Host fidelity of the ascidian microbiota

The vast majority of symbiont OTUs (71.2%) 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 3,321 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 Great Barrier Reef 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 particular 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. 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, 2001; Hirose, 2000; Hirose *et al*., 2006; Hirose and Hirose, 2007; 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

495 component of the ascidian microbiota was notably comprised of lineages with known
496 ammonia-oxidization capabilities. In particular, sequences affiliated with the genus
497 *Nitrosopumilus* dominated the archaeal communities in Great Barrier Reef ascidians and
498 several *Nitrosopumilus* OTUs exhibited a widespread distribution among hosts and high
499 relative abundance within hosts. In coral reef waters, observations of high nitrite/nitrate
500 concentrations compared to adjacent, open water habitats have long suggested active
501 nitrification among reef-associated microbes (Webb *et al*., 1975). More recent studies have
502 reported that host-associated microbes in sponges and corals contributed to nitrification in
503 these reef habitats to a larger extent than reported for free-living communities in sediments
504 and seawater (Diaz and Ward, 1997; Southwell *et al*., 2008). The finding herein of
505 widespread ammonia-oxidizing Archaea in coral reef ascidians suggests an additional and
506 potentially important source of nitrification in reef habitats.
507 In fact, the most dominant of all OTUs in the ascidian microbiota (16.7 % of total
508 reads) was classified in the genus *Nitrosopumilus* and matched nearly identically (>99%
509 sequence identity) to a symbiotic AOA previously described in the Mediterranean ascidian
510 *Cystodytes dellechiajei*, where active nitrification was detected in the tunic layer by
511 expression of the alpha-subunit of ammonia monooxygenase (*amoA*) gene and net NO_x
512 production (Martínez-García *et al*., 2008). Another common OTU was classified in the genus
513 *Cenarchaeum*, a candidate taxon erected for the sponge-associated symbiont *Cenarchaeum*
514 *symbiosum* (Preston *et al*., 1996) whose genome includes homologues of genes associated
515 with chemolithotrophic ammonia oxidation (Hallam *et al*., 2006). Several phylotypes of
516 *Cenarchaeum* have been reported from marine sponges (Schleper *et al*., 1998; Margot *et al*.,
517 2002) and all to date exhibit specificity for a single host genus (*Axinella*). Similarly, the
518 *Cenarchaeum* OTU detected herein was only recovered from two individuals of the ascidian
519 host *Leptoclinides madara*, at high relative abundance (16.6 – 27.8%), and was absent from

520 all other ascidian hosts and ambient seawater samples. Thus, ascidians appear to associate
521 with both generalist and specialist lineages of AOA. Finally, some ascidians (e.g.,
522 *Lissoclinum badium*) hosted *Nitrospina* symbionts, a genus of *Deltaproteobacteria* whose
523 members are capable of nitrite-oxidation, in addition to dominant AOA lineages, suggesting
524 that the complete nitrification process may occur in the ascidian tunic of at least some species.
525
526 Ascidians as microbial habitat
527 The rich microbiota of coral reef ascidians indicates a fertile habitat for marine
528 microorganisms and prompts further studies of the microenvironmental conditions in ascidian
529 hosts that support such diverse microbial communities. Previous research in this area has
530 already revealed dynamic chemical landscapes in and around ascidians (Kühl and Larkum,
531 2002; Behrendt *et al*., 2012; Kühl *et al*., 2012), including fluctuations in oxygen
532 concentrations from supersaturation to anoxia and pH conditions from neutral to strongly
533 alkaline within minutes. These rapid changes and steep gradients in oxygen and pH may offer
534 periodic windows of optimal conditions for diverse metabolic pathways, thereby maintaining
535 the complex microbiota that occurs in ascidian tunics. In addition, ammonia is the primary
536 form of nitrogenous waste produced by ascidians (Goodbody, 1974) and may be recycled via
537 uptake or oxidation by resident microbes. For example, the widespread AOA reported herein
538 may utilize the ammonia-rich waste products of their host ascidians as substrate for
539 nitrification reactions. Indeed, nitrifying microbes require not only a reduced form of
540 inorganic nitrogen, but also high oxygen and low irradiance levels, as marine AOA are
541 particularly susceptible to photoinhibition at higher irradiance levels (Merbt *et al*., 2012).
542 Thus, the ascidian tunic habitat not only satisfies the ammonia and oxygen requirements of
543 AOA, but may also shelter these populations from the high irradiance levels characteristic of

544 shallow water reefs (e.g., Vermeij and Bak, 2002) and represent important habitats for
545 nitrite/nitrate regeneration in coral reef environments.

546 While the taxonomic scope of the ascidian species examined herein was broad, the
547 geographic scope was restricted to shallow water habitats of the Great Barrier Reef. Yet even
548 within this single biome, our results show a remarkably rich and diverse microbial
549 community associated with coral reef ascidians. Given the broad distribution of ascidians in
550 the marine environment (Lambert, 2005), it is likely that additional microbial diversity awaits
551 discovery as future studies target ascidians species from different latitudes and marine
552 habitats. In addition, further studies characterizing the physical and chemical aspects of the
553 ascidian host, coupled with expanded efforts to document the diversity of the ascidian
554 microbiota, will continue to reveal the role of ascidians as habitats for novel microbial
555 communities and bioreactors for microbial-mediated processes in marine biogeochemical
556 cycles.

557

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Figures Legends

Figure 1. Taxonomic diversity of the ascidian microbiota. **(a)** Phylum level distribution of the 3,321 microbial $OTU_{0.03}$ recovered from 42 Great Barrier Reef 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, *Caldithrix*, 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.

Figure 2. Microbial community similarity and composition in 42 samples of Great Barrier Reef 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 biosphere OTUs among ascidian hosts.

804 **Figure 4.** Transmission electron microscopy images of bacteria observed in the inner tunic of
805 the ascidians: (A) *Phallusia julinea*, (B) *Polycarpa aurata*, (C) *Pycnoclavella* sp., (D)
806 *Clavelina meridionalis*, (E) *Lissoclinum badium*, and (F) *Synoicum castellatum*. Arrowheads
807 point to bacterial cells, (AC) ascidian cell.
808

Tables

Table 1. Taxonomic classification of ascidian hosts and sequence data summary for ascidian and seawater samples.

			Total		Archaea		Bacteria	
Species	**Order**	**Family**	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 =	**67826**	**3321**	**12128**	**104**	**55698**	**3217**
Filtered Seawater	n.a.	n.a.	9573	221	289	24	9284	197
Filtered Seawater	n.a.	n.a.	14441	248	134	21	14307	227
Filtered Seawater	n.a.	n.a.	2797	129	3	3	2794	126
		Ambient Seawater Total =	**26811**	**385**	**426**	**26**	**26385**	**359**
		Grand Total =	**94637**	**3604**	**12554**	**124**	**82083**	**3480**

814 **Table 2**. Intra-specific variation in the ascidian microbiota highlighting the shared

815 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	2,479	697	1,338 (54.0)	26 (3.7)
Pycnoclavella sp.	N	2	1,565	341	1,077 (68.8)	19 (5.6)
Pycnoclavella diminuta	N	3	3,575	673	1,731 (48.4)	35 (5.2)
Didemnum multispirale	Y	3	8,813	367	6,192 (70.3)	24 (6.5)
Leptoclinides madara	Y	2	1,260	81	1,116 (88.6)	11 (13.6)
Lissoclinum badium	Y	2	7,894	41	7,848 (99.5)	15 (36.6)
Eudistoma amplum	Y	3	1,786	491	809 (45.3)	31 (6.3)
Synoicum castellatum	N	3	10,413	620	5,237 (50.3)	17 (2.7)
Phallusia arabica	N	3	497	82	104 (20.9)	2 (2.4)
Polycarpa aurata	N	3	636	39	514 (80.8)	3 (7.7)

816

817

818 **Table 3.** Abundant OTUs in the ascidian microbiota, showing their representation in ascidian
819 (ASC) and seawater (SW) datasets, number of host species, closest known relative and
820 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	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*

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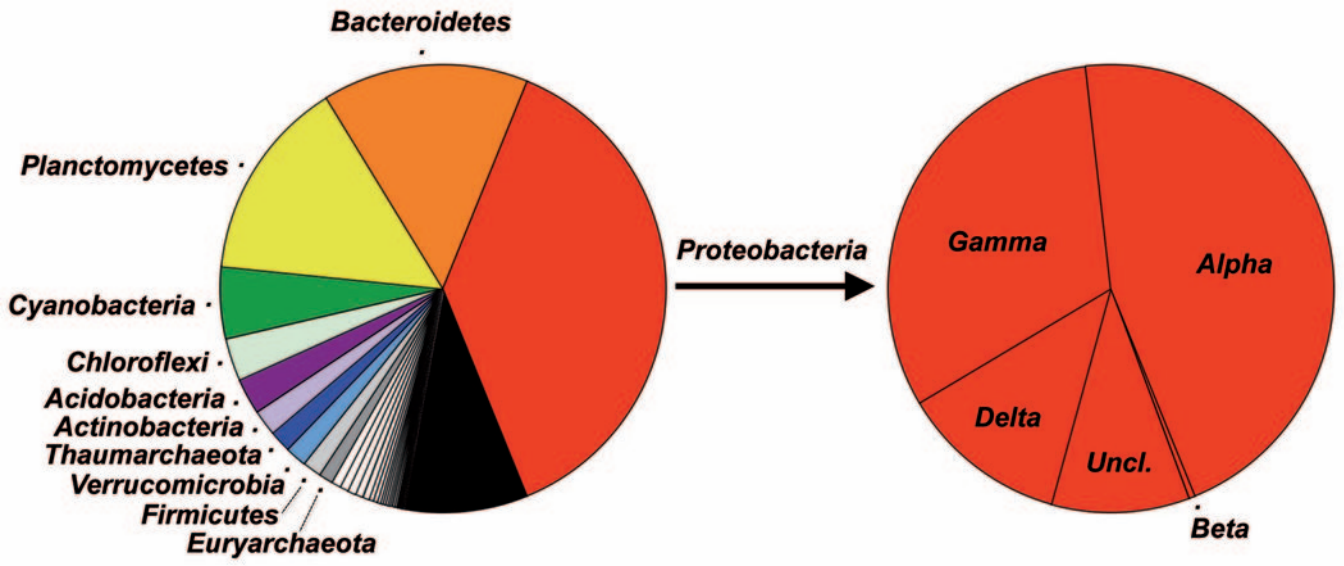
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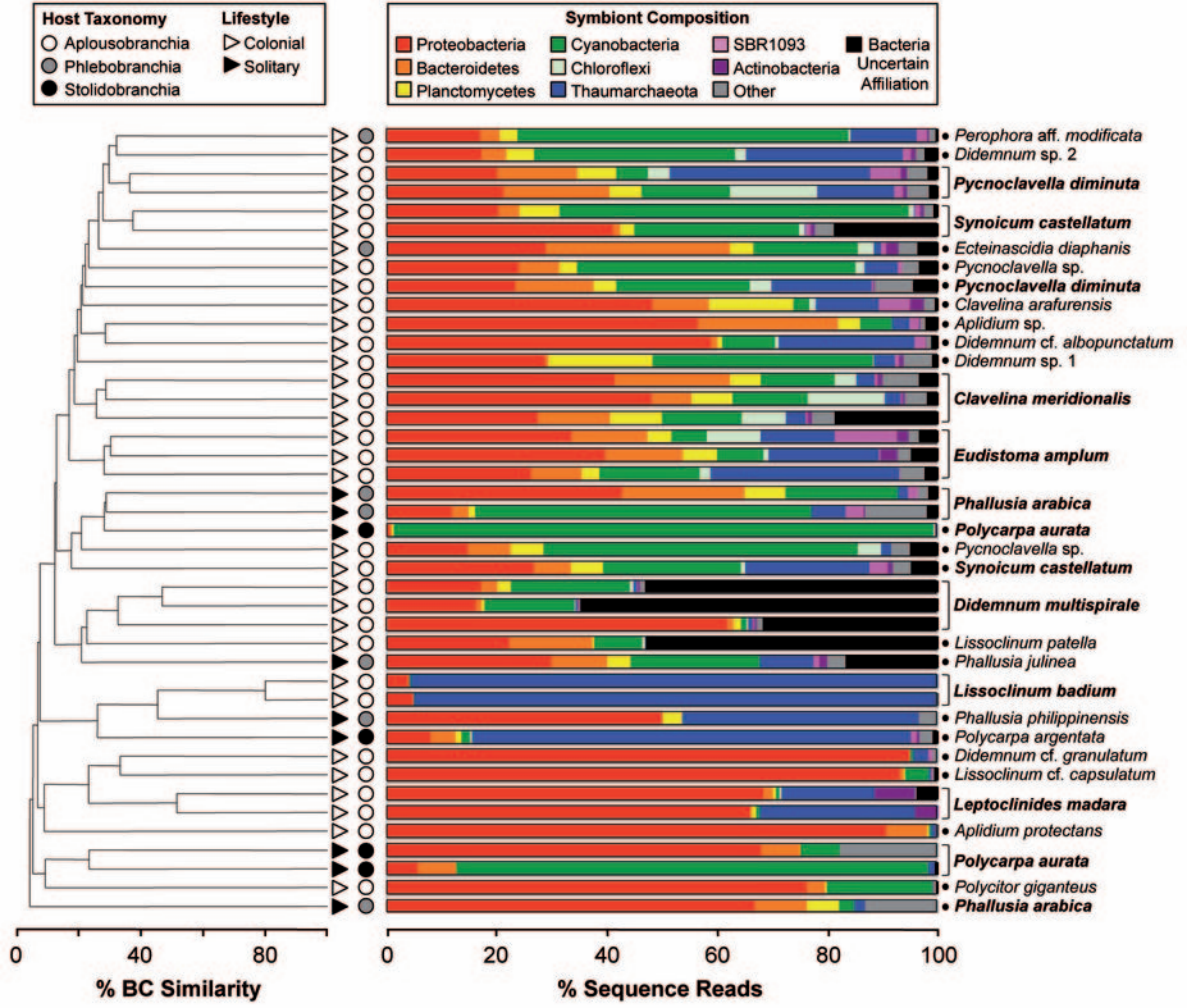
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Solitary
Symbiont Composition
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Planctomycetes
Cyanobacteria
Chloroflexi
Thaumarchaeota
SBR1093
Actinobacteria
Other
Bacteria Uncertain Affiliation
Perophora aff. modificata
Didemnum sp. 2
Pycnoclavella diminuta
Synoicum castellatum
Ecteinascidia diaphanis
Pycnoclavella sp.
Pycnoclavella diminuta
Clavelina arafurensis
Aplidium sp.
Didemnum cf. albopunctatum
Didemnum sp. 1
Clavelina meridionalis
Eudistoma amplum
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Polycarpa aurata
Pycnoclavella sp.
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Lissoclinum patella
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Lissoclinum badium
Phallusia philippinensis
Polycarpa argentata
Didemnum cf. granulatum
Lissoclinum cf. capsulatum
Leptoclinides madara
Aplidium protectans
Polycarpa aurata
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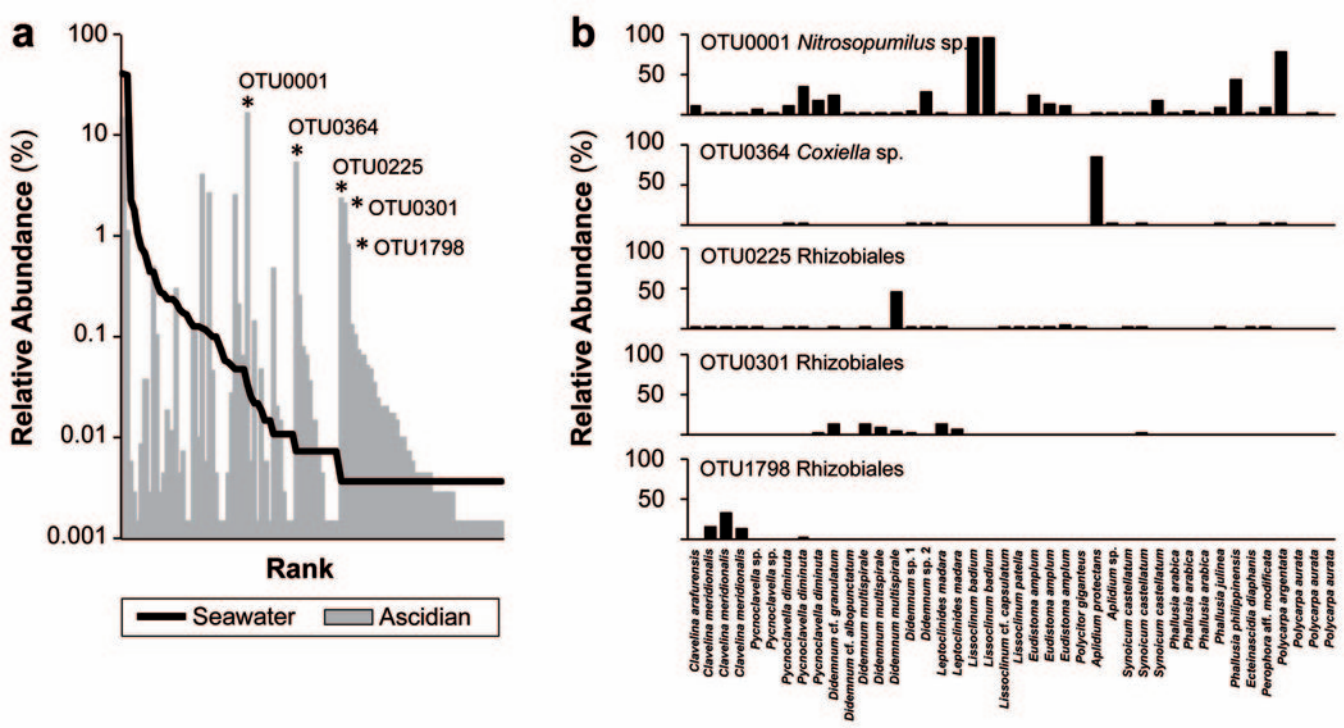
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OTU0225
OTU0301
OTU1798
Rank
Seawater
Ascidian
b
Relative Abundance (%)
OTU0001 Nitrosopumilus sp.
OTU0364 Coxiella sp.
OTU0225 Rhizobiales
OTU0301 Rhizobiales
OTU1798 Rhizobiales
100
50
Clavelina arafurensis
Clavelina meridionalis
Clavelina meridionalis
Clavelina meridionalis
Pycnoclavella sp.
Pycnoclavella sp.
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Pycnoclavella diminuta
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Aplidium sp.
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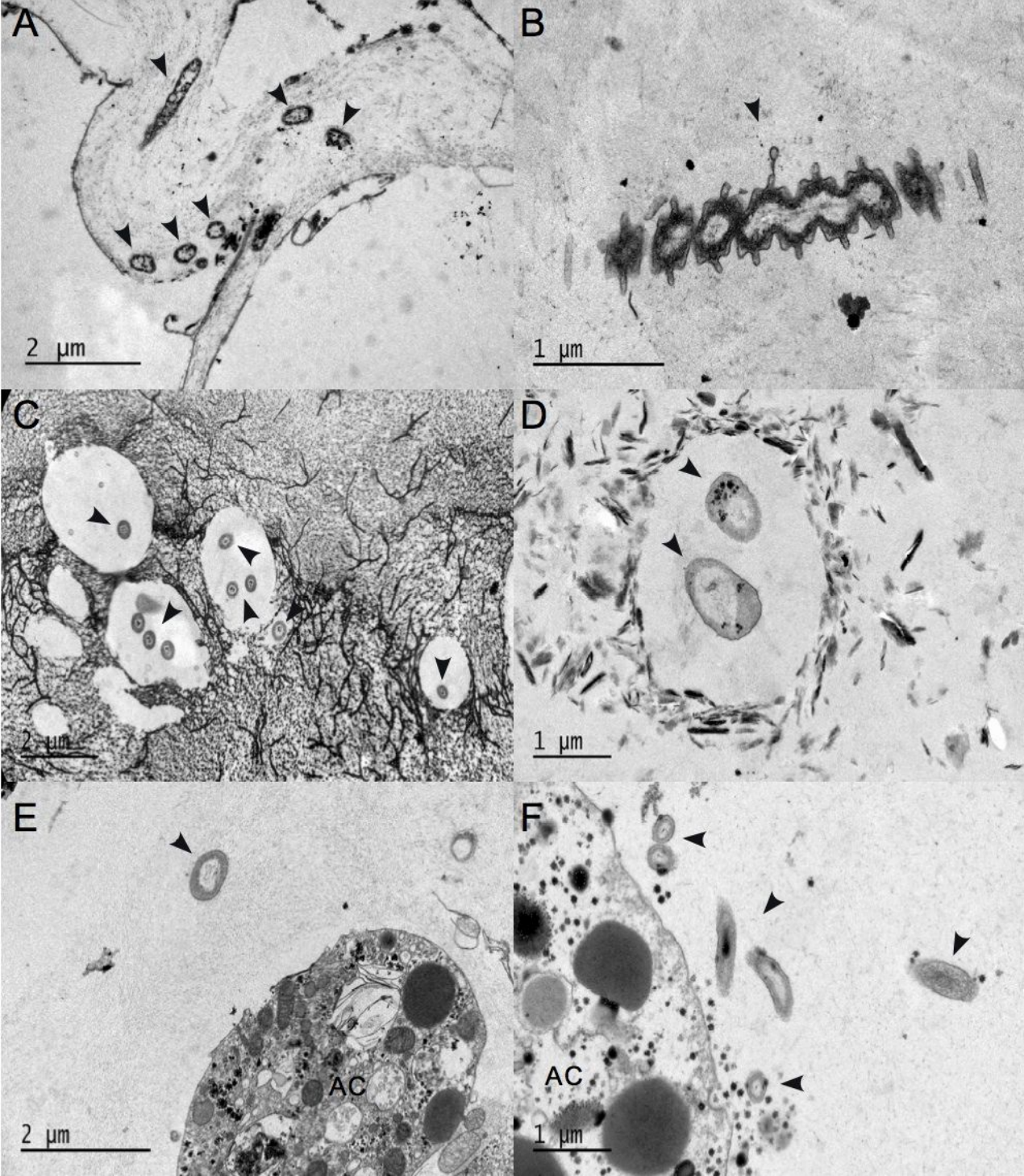
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