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SHORT COMMUNICATION

Diversity of *Actinobacteria* Associated with the Marine Ascidian *Eudistoma toealensis*

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Abstract Ascidians have yielded a wide variety of bioactive natural products. The colonial ascidian *Eudistoma toealensis* from Micronesia has been identified as the source of a series of staurosporine derivatives, though the exact origin of these derivatives is still unknown. To identify known staurosporine-producing microbes associated with *E. toealensis*, we analyzed with 16S rRNA gene tag pyrosequencing the overall bacterial community and focused on potential symbiotic bacteria already known from other ascidians or other marine hosts, such as sponges. The described microbiota was one of very high diversity, comprising 43 phyla: two from archaea, 34 described bacterial phyla, and seven candidate bacterial phyla. Many bacteria, which are renowned community members of other ascidians and marine holobionts, such as sponges and corals, were also part of the *E. toealensis* microbial community. Furthermore, two known producers of indolocarbazoles, *Salinispora* and *Verrucosispora*, were found with high abundance exclusively in the ascidian tissue, suggesting that microbial symbionts and not the organism itself may be the true producers of the staurosporines in *E. toealensis*.

Keywords Ascidian · *Actinobacteria* · *Eudistoma toealensis* · Microbial diversity · Symbiosis · 16S rRNA

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Introduction

Ascidians (Tunicata) are an important source of marine natural products, with over 1000 natural products identified from ascidians so far (Schmidt and Donia 2010) and some 40 novel ascidian-derived natural products still isolated every year (Blunt et al. 2013). The colonial ascidian *Eudistoma toealensis* is a highly abundant species within mangrove root habitats in Micronesia and, despite lacking morphological defenses, only the flatworms *Pseudoceros indicus* and *Pseudoceros tristiatus* are known to feed upon this species (Schupp et al. 1999, 2002). A series of staurosporine derivatives, belonging to the group of indolocarbazole alkaloids, has been isolated from *E. toealensis*. Staurosporines have received considerable attention due to their pronounced cytotoxic activity resulting from inhibition of protein kinases (Blunt et al. 2012; Sánchez et al. 2006; Tamaoki et al. 1986). In addition, several staurosporine derivatives have entered phase I/II clinical trials for treating various cancer types (e.g., leukemia, lymphomas, advanced solid tumors, and melanoma), emphasizing their role as highly bioactive secondary metabolites (Sánchez et al. 2006). Besides being isolated from several marine macroorganisms (e.g., nudibranchs, ascidians), staurosporines have long been known to be produced by terrestrial *Streptomyces* strains and, more recently, from various marine actinomycetes (Schmidt and Donia 2010). However, the source of the *E. toealensis*-associated staurosporines is still unknown. Since *E. toealensis* is a filter feeder and ingests diverse marine microbes from seawater, it is possible that these compounds are of microbial origin and are actually taken up via the food chain (Schupp et al. 1999, 2009). Such metabolic associations and interactions between marine filter feeders and microbes are currently best known from marine sponges (Hentschel et al. 2006; Taylor et al. 2007; Webster and Taylor 2012; Wilson et al. 2014). Although sponges and ascidians are phylogenetically not closely related, the identical

lifestyle of filter-feeding in often shared habitats has presumably led to similar symbiotic interactions with microorganisms. Recent studies highlighted the status of ascidians as marine holobionts capable of hosting highly diverse microbial communities with great potential for specific biosynthetic pathways and microbially derived secondary metabolites (Behrendt et al. 2012; Donia et al. 2011; Erwin et al. 2013, 2014; López-Legentil et al. 2011; Schmidt and Donia 2010).

The aim of this study was to identify known staurosporine-producing microbes associated with *E. toealensis* from two Micronesian islands. While analyzing the overall bacterial community, a focus was set on (a) potential symbiotic bacteria already known from other ascidians and sponges and (b) the ascidian-associated *Actinobacteria*, due to their possible staurosporine production in *E. toealensis*.

Materials and Methods

In 2006, whitish and slightly transparent *E. toealensis* specimens were collected via snorkeling on the Micronesian Islands of Chuuk (EtCI 1–3) (7° 26′ N, 151° 51′ E) and Pohnpei (EtPI 1–5) (6° 51′ N, 158° 13′ E) from mangrove roots at depths of 1 to 2 m. Ascidians were compared with previously collected vouchers from Schupp et al. (1999), which have been identified by ascidian taxonomists Monniot and Monniot at the Museum National d′Histoire Naturelle, Paris, France. During sampling on Pohnpei, environmental samples (rootPI 1–3) were also collected from the surface of the mangrove roots by swabbing. All samples were frozen immediately, freeze-dried and stored at −20 °C until sample analysis.

Genomic DNA was extracted from ascidian tissue and root surface swab samples using a bead-beating method previously described for sponges (Taylor et al. 2004). Additionally, root surface swab samples were incubated for 30 min at 94 °C after initial bead-beating following a modified DNA extraction protocol for swab samples (modified after Waite et al. 2012). 16S rRNA gene amplification with primers 454MID_533F (GTG CCA GCA GCY GCG GTM A) and 454_907RC (CCG TCA ATT MMY TTG AGT TT) and purification for pyrosequencing were performed as previously described (Simister et al. 2012b). The resulting flowgram data can be accessed via the Sequence Read Archive (SRA) of the National Center for Biotechnology Information under the accession number SRX682233.

Sequences were initially processed using mothur v.1.33.0 (Schloss et al. 2009, 2011). Pyrosequencing flowgrams were filtered and denoised using the mothur implementation of AmpliconNoise (Quince et al. 2011). Sequences were removed from the analysis if they were <200 bp, contained ambiguous characters, had homopolymers longer than 8 bp, more than one MID mismatch, or more than two mismatches to the reverse primer sequence. Denoised and trimmed sequences (mothur v.1.33.0) were uploaded and processed via SILVAngs v.1.3.0 (https://www.arb-silva.de/ngs/) as described in Krupke et al. (2014). SILVAngs classification was performed two times, for each individual sample (*E. toealensis*, EtCI 1–2 and EtPI 1–5; rootPI 1–3) and additionally as a pooled dataset for each combination site/sample (EtCI, EtPI, and rootPI).

The SILVAngs fingerprint results, which provided detailed comparative information about the classification of the 0.03 operational taxonomic units (OTUs) (i.e., >97 % 16S rRNA gene sequence similarity) for each sample at maximum taxonomic depth (setting: max. taxonomic depth ‘20’), were subsequently used for multivariate nonmetric multidimensional scaling (nMDS, Bray-Curtis dissimilarity) using the metaMDS command from the vegan package (Oksanen et al. 2011) in R (v. 3.0.2) (R Development Core Team 2013). Hypothesis-based community treatments were drawn with the vegan command ‘ordieellipse’ (0.95 confidence interval). Treatments were as follows: (a) source—A ‘EtCI’, B ‘EtPI’, and C ‘rootPI’, (b) habitat—‘ascidian’ and ‘environmental’, and (c) location—‘Pohnpei’ and ‘Chuuk’. These treatments were used for hypothesis-based multivariate analysis of variance by the ‘adonis’ command from the vegan package. The same dataset was used to generate heatmaps with JColorGrid v1.860 (Joachimiak et al. 2006) for *Actinobacteria*. Dendrograms were generated using the vegan package in R via the commands “vegdist” (Bray-Curtis dissimilarity) and “hclust” (method=average) and subsequently added onto the heatmaps. OTU and sequence statistics, taxonomic fingerprint, and krona charts were provided by SILVAngs v.1.3 (Ondov et al. 2011; Quast et al. 2013). Eukaryotes and sequences classified as “no relatives” found in our samples (n=792) were excluded from all statistical analyses (Suppl. Table 1).

Results and Discussion

OTU Statistics and Microbial Diversity

The analyzed ascidian and environmental microbiota displayed a very high operational taxonomic unit (OTU) diversity within all sites and samples. The microbial community associated with *E. toealensis* comprised 2967 OTUs (0.03 cutoff) in total among the three individuals from Chuuk Island (EtCI 1–3) and 3405 OTUs among the five individuals from Pohnpei Island (EtPI 1–5) (Table 1).

Overall, at phylum level, the *E. toealensis* microbial composition is comparable to that described in other recent

Table 1 Sequence and OTU summary—with number of total sequence available for each individual sample and pooled samples, number of OTUs for individual and pooled samples, frequencies of classified sequences and sequences considered as unclassified (No Relative-BAST alignment coverage and alignment identity<93 %) and sampling coverage

Sample name	No. of sequences	No. of OTUs	Classified (%)	No relative (%)	Good's coverage
EtCI1	7891	675	95.08	4.82	0.95
EtCI2	14,296	1932	99.02	0.85	0.95
EtCI3	8447	1243	97.67	2.24	0.93
EtCI pooled	30,634	2967	98.19	1.70	0.96
EtPI1	4372	858	99.91	0.05	0.91
EtPI2	8785	1686	99.57	0.43	0.91
EtPI3	9128	1566	99.64	0.19	0.93
EtPI4	6327	751	99.83	0.08	0.94
EtPI5	6910	1252	99.58	0.23	0.92
EtPI pooled	35,522	3405	99.68	0.21	0.96
rootPI1	9442	2112	99.75	0.13	0.89
rootPI2	6952	1806	99.61	0.07	0.87
rootPI3	7969	1868	99.77	0.06	0.89
rootPI pooled	24,363	3953	99.73	0.07	0.93

ascidian microbiology studies (Behrendt et al. 2012; Erwin et al. 2013, 2014). Here, we report 43 ascidian-associated phyla: two from archaea, 34 described bacterial phyla, and seven candidate bacterial phyla. The dominant phylum was *Proteobacteria*, which accounted for over 50 % of all classified sequences found in every sample (Fig. 1, Suppl. Table 1). Within *Proteobacteria*, the *Alphaproteobacteria* were, on average, most dominant (20.1 % averaged across all samples), followed by *Gammaproteobacteria* (18.7 %) and *Deltaproteobacteria* (11.2 %). Other abundant phyla throughout all samples included *Planctomycetes*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, and *Cyanobacteria* (Fig. 1, Suppl. Table 1 and 2). In comparison to the known dominant phyla in *Eudistoma amplum* (Erwin et al. 2014), only the low abundance of *Thaumarchaeota* in *E. toealensis* deviates noticeably from the general dominant phyla within the two *Eudistoma* species. However, due to low sequence numbers and possible sequencing errors or primer biases in the targeted 16S rRNA region, caution is required in order to not overestimate the abundance and diversity for the archaeal lineages in our data.

In our study, 31 phyla are shared between *E. toealensis* and environmental samples (Suppl. Figure 1 & Suppl. Table 1). While 12 phyla were recovered exclusively from *E. toealensis*, two phyla were obtained from the environment only (BHI80-139 and *Synergistetes*). An example of the shared community (found in both *E. toealensis* and on the root surface) is a strictly anaerobic described *Chloroflexi* lineage which was also found in other recent studies of ascidian-associated microbiota and which has been described as a sponge and coral symbiont (Behrendt et al. 2012; Erwin et al. 2013, 2014; Simister et al. 2012a; Taylor et al. 2013). Two other sponge symbionts within the shared phyla dataset were the Deltaproteobacteria Candidatus *Entotheonella* (Brück et al. 2008; Schmidt et al. 2000; Wilson et al. 2014) and *Nitrospina* (Hentschel et al. 2006; Schmitt et al. 2012). The candidate genus *Entotheonella* is a renowned symbiotic genus in the marine sponge *Theonella swinhoei* with a remarkably diverse natural products repertoire. Almost all bioactive polyketides and peptides from *T. swinhoei* have been attributed to one of the two chemically distinct *Entotheonella* phylotypes inhabiting this sponge (Wilson et al. 2014). While *Entotheonella* spp. is widely distributed in sponges, we observed members of this candidate genus in *E. toealensis* from both locations (1 % EtCI and 8 % EtPI of all *Desulfobacterales*) and our environmental samples (0.2 %). The presumed nitrite-oxidizing *Nitrospina* symbionts, which were recently found in some ascidians (Erwin et al. 2014), comprise 11 % of all *Desulfobacterales* in EtCI, 3 % in EtPI, and 0.6 % in environmental root surface swabs (Suppl. Table 2).

Among the microbiota occurring exclusively within *E. toealensis* was the ammonia-oxidizing *Thaumarchaeota* (i.e., Marine Group I, Candidatus *Nitrosopumilus*, and the Soil Crenarchaeota Group), but apparently at lower abundance than that recently described by Erwin et al. (2014) (Fig. 1, Suppl. Table 1). However, finding evidence of *Thaumarchaeota* occurrence only in *E. toealensis* specimens and not in our environmental samples highlights this genus as a potential ascidian symbiont (Martínez-García et al. 2008).

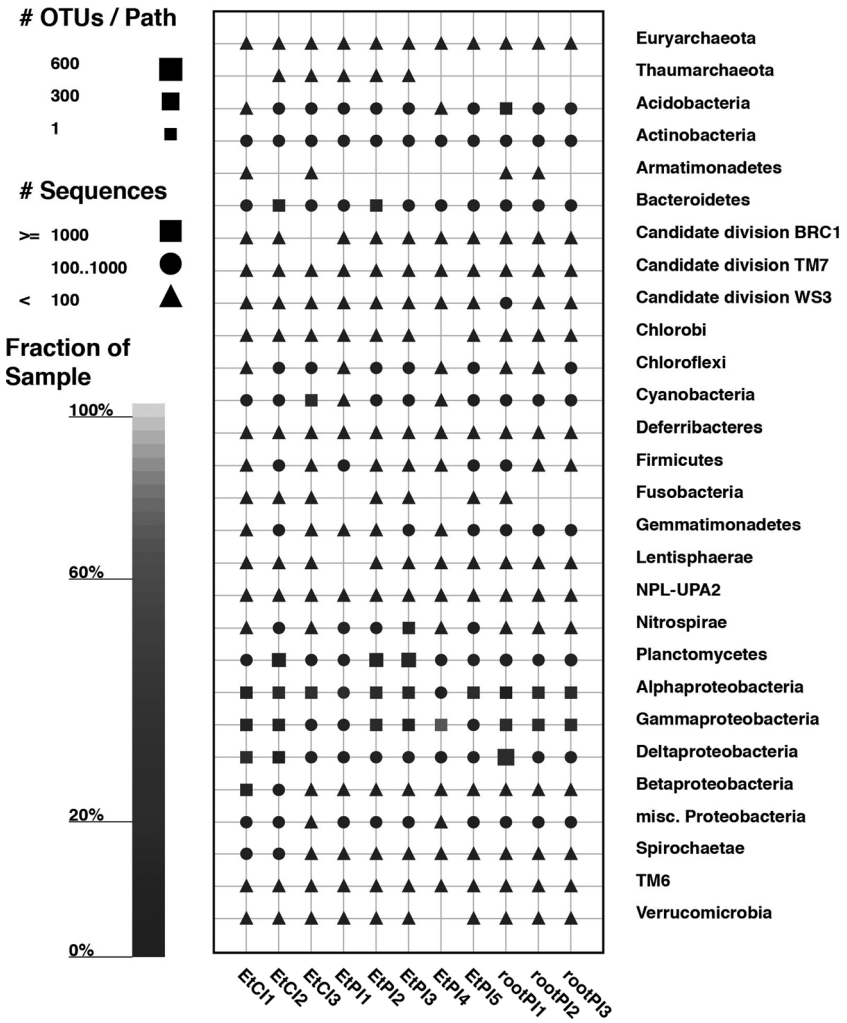

Fig. 1 Taxonomic breakdown per sample at phylum level—showing only phyla with ≥10 % relative abundance

Moreover, 4 % of the *E. toealensis Gammaproteobacteria* community from Chuuk Island (and 0.2 % from Pohnpei Island) was associated with the genus Candidatus *Endoecteinascidia*, which was previously described as species specific for the ascidian *Ecteinascidia diaphanis* (Great Barrier Reef) and *E. turbinata* (Mediterranean and Caribbean Sea) (Erwin et al. 2014; Moss et al. 2003; Pérez-Matos et al. 2007). To the best of our knowledge, this is the first time that this symbiont lineage, with an assumed role as a secondary metabolite producer (Rath et al. 2011), has been reported from another ascidian genus.

By using the 0.03 OTU community data (Suppl. Table 2) for nonmetric multidimensional scaling, the resulting ordination and multivariate analysis of variance (adonis) showed significant differences between ascidian and environmental samples (Suppl. Figure 2). The distinct grouping of environmental and *E. toealensis* samples supports recent findings that ascidians host very specific microbial communities with potential symbiotic relationships (Donia et al. 2011; Erwin et al. 2014; Martínez-García et al. 2007; Piel 2009; Schmidt and Donia 2010). As with sponges, the maintenance of symbiont communities presumably represents a combination of horizontal and vertical transmission (Erwin et al. 2014; Schmitt et al. 2012). While vertical transmission is usually associated with colonial ascidians, horizontal acquisition from the environment is assumed for solitary ascidians, e.g., *Styela plicata* (Erwin et al. 2013). However, for the colonial ascidian *E. toealensis*, the large number of microbial phyla that are shared with the environment suggests that the transmission of associated bacteria is presumably a mix of vertical and horizontal transmission, as observed and discussed for sponges (Reveillaud et al. 2014; Schmitt et al. 2012; Taylor et al. 2013) and ascidians (Erwin et al. 2013, 2014).

Actinobacteria Diversity

Several staurosporine derivatives have been isolated from *E. toealensis* samples in the past (Proksch et al.

2003; Schupp et al. 1999, 2001) with high structural similarity between compounds found in *E. toealensis* and in *Actinobacteria* suggesting a microbial origin (Schmidt and Donia 2010). Since *Actinobacteria* are well known producers of secondary metabolites (e.g., staurosporines and other indolocarbazoles) in marine eukaryotes and are, furthermore, often associated with marine sponge and coral holobionts, we focused on the diversity of *Actinobacteria* associated with *E. toealensis* (Sánchez et al. 2006; Piel 2009; Schmidt and Donia 2010; Simister et al. 2012a; Schmitt et al. 2012; Webster and Taylor 2012; Blunt et al. 2013).

Actinobacteria constitute between 2 and 10 % of all bacteria within the dataset and are comprised of 51 *Actinobacteria* genera (Fig. 2 and Suppl. Table. 3). The Krona charts of the three pooled datasets showed distinct community structures, in which the *E. toealensis* samples from Chuuk and Pohnpei Islands (Fig. 3a, b) exhibited greater diversity than the environmental samples (Fig. 3c). Among the 51 *Actinobacteria* genera, 16 were shared between *E. toealensis* and environmental samples (Fig. 2 and Suppl. Table 3). Additionally, nMDS analysis and adonis hypothesis testing based on the *Actinobacteria* community data revealed a significant difference between the *E. toealensis* and environmental samples, while the overlapping ordination of most of the ascidian samples tentatively suggests an *E. toealensis*-specific *Actinobacteria* community within geographically different sampling sites (Suppl. Figure 2). The shared *Actinobacteria* made the greatest contributions, with two dominant marine groups OCS155 and Sva0996 and two uncultured *Acidimicrobiale*s and *Gaiellales* clades (Fig. 2). Most notable were the genera *Salinispora* and *Verrucosispora*, which were only found in *E. toealensis* but not the environmental samples (Figs. 2 and 3). Both are members of the *Micromonosporaceae*, and these two have been described as potential indolocarbazole producers (Sánchez et al. 2006). Bacteria of the marine genus *Salinispora* have been cultured from sponges (e.g., Great Barrier Reef sponge *Pseudoceratina clavata*; Kim et al. 2005) and are known for their production of bioactive secondary metabolites, such as salinosporamide A, sporolide A, and also staurosporine derivatives (Blunt et al. 2013; Freel et al. 2011; Jensen et al. 2007; Udwary et al. 2007). *Verrucosispora* are known producers of numerous ascidian (Blunt et al. 2012) and sponge (Blunt et al. 2013; Jiang et al. 2007) secondary metabolites. Both *Salinispora* and *Verrucosispora* have also been recently cultured from the colonial ascidian *Lissoclinum patella* (Donia et al. 2011). Furthermore, two new staurosporine derivatives have been isolated from the Brazilian ascidian *Eudistoma vannamei* (Jimenez et al. 2012). Subsequently, 20 actinomycetes strains were isolated from *E. vannamei*, indicating that ascidians of the genus *Eudistoma* seem to host diverse actinomycetes communities, which produce biologically highly active secondary metabolites (Jimenez and Ferreira 2013).

The exclusive low-abundance *Actinobacteria* members in our data (Fig. 2) exhibit also an intriguing spectrum of marine-invertebrate associations. Many of them are known for potential symbiotic relationships and/or microbial secondary metabolite production within their hosts. For example, *Acidimicrobium*, *Brachybacterium*, *Corynebacterium*, *Leucobacter*, and *Solirubrobacter* representatives were found in various sponge species (Hentschel et al. 2006; Khan et al. 2012; Sfanos et al. 2005; Taylor et al. 2007). The genus *Microbacterium*, which already showed antitumor properties (Wicke et al. 2000), has been recovered from sponges (Lafi et al. 2005; Muscholl-Silberhorn et al. 2008; Sfanos et al. 2005; Taylor et al. 2007), sea anemones (Du et al. 2010), and sediments (Bollmann et al. 2010; Gavrish et al. 2008). Nitrogen-fixing *Sporichthya* are potential symbionts located in the nidamental glands of the squid *Sepia officinalis* (Grigioni et al. 2000). Finally, the genus *Nocardioides* (family Nocardiopsaceae) was found in culture-dependent and independent studies in the sponges *Haliclona* sp. and *Hymeniacidon perleve* (Khan et al. 2011; Sun et al. 2010).

Concluding Remarks

This study revealed exceptionally high microbial diversity within the ascidian species *E. toealensis*. Many known symbiotic microbes, which previously had been described from sponges and ascidians (e.g., Candidatus *Entotheonella*, *Nitrospina*, *Thaumarchaeota*), were also part of the *E. toealensis*-associated microbiota. Some of these microbes may contribute to the ascidians' metabolic pathways, for example, with nitrification abilities, while others are able to synthesize highly biologically active secondary metabolites, with bioactivities ranging from anticancer, antimicrobial, and antiviral activities to chemical defenses. Altogether, *E. toealensis* seems to be an important holobiont, able to host a diverse and rich microbial biota with a great potential to act as a source of bioactive compounds of microbial origin. Moreover, with the occurrence of *Salinispora* and *Verrucosispora*, two known producers of indolocarbazoles, such as staurosporines, were found with high abundance exclusively in the ascidian tissue, hinting that microbial symbionts and not the organism itself may be the true producers of these derivatives.

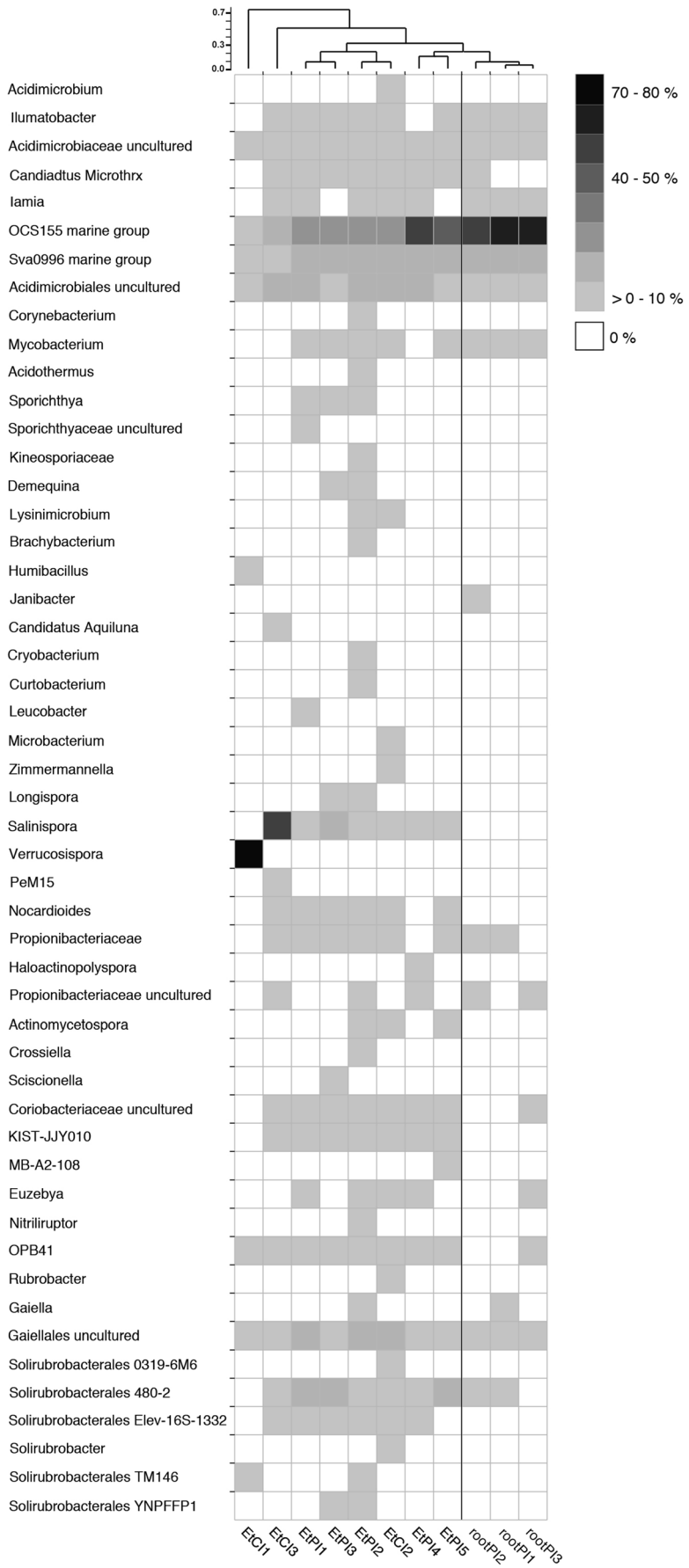

Fig. 2 Occurrence of *Actinobacteria* in Ascidian and root surface samples. The *grayscale* code indicates relative abundance, ranging from *light gray* (low abundance) to *black* (high abundance). *White* indicates that no sequence was assigned to the specific *Actinobacteria* genera. Samples are clustered using Bray-Curtis dissimilarity and group averages

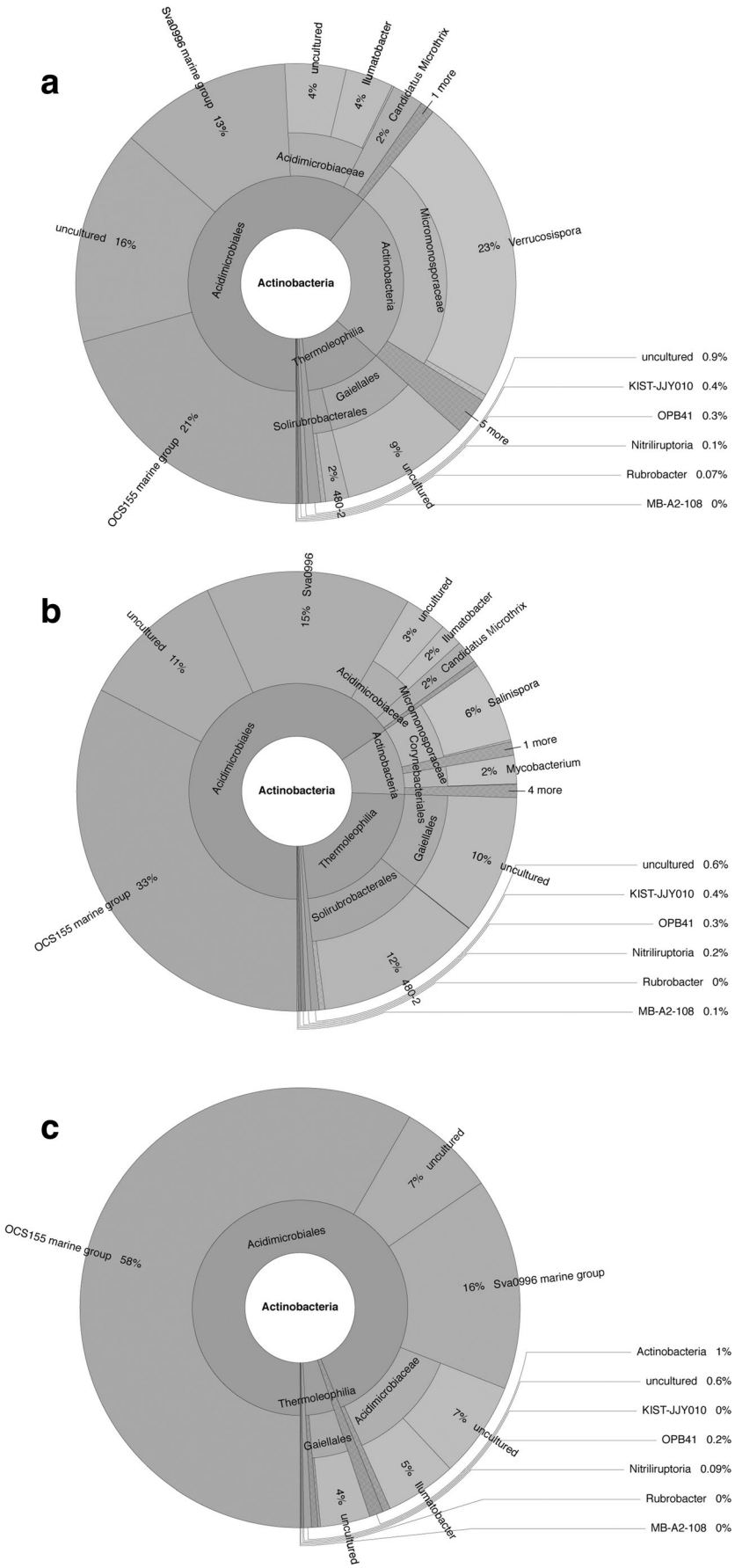

Fig. 3 Overview of the diversity and relative abundance of *Actinobacteria* groups within the pooled Ascidian samples from **a** Chuuk Island (EtCI), **b** Pohnpei (EtPI), and **c** root surface samples from Pohnpei (rootPI) visualized in a hierarchical structure

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