Microbial diversity of coralline sponges

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Summary

Coralline sponges, extraordinary members of the phylum Porifera, form a solid basal skeleton of calcium carbonate. These sponges are not closely related and show differences in their basal skeletons. Coralline sponges were dominant and abundant reef-building organisms during long periods of the Earth's history. They belong to the most understudied sponges in terms of associations with microbial symbionts, although their Silurian fossils point to close interactions with microorganisms and might indicate an early stage of sponge-microbial symbiosis. Moreover, the coralline sponge *Astrosclera willeyana* uses the degraded remains of bacteria to seed growth of its skeleton in the biomineralization process.

Here we employed molecular methods for a detailed study of microorganisms associated with distantly related sponges of the genus *Astrosclera* and *Vaceletia* in order to explore the hitherto unknown microbial diversity in coralline sponges. We also aimed to determine whether the microbial communities of these 'living fossils', likely living representatives of a long-extinct ancient groups, differ from those reported for other sponges, or whether they show some specific microbial patterns. Furthermore, we expected to gain some insight into the mechanisms of maintenance and evolution of microbial symbiosis in sponges. By first constructing an extended 16S rRNA gene clone library of microbiota associated with *Vaceletia crypta*, we revealed a highly diverse symbiotic community with a complex composition of phyla commonly affiliated with marine sponges. Due to the high similarity of the obtained sequences related to other sponge-derived sequences and their prevalent affiliations to sponge-specific clusters, we showed that the 'living fossil' coralline sponge *V. crypta* shares features of its microbial community with other sponges. By employing denaturing gradient gel electrophoresis (DGGE) cluster analysis we were then able to confirm the high microbial diversity associated with the *Vaceletia* species and, moreover, to indicate distinct microbial communities in the different growth forms (solitary and colonial).

By having a detailed characterization of microbial communities associated with *Astrosclera willeyana* from the Great Barrier Reef and the Red Sea (GBR), and based on further 16S rRNA gene clone libraries, we also exhibited complex and

abundant consortia of microorganisms with high resemblance to sequences obtained from other sponges. The *A. willeyana*-associated sequences formed numerous sponge-specific clusters confirming the uniqueness of the microbial associations in the sponges. A comparison of the clone libraries revealed, despite the many similarities, a less complex structure of the microbiota hosted by the Red Sea *Astrosclera* specimen.

Primary DGGE analysis of microbial communities associated with *A. willeyana* samples from different sites at the GBR indicated closer relationships between the microbial communities with respect to geographic origin (northern vs. southern GBR) and suggested that the differences in symbiotic community composition might be an additional indicator of cryptic species. We could confirm this finding with further DGGE analysis of numerous *Astrosclera* specimens from nearly the entire area of occurrence of this coralline sponge, i.e. from the Red Sea to the central Pacific.

Finally, through a comparison of the 16S rRNA gene clone libraries constructed from co-occurring *V. crypta* and *A. willeyana* from the GBR, we were able to demonstrate that, despite some differences, very high similarity exists in the phylogenetic composition of both symbiotic consortia. Moreover, in contrast to other sponges, distantly related coralline sponges shared a much higher degree of microbial species, thus suggesting specific patterns for the constitution of microbial communities in this unique group of sponges.

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4.Microbial duel between coralline sponges – a comparison of the symbiotic communities of Astrosclera willeyana and Vaceletia crypta

Introduction

Sponges

Sponges (phylum Porifera), which have a fossil record dating back nearly 700 million years (Erwin et al. 2011), arguably belong to the earliest branching Metazoa (Philippe et al. 2009, Philippe et al. 2011). The phylum Porifera consists of four extant classes: the Hexactinellida (glass sponges), Calcarea (calcareous sponges), Demospongiae (demosponges), and Homoscleromorpha (Wörheide et al. 2012) and contains most of so far 8,000 described species out of an estimated number of more than 15,000 sponge species living today (Hooper & Van Soest 2002). Sponges predominantly inhabit tropical and subtropical oceans as well as polar regions, the deep sea, and freshwater lakes and streams (Hooper & Van Soest 2002), where they belong to important members of benthic communities in terms of their biomass and function (Bell 2008). Their activity not only influences the sea floor, but also influences pelagic processes, as sessile filter feeders sponges process great volumes of seawater. A 1 kg sponge can pump up to 24,000 L per day (Vogel 2008) in order to feed on microorganisms and food particles taken up from the seawater.

Most physiological functions of sponges depend on the flow of ambient water, which enables nutrition, respiration and gas exchange, the removal of digestion residuals and excretes, the release and intake of gametes and other reproductive products, etc. Sponges comprise several different cell layers, which form a body plan built around an aquiferous system of pores, canals, and chambers (Fig. 0.1) through which surrounding water is pumped in and out. Water enters a sponge through the ostia (pores) in the outer pinacoderm layer composed of cells called pinacocytes, which also cover interior canals that penetrate the sponge body and lead to the chambers. The chambers are covered with choanoderm, a layer of special flagellated cells called choanocytes that beat their flagellum to pump the water from ostia to the exits by the osculum. The choanocytes not only produce water current, but also filter out from the water food particles including bacteria, unicellular algae, and even viruses (Hentschel et al. 2012) and transfer them to the inner mesohyl layer. The mesohyl, a glycosidic matrix, contains several types of cells; among others archaeocytes, which digest food particles and as totipotent cells can give rise to any of other sponge cell types. In many demosponges, the

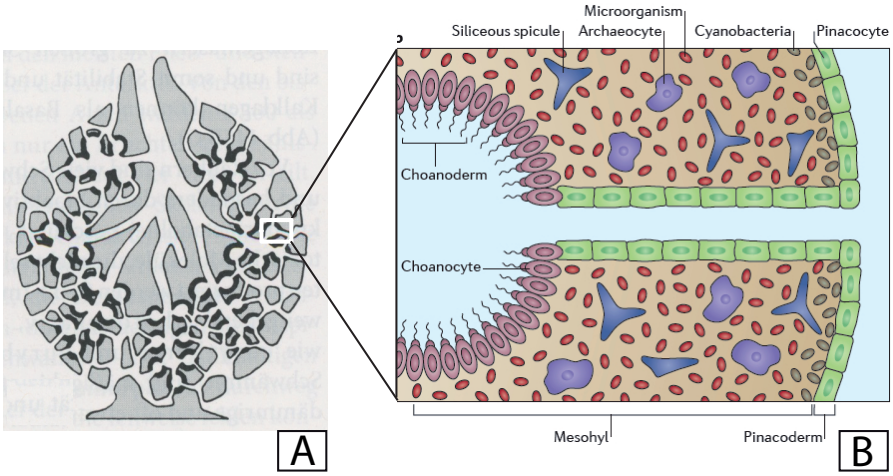

Figure 0.1. Body plan of sponges: (A) schematic overview of a sponge. Adopted from Westheide &Rieger (2013) , (B) an enlargement of the internal structure of a typical demosponge. Adopted from Hentschel et al. (2012).

mesohyl also contains dense and various communities of symbiotic microorganisms, which may include cyanobacteria mostly restricted to the light-exposed outer regions (Taylor et al. 2007b, Hentschel et al. 2012). Despite the simple body plan, sponges exhibit different shapes and sizes. They vary from a few millimeters, thin encrusting species, to giant sponges of a few meters in size (Hooper & Van Soest 2002). The structure of most sponges is supported by skeleton of siliceous or calcareous spicules, which have an enormous range of shapes, sizes, and patterns of organization (Bergquist 2001). Together with collagenous tissues, such as spongin, these enable the development of large individuals (Hooper & Van Soest 2002).

Sponges, which reproduce sexually or asexually through a variety of strategies, may be hermaphrodite or gonochoristic (Maldonado & Riesgo 2008). In terms of development, sponges can either be oviparous with external embryonic development and a free-swimming larval stage or viviparous with embryos brooded in the mesohyl, where larvae are formed before they are subsequently released into ambient water (Maldonado & Riesgo 2008). In the development cycle of a few demosponges, embryos grow directly into juveniles without the free-swimming larvae stage

For several decades, sponges have attracted attention as the most prolific marine producers of biologically active natural products (Taylor et al. 2007b). Sponge-

derived compounds fulfill in host a variety of functions, including their use as predator repellents, anti-pathogen and anti-fouling agents, competition facilitation, involvement in communication between individuals, and involvement in sponge reproduction (Hay & Fenical 1996, Thoms et al. 2006, Hay 2009, Turon et al. 2009). Each year, more then 200 new bioactive secondary metabolites with a wide range of biotechnologically relevant properties are reported from sponges (Blunt et al. 2013) due to the wide range of their activities including anticancer, anti-inflammatory, antimicrobial, antifungal, antiviral, and antimalarial functions (Faulkner 2002, Blunt et al. 2003, Proksch et al. 2003, Newman & Cragg 2004, Sipkema et al. 2005, Piel 2009). Numerous studies have shown that many secondary metabolites obtained from sponges are, in fact, produced by microorganisms harbored by those hosts (Kobayashi & Ishibashi 1993, Bewley & Faulkner 1998, Schmidt et al. 2000, Proksch et al. 2003, Piel et al. 2004a, Piel et al. 2004b, Glaser & Mayer 2009).

Microbial diversity in sponges

Marine sponges host abundant and diverse microbial communities (Taylor et al. 2007b, Webster & Taylor 2012), and those having the most ancient symbiotic associations between microorganisms and metazoa are estimated to have existed for 600 million years (Wilkinson 1984). The terms "symbiosis" and "symbiont," which are used throughout this thesis with their broadest possible definition, refer simply to any close, permanent, and long-term relationship between two or more different organisms (similar as by Taylor et al. 2007b). Sponges differentiate between bacterial symbionts and "food bacteria" (Wilkinson et al. 1984, Wehrl et al. 2007); the density of symbiotic communities in sponges exceeds 3-4 orders of magnitude the density of microorganisms in the surrounding seawater (see review Taylor et al. 2007b). Sponges with bacterial population density of 10^8-10^{10} bacteria per gram of wet weight were defined as "bacteriosponges" or "high-microbial-abundance sponges," whereas up to 70% biomass could consist of microbial symbionts (Wörheide 1998). In the same habitat, "low-microbial-abundance sponges" may coexist with distinctly lower bacterial population density of 10^5-10^6 bacteria per gram of sponge wet weight (Vacelet & Donadey 1977, Hentschel et al. 2002, Hentschel et al. 2006). Advances in molecular techniques over the past three decades have greatly improved our knowledge of microbial

diversity in sponges. To date, at least 28 bacterial phyla (18 formerly described and 10 bacterial candidate phyla), as well as major archaeal linages, have been reported from sponges (Fig. 0.2) based on cultivation and/or conventional molecular approaches such as 16S rRNA gene library construction (Hentschel et al. 2012, Webster & Taylor 2012, Webster et al. 2013). The application of next generation sequencing methods have allowed the detection of several more phyla (Lee et al. 2011, Schmitt et al. 2012a). Phylogenetic analyses have shown that members of the phyla *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Proteobacteria*, (especially the *Alpha*, *Delta*, and *Gamma* classes), as well as the candidate phylum "*Poribacteria*," occur most frequently among sponge microbiota and therefore are recognized as "core" taxa and the dominant sponge symbionts (Taylor et al. 2007b, Hentschel et al. 2012, Webster et al. 2013).

Microbial associations in sponges reveal low temporal variability and appear to be fairly stable in individuals and through time (Taylor et al. 2004, Webster et al. 2004, Hentschel et al. 2006). Numerous studies have provided evidence for specific microbial consortia in sponges from different oceans and for their differences from those in the surrounding water (Wilkinson 1978, Santavy et al. 1990, Hentschel et al. 2002, Taylor et al. 2004, Taylor et al. 2007b). Moreover, a new candidate phylum "*Poribacteria*" of sponge-specific bacteria, which do not occur in seawater or sediment samples and are not yet cultivable in the laboratory, have been reported (Fieseler et al. 2004). In the original study using phylogenetic analyses, Hentschel et al. (2002) indicated the existence of monophyletic, sponge-specific 16S rRNA sequence clusters, where 70% of the 190 sponge-derived sequences belonged. Moreover, Hentschel et al. (2002) established criteria for the definition of monophyletic, sponge-specific clusters: a group of at least three sequences that (i) are found in different host sponge species and/or from different geographic locations, (ii) are more similar to each other than to any other sequence from non-sponge source, and (iii) cluster together independently of the tree construction method. Subsequent studies reported further sponge-specific 16S rRNA sequence clusters from different sponges (Hill 2004, Schirmer et al. 2005, Hill et al. 2006, Taylor et al. 2007b). However, the next generation sequencing analysis of several samples recently detected the putatively sponge-specific bacteria in different marine environments, although generally at extremely low

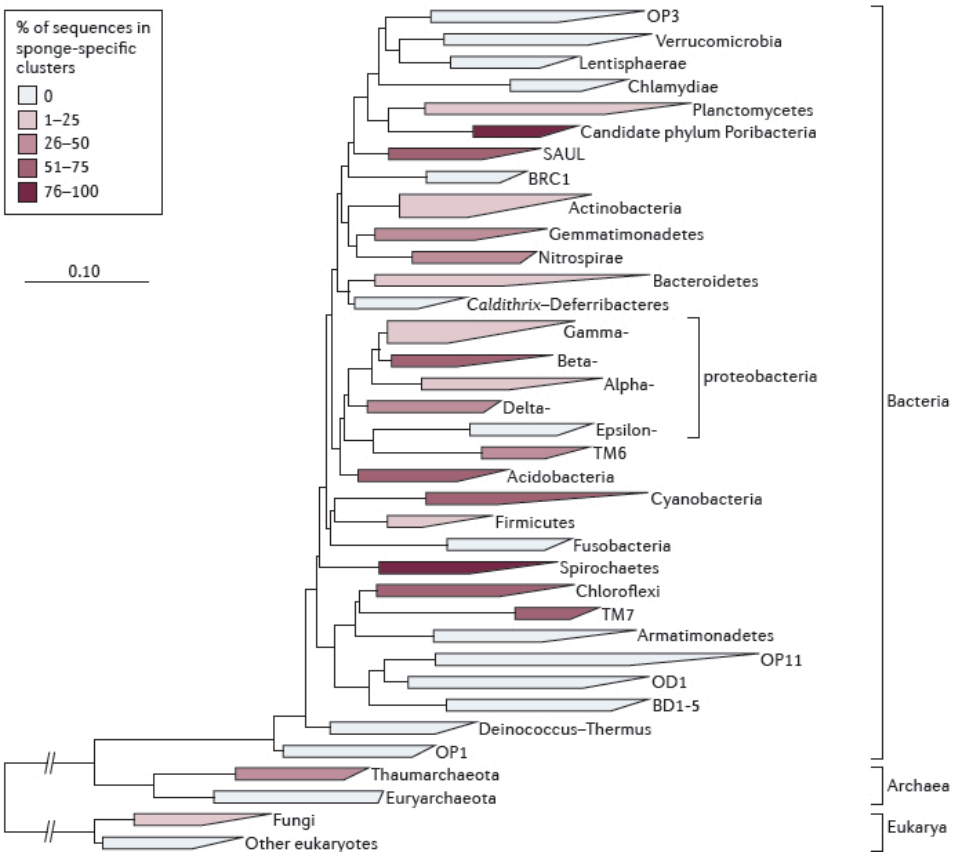

Figure 0.2. Diversity and specificity of marine sponge-associated microorganisms. Several phyla detected using pyrosequencing are not included. Adopted from Hentschel et al 2012.

abundances. Yet the studies also suggested that those bacteria might survive outside of a sponge host (Webster et al. 2010, Taylor et al. 2013). Nevertheless, Gloeckner and colleagues (2013) have produced new evidence that spawning leads to a 50% reduction of bacteria cells in the mesohyl of adult *Ectyoplasia ferox* and that in addition to symbiotic microorganisms found in embryos, a fraction of bacteria might be released into seawater. These findings might therefore explain the presence of symbiont-specific 16S rRNA sequences detected in the seawater sampled, e.g., around *Rhopaloides odorabile* during the spawning season (Webster et al. 2010).

The existence of sponge-specific microbes was recently confirmed through comprehensive phylogenetic analyses of 7546 sponge-derived 16S and 18S rRNA sequences, as nearly one-third of the analyzed sequences fell into monophyletic,

sponge-specific sequence clusters (SSC/SCC) (Simister et al. 2012). However, due to the generally short length of the sequences, the 454 data was excluded from this analysis; therefore, the existence of "sponge-specific" microbes in non-sponge samples cannot be ruled out (Simister et al. 2012). The most abundant sponge-specific clusters occurred among *Chloroflexi*, *Cyanobacteria*, "*Poribacteria*," *Betaproteobacteria*, and *Acidobacteria* (Fig. 0.2) (Simister et al. 2012). Despite the evidence of sponge-specific clusters, recent analysis has demonstrated the structure and composition of microbial communities as specific for particular sponge species and has excluded a correlation between host phylogeny and arrangement of symbionts (Webster et al. 2010, Lee et al. 2011, Montalvo & Hill 2011, Erwin et al. 2012). Erwin et al. (2012) classified numerous physical, chemical and biological conditions that may have an impact on the structure of symbiotic communities in marine sponges and showed that host-specific factors, such as mesohyl conditions, shape the structure of sponge-associated microbiota.

Questions about the origin, evolution, and maintenance of sponge-microbe associations constitute one of the important future directions of the research on microbial symbiosis in sponges (Taylor et al. 2007a, Vogel 2008, Webster & Blackall 2008, Hentschel et al. 2012, Webster & Taylor 2012). In a comprehensive review predicated on classical methods and molecular approach data, Taylor and colleagues (2007b) considered various scenarios of evolution of microbial symbiosis in sponges including ancient symbiosis maintained by vertical transmission, parental and environmental symbiont transmission, and environmental acquisition. The passage of complex assemblages of symbionts from adult sponge to next generations was first observed fifty years ago by Lévi and Porte (1962) using electron microscopy. Since then, numerous studies using microscopic methods have proven a vertical transmission of sponge symbionts (Gaino et al. 1987, Kaye 1991, Sciscioli et al. 1991, Usher et al. 2001, Ereskovsky et al. 2005, de Caralt et al. 2007), and more recently, have used molecular techniques including 16S rRNA gene library sequencing, denaturing gradient gel electrophoresis (DGGE), and fluorescence in situ hybridization (FISH) (Enticknap et al. 2006, Schmitt et al. 2007, Sharp et al. 2007, Schmitt et al. 2008, Steger et al. 2008, Lee et al. 2009, Gloeckner et al. 2013). The high sponge-specificity of microbial symbionts, absent from the surrounding seawater, served in the ongoing discussion on the origin and maintenance of microbial communities as one further argument for vertical transmis-

sion (Taylor et al. 2007b). The second strategy, where sponges acquired their symbionts from the surrounding sea water during filter-feeding process, could be evidenced only indirectly (Hentschel et al. 2012, Schmitt et al. 2012a). Uniform distribution of symbionts and general conformity in the microbial signatures in taxonomically distantly related sponges, with geographically non-overlapping distributions patterns (Hentschel et al. 2002, Olson & McCarthy 2005, Hill et al. 2006, Taylor et al. 2007b) and relatively small divergence between the sponge-derived microbial 16S rRNA gene sequences in SSC/SCC (Taylor et al. 2007b) were cited as arguments in support of this thesis. Detection of sponge-specific microorganisms in several marine environments (Webster et al. 2010, Taylor et al. 2013) suggested that members of the rare seawater biosphere can act as seed organisms for sponge-specific microbes (Webster et al. 2010). Support for this hypothesis is provided by the fact that sponges can differentiate between the functional categories food bacteria and bacterial symbionts taken from seawater (Wilkinson et al. 1984, Wehrl et al. 2007). Recent studies have indicated that microbial communities in marine sponges are being shaped instead through a combination of both strategies (Hentschel et al. 2012, Schmitt et al. 2012a). Nevertheless, several issues concerning the evolutionary origin and timing of the strategies shaping sponge-microbe associations remain unresolved.

Coralline sponges

Coralline sponges, also called sclerosponges, are unique members of the phylum Porifera (Reitner 1992, Wörheide 2008). In addition to a spicular skeleton, which is characteristic for recent sponges, coralline sponges build an unusual solid calcareous skeleton (Reitner 1992, Chombard et al. 1997), similar in appearance to some reef building corals. During long periods of the earth's history, sclerosponges (e.g., "Stromatoporoids," "Chaetetids," and "Sphinctozoans") dominated as diverse and abundant reef-building organisms (Vacelet 1985). Beginning in the late Jurassic period, hermatypic corals replaced them in their reef building function (Reitner 1992). Coralline sponges were thought to be extinct until their rediscovery in the late 1960s (Hartman 1969). Today, only approximately 15 taxa live. They are mainly restricted to the cryptic niches of coral reefs with reduced light and strict oligotrophic conditions such as caves, and deeper fore-reef areas (Reitner 1992, Wörheide 1998). Coralline sponges are long-lived and grow slowly

(0.15 to 1.2mm year^{-1}) (Fallon & Guilderson 2005). Their skeletons enable reconstructions of the paleoclimate from proxy records of salinity and water temperature over the 100 to 1000 year time range (Fallon et al. 2005) and also have provided insight into early mechanisms of biomineralization (Jackson et al. 2007). Sclerosponge genera, such as *Acanthochatetes*, *Vaceletia* or *Astrosclera*, are regarded as "living fossils" due to their occupation of the same ecological niches for hundreds of millions of years. In addition, these organisms display very similar morphological characteristics when compared to their fossil relatives that lived millions of years ago (Reitner et al. 2001). Fossil records of Silurian stromatoporoids found near ubiquitous microbial laminae, or less commonly encrusted by cyanobacteria, denote close associations (Soja et al. 2003) and might indicate an early stage of sponge-microbial symbiosis. Therefore, coralline sponges might provide insight into the evolution of sponge-microbial associations. Microbial communities in coralline sponges have not hitherto been investigated using molecular techniques.

Vaceletia crypta

The coralline sponge *Vaceletia* is the only recent member of a sphinctozoan-like sponges, which were reef-building organisms in the Permo-Triassic (Wörheide & Reitner 1996, Vacelet 2002). The discovery of *Vaceletia crypta* by Vacelet (1977) reversed the common belief that sphinctozoan-type coralline sponges were long extinct (see also Wörheide and Reitner 1996, Wörheide 2008). *Vaceletia* widely occurs throughout the Indo-Pacific in semi-closed cavities of coral reefs, front reef caves, and bathyal environments; it has been reported at depth ranging from 10 m to 530 m (Vacelet 2002). Based on the analyses of partial 28S and full-length 18S rDNA sequences, Wörheide (2008) showed that monophyletic taxon *Vaceletia* belongs to the Keratosa. *Vaceletia* had the highest affinities to the (possibly paraphyletic) extant order Dictyoceratida, which includes the commonly known bath sponges (Wörheide 2008).

Vaceletia has long been considered a widespread monotypic genus with a single species, *V. crypta*. However, several morphotypes with different growth modes (solitary vs. colonial) of *Vaceletia* have been discovered in the Indo-Pacific, although their taxonomic status remained unclear (Vacelet *et al.* 2002; Wörheide & Reitner 1996). The colonial form, thus far only found in shallow water reef caves

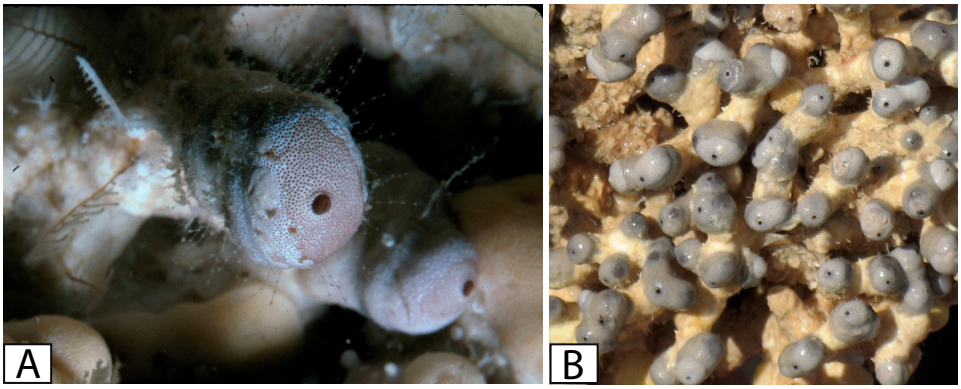

Figure 0.3. (A) *Vaceletia crypta* from Guam, dimension unknown, photo adopted from www.flmnh.ufl.edu; (B) colonial *Vaceletia* from Coral Sea, diameter approx. 5 cm, photo Karlińska-Batres

in the western Pacific, has a reef-building capability. The solitary *V. crypta* (Fig 0.3), non-colonial form, has no reef building potential (Vacelet 2002) and is more widespread in the darkest areas of reef caves of Indo-Pacific (Wörheide & Reitner 1996, Wörheide 2008). The living part reaches 5-9 mm (height) and 3 mm (diameter), and has a grey color (Vacelet 2002).

Astrosclera willeyana

Astrosclera willeyana (Fig. 0.4) is considered to be a living relative of the long-extinct "Stromatoporoidea," which formed extensive reefs during the Paleozoic and Mesozoic eras (Wood 1987, Chombard et al. 1997). Genus *Astrosclera* was thought to be extinct until it was rediscovered in the Pacific by Lister (1900) (Wörheide 1998). In today's coral reefs, *A. willeyana* is the most common coralline sponge throughout the Indo-Pacific, from the northern Red Sea to Tahiti (Wörheide 1998, 2008). *A. willeyana* occurs at depths from 1 m to 185 m (Hartman 1980); in shallow waters, it can be found mainly in caves, sometimes at the dimly lit cave entrances and under overhangs less than 10 m (Wörheide 1998). The very darkest areas of caves almost entirely lack *A. willeyana* (Wörheide 1998). As with all coralline sponges, *A. willeyana* grows slowly, at a rate of 0.2-1.2 mm/a (Wörheide 1998, Fallon & Guilderson 2005) and has a pyriform-half spherical (mushroom) growth form (Wörheide 1998). The color of *A. willeyana* depends on the light intensity and varies from bright salmon orange in dark areas to greenish (caused by green algae) and/or red by overhangs and cave entrances with dim

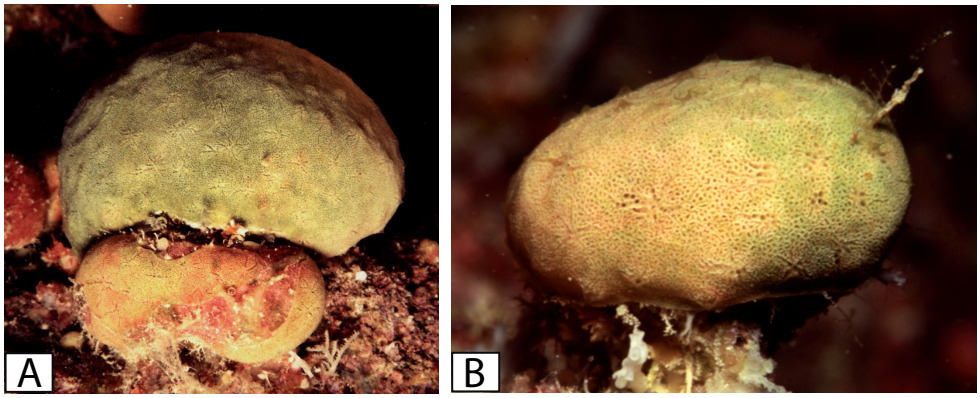

Figure 0.4. *A. willeyana* from Osprey Reef; diameter approx. 9 cm (A) and approx. 6 cm (B). Pictures adopted from Wörheide (1998)

lighting (Wörheide 1998). The living tissue of *A. willeyana* that contains the associated microorganisms, penetrates the basal skeleton to a maximum depth of 50% in small specimens; this ratio decreases with increasing specimen size (Wörheide et al. 2007). In a detailed characterization of the *A. willeyana* from the Indo-Pacific, Wörheide (1998) described the large bacterial populations in their soft tissue, mostly rod- or coccoid-shaped, although he also noted an unequal distribution of bacteria in all of the tissue zones. The choanosome contained a large number of symbiotic bacteria; here, the number in some areas exceeded 70% of total biomass (Wörheide 1998). However, some parts of the sponges' tissues were nearly free of symbiotic bacteria (Wörheide 1998). Based on TEM studies, Wörheide (1998) distinguished four major bacterial morphotypes: rod-shaped; spherical to ovoid with a dense membrane; ellipsoid with a dense membrane and surrounded by loosely bound EPS sheets; and larger bacteria with a diffuse protoplasm and outer "capsule" (supposed EPS capsule). Jackson et al. (2010) employed *A. willeyana* as a model to elucidate the early mechanisms of biocalcification. This study showed that in *A. willeyana* remaining bacterial matter are entrapped to seed the growth of $CaCO_3$ crystals during the process of biomineralization. Moreover, based on fossil evidence, this study implied that the reef-building stromatoporoids from the Paleozoic and Mesozoic eras conducted similar processes of bacterially induced skeleton formation (Jackson et al. 2010). Therefore, this microbial–metazoan relationship might have established some ancient reef ecosystems (Jackson et al. 2010). Jackson et al. (2011) recently published further data suggesting ancient origins of the sponge-microbial association. This study indicated a

horizontal transfer of a gene encoding a protein that is most likely involved in skeletogenesis in *A. willeyana* from a bacterium into the *A. willeyana* genome. This horizontal gene transfer (HGT) event may have contributed to the evolution of *A. willeyana*'s bodyplan (Jackson et al. 2011). This first example of an HGT event into a sponge genome from a prokaryote provided other evidence supporting an ancient origin for the *A. willeyana*-microbial association (Jackson et al. 2010); however, the identity of the microbial associates of this coralline sponge was still undetermined.

Aims of this study

Through the application of molecular methods for the detailed study of microorganisms associated with sponges of the genus *Astrosclera* and *Vaceletia*, the aim of the study was (1) to explore hitherto unknown microbial diversity in coralline sponges, (2) to determine whether the microbial communities of extant coralline sponges which represent long-extinct ancient groups, differ from those reported from other sponges or show some specific microbial patterns, and (3) to gain insight into the mechanisms of maintenance and evolution of microbial symbiosis in sponges.

In this project microbial 16S rRNA gene, a standard marker to examine the richness and diversity of microorganisms in the environment (Woese 1987, Pace 1997), was amplified from numerous samples of coralline sponges *Vaceletia crypta* and *Astrosclera willeyana*. Subsequently three extended clone libraries obtained from different specimens were constructed and sequenced, and denaturing gradient gel electrophoresis (DGGE) analyses were performed with the remaining samples. These methods allowed us to complete a detailed characterization of microbiota associated with *V. crypta* and to conduct an investigation into whether growth mode and/or putative sister-species relationships lead to differences in microbial diversity (**Chapter 1**). Furthermore we explored the spatial variability in sponge-derived microbial communities between *A. willeyana* from diverse sites along the GBR, and we surveyed the taxonomic composition of microbial associates from one of *A. willeyana* from the GBR (**Chapter 2**). We subsequently compared this community with a microbial community associated with *A. willeyana* from the northern Red Sea, and we explored the differences in the symbiotic

communities of *A. willeyana* over a wide geographic range from the Red Sea to the central Pacific (**Chapter 3**). Finally, we examined the differences between the microbial associates of two co-occurring *A. willeyana* and *V. crypta* (**Chapter 4**).

Chapter 1

Microbial diversity in the coralline sponge *Vaceletia crypta*

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Microbial diversity in the coralline sponge *Vaceletia crypta*

Abstract

Coraline sponges of the genus *Vaceletia* are regarded as a 'living fossils', the only recent members of the so-called 'sphinctozoan-type' sponges that contributed to reef-building during the Palaeozoic and Mesozoic eras. *Vaceletia* species were thought to be extinct until the discovery of *V. crypta* in the 1970's. Here, we used molecular methods to provide first insights into the microbial diversity of these coralline sponges. Both denaturing gradient gel electrophoresis (DGGE) analyses of 19 *Vaceletia* specimens and the analysis of 427 clones from a bacterial 16S rRNA gene clone library of a specimen of *V. crypta* from the Great Barrier Reef (Australia) revealed high diversity and a complex composition with a relatively homogeneous phylogenetic distribution. Only a single archaeal 16S rRNA phylotype was recovered. The most abundant bacteria were the *Chloroflexi* (35%). Of the microbial community, 60% consisted of the *Gammaproteobacteria, Gemmatimonadetes*, *Actinobacteria, Nitrospira, Deltaproteobacteria*, *Deferribacteres*, and *Acidobacteria*, with nearly equal representation. Less abundant members of the microbial community belonged to the *Alphaproteobacteria* (3%), as well as to the *Poribacteria, Betaproteobacteria, Cyanobacteria, Spirochaetes*, *Bacteroidetes*, *Deinococcus-Thermus* and *Archaea* (all together 4%). Of the established 96 OTUs, 88% were closely related to other sponge-derived sequences, and thereof 71 OTUs fell into sponge- or sponge-coral specific clusters, which underscores that the "living fossil" coralline sponge *Vaceletia* shares features of its microbial community with other sponges. The DGGE cluster analysis indicated distinct microbial communities in the different growth forms (solitary and colonial) of *Vaceletia* species.

1.1. Introduction

Sponges (phylum Porifera) are arguably the earliest branching Metazoa (Philippe et al. 2009, Philippe et al. 2011), with a fossil record dating back nearly 700 million

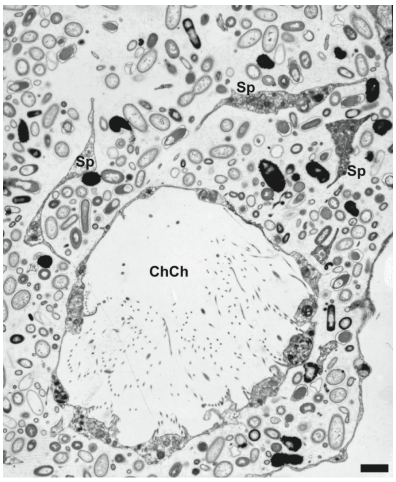


Figure 1.1. TEM micrograph of the choansosome of *V. crypta* with numerous bacterial cells and a few sponge cells (Sp) in the mesohyl, as well as in the choanocyte chamber (ChCh). Scale bar = 2μm

years (Erwin et al. 2011). Sponges harbor rich and diverse microbial communities in their tissues (for a comprehensive review see Taylor et al. 2007b). In the so-called 'high microbial abundance sponges' or 'bacteriosponges', microbes can make up to 40% of the biomass of the host, whereas the 'low microbial abundance sponges' harbor relatively small numbers of microorganisms (Reiswig 1981). Currently, 30 bacterial phyla, two major lineages of the *Archaea*, and several types of eukaryotic microbes associated with sponges have been identified (Hentschel et al. 2002, Hentschel et al. 2003, Taylor et al. 2007b, Hardoim et al. 2009, Webster et al. 2010, Schmitt et al. 2012b). Phylogenetic analyses have indicated a low temporal variability in marine sponge-associated microbial communities, which are fairly stable within individuals and through time (Taylor et al. 2004, Webster et al. 2004, Hentschel et al. 2006). Hentschel et al. (2002) established criteria to define monophyletic, sponge-specific "clusters": a group of at least three sequences that (i) are found in different host sponge species and/or from different geographical locations, (ii) are more similar to each other than to any other sequence from a non-sponge source, and (iii) are grouped together into one clade independent of the tree construction method. This and further studies have shown that taxonomically distantly related sponges with geographically non-overlapping distribution patterns and with host-specific secondary metabolite profiles contain surprisingly uniform microbial signatures (for a review see

Taylor et al. 2007b). However, a recent pyrosequencing study by Webster and colleagues reported the presence of *Poribacteria*, and 17 of the other 33 currently reported sponge-specific groups in seawater (Webster et al. 2010, Taylor et al. 2013). Therefore, this study questions the hypothesis that some groups of microbes are restricted to the sponge host and distinct from those in the surrounding seawater (Taylor et al. 2004).

Coralline sponges, also known as sclerosponges, are unique members of phylum Porifera (Reitner 1992, Wörheide 2008) because they build a solid secondary calcareous skeleton (Reitner 1992, Chombard et al. 1997) in addition to a primary, often spicular, skeleton. During long periods of the Earth's history, sclerosponges were dominant, diverse and abundant reef-building organisms (Vacelet 1985). These organisms were thought to be extinct until their rediscovery in the late 1960s (Hartman 1969). Today, only approximately 15 taxa live, mainly restricted to the cryptic niches of coral reefs with reduced light and oligothrophic conditions, such as caves and deeper fore-reef areas (Reitner 1992, Wörheide 1998). Sclerosponge genera, such as *Acanthochatetes*, *Vaceletia* or *Astrosclera*, are regarded as "living fossils" due to their occupation of the same ecological niches for hundreds of millions of years. In addition, these organisms display very similar morphological characteristics when compared to their fossil relatives that lived millions of years ago (Reitner et al. 2001). Therefore, coralline sponges might provide insight into the evolution of sponge-microbial symbioses. Fossil records from Silurian microbial reefs, with stromatoporoids neighboring ubiquitous microbial laminae or less commonly encrusted by cyanobacteria, might already indicate those close associations (Soja et al. 2003).

Sponges of the genus *Vaceletia*, which was thought to be extinct until its rediscovery in the 1970's (Vacelet 1977), systematically belong to the Keratosa, a group of sponges devoid of a primary mineralized skeleton (Wörheide 2008). Bacteria may make up more than 50% of the entire biomass of the sponge (Reitner & Wörheide 2002). *Vaceletia* species occur in two putative sister-species with different growth modes (solitary vs. colonial; Wörheide & Reitner 1996). For the detailed descriptions and definitions of the solitary and colonial forms see Vacelet (1988), Vacelet et al. (1992), Wörheide & Reitner (1996).

The microbial communities in coralline sponges have yet to be investigated in detail. Here, we aimed to perform detailed characterizations and sequenced a 16S

rRNA clone library of a randomly picked *Vaceletia crypta* specimen, the only validly described recent species of the genus. To further investigate whether growth mode and/or putative sister-species relationships lead to differences in microbial diversity, we additionally performed denaturing gradient gel electrophoresis (DGGE). We aimed to determine whether the microbial communities of these "living fossil" sponges differ from those reported from other sponges and, by phylogenetic analyses, contribute to the question of the maintenance of sponge-microbe symbioses.

1.2. Methods

1.2.1. *Sample collection*

Seventeen samples were collected by SCUBA diving at depths from 7 to 30 meters at several sampling sites in the Coral Sea and Pacific Ocean. Sponges were excised from the substrate using a chisel and a hammer and transferred directly (underwater) to plastic bags. Two samples were collected by a Remotely Operated Vehicle (ROV) at depths from 200 to 250 meters. The sampling details for all samples used are listed in Table 1.1. After collection, sponge samples were preserved either in silica gel (Erpenbeck et al. 2004), DMSO buffer (20% DMSO, 0.25 M EDTA, and NaCl to saturation, pH 8.0; adapted from Seutin et al. (1991) or 95% ethanol.

1.2.2. *DNA extraction*

Samples were rinsed with autoclaved Millipore water, and the preserved living tissue was cut and crushed aseptically with a sterile scalpel on a Petri dish. Total DNA was extracted from 3 mg of tissue using a Qiagen DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

1.2.3. *PCR amplification and cloning of the 16S rRNA genes genes of V. crypta from Yonge Reef, Great Barrier Reef, (sample no. GW947)*

The bacterial 16S rRNA genes were amplified from the DNA extract obtained by PCR using GoTaq polymerase (Promega GmbH, Mannheim, Germany) and universal bacterial primers (616F: 5'- AGA GTT TGA TYM TGG CTC AG -3' and 1525R: 5'- AGA AAG GAG GTG ATC CAG CC -3') (Lane 1991). Cycling condi

Table 1.1. Sample data of investigated *Vaceletia* sp. specimens, with collection sites details. * As the exact coordinates for the sampling sites in Palau and Solomon Islands were not available, the given coordinates are based on the Gazetteer of Conventional Names, Third Edition, August 1988, US Board on Geographic Names

	Sample No.	Location	Site (location)	Depth	Date	Latitude	Longitude
Solitary							
V. crypta	GW947	Coral Sea	Yonge Reef	8m	2006	14°57.212 S	145°61.489 E
V. sp.	GW5147.1	Palau	Siaes Tunnel #1	6m	2000	7°30 N*	134°30 E*
V. sp.	GW5147.2	Palau	Siaes Tunnel #2	6m	2000	7°30 N*	134°30 E*
V. sp.	GW5147.3	Palau	Siaes Tunnel #3	6m	2000	7°30 N*	134°30 E*
V. sp.	GW727		Solomon Islands	200m	2000	8°00 S*	159°00 E*
V. crypta	GW5450	Coral Sea	Osprey Reef #1	12m	1995	13°53.392 S	146°33.267 E
V. crypta	G 313971	Coral Sea	Osprey Reef #2	10m	1999	13°48.063 S	146°32.731 E
V. crypta	G 313989	Coral Sea	Osprey Reef #3	9m	1999	13°53.392 S	146°33.267 E
V. crypta	G 316280	Coral Sea	Osprey Reef #4	30m	2002	13°50.09 S	146°33.07 E
V. sp.	G 316297	Coral Sea	Holmes Reef	12m	2002	16°30.629 S	147°50.400 E
Colonial							
V. sp.	G 318578	Norfolk Ridge	Jumeau-West	240m	2001	23°40.766 S	168°00.602 E
V. sp.	G 313956	Coral Sea	Bougainville Reef #1	10m	1999	15°28.934 S	147°06.076 E
V. sp.	G 316289	Coral Sea	Bougainville Reef #2	25m	2002	15°28.934 S	147°06.076 E
V. sp.	G 316284	Coral Sea	Osprey Reef #5	14m	2002	13°53.5 S	146°33.1 E
V. sp.	G 316001	Coral Sea	Osprey Reef #4	8m	1999	13°56.594 S	146°35.909 E
V. sp.	G 313993	Coral Sea	Osprey Reef #6	10m	1999	13°53.428 S	146°33.300 E
V. sp.	G 313986	Coral Sea	Osprey Reef #7	7m	1999	13°49.803 S	146°33.940 E
V. sp.	G 313979	Coral Sea	Osprey Reef #8	15m	1999	13°49.744 S	146°33.958 E
V. sp.	G 313972	Coral Sea	Osprey Reef #9	10m	1999	13°48.063 S	146°32.731 E

tions for the Biometra thermocycler were as follows: an initial denaturation step (2 min at 95°C) followed by 35 cycles of denaturation (30 s at 94°C), primer annealing (1 min at 55°C), elongation (2 min + 4 s at 72°C) and a final extension step (5 min at 72°C). After purification using the mi-PCR Purification kit (metabion GmbH, Martinsried, Germany), the DNA was subsequently cloned into the plasmid cloning vector using an Invitrogen TOPO® TA Cloning Kit for Sequencing according to the manufacturer's instructions (Life Technologies GmbH, Darmstadt, Germany). The inserts of 427 clones were PCR reamplified using vector specific primers (M13) (Sambrook & Russell 2001) and Promega GoTaq polymerase. Analytical digestions of PCR products of 1500 base pairs length were performed in single reactions using the restriction enzyme *Msp*I (Fermentas GmbH, St. Leon-Rot, Germany) following the manufacturer's instructions. Based on the restriction patterns, similar clones were grouped together and chosen ran-

domly for sequencing. Clones with undefined restriction patterns were additionally taken for sequencing. Prior to sequencing, amplified inserts were purified using a silica-based protocol modified after Boyle and Lew (1995).
For the amplification of archaeal 16S rRNA gene from the DNA extract, touchdown PCR using universal archaeal primers (21F: 5'- TTC CGG TTG ATC CYG CCG GA - 3' and 915R 5'- GTG CTC CCC CGC CAA TTC CT -3') (DeLong 1992, Raskin et al. 1994) and an annealing temperature decreasing from 60 to 50.5°C (30 s each) in 0.5°C increments was employed. The cycling conditions for the Biometra thermocycler using Promega GoTaq were as follows: one cycle of initial denaturation (2 min at 95°C); 35 cycles of denaturation (30 s at 94°C), primer annealing (30 s from 60°C minus 0.5°C), and elongation (2 min + 4 s at 72°C) followed by 25 cycles of denaturation (30 s at 94°C), primer annealing (30 s at 51°C), and elongation (2 min + 4 s at 72°C) and a final extension step (5 min at 72°C). A strong band of approx. 900 base pairs was excised, and the DNA was purified from an agarose gel using the E.Z.N.A Gel Extraction Kit (VWR International GmbH, Darmstadt, Germany) following the manufacturer's instructions and subsequently taken for sequencing.

1.2.4. *Sequencing*

Sequencing was performed by the Genomics Service Unit (Ludwig-Maximilians-Universität München) using the BigDye® Terminator v3.1 on a 48-capillary sequencer (ABI 3730, Applied Biosystems). For the cloned bacterial inserts, the primers: 610RII (5'- ACC GCG/T A/GCT GCT GGC AC -3') (Dotzauer et al. 2002), 616F and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') (Lane 1991), or 614F (5'- GTG CAT GGC TGT CGT CAG CTC G -3') (this study) were used. The archaeal PCR product was sequenced with AR20F primer (5'- TTC CGG TTG ATC CYG CCRG -3') (Moyer et al. 1998). CodonCode Aligner (http://www.codoncode.com/aligner/) was used for sequence editing and assembly. Sequences were checked for chimeras using the Bellerophon web applications (Huber et al. 2004). Chimera sequences were removed before further analyses.

1.2.5. *Phylogenetic analyses*

Phylogenetic analyses were performed using ARB (Ludwig et al. 2004). The ini-

tial ARB database was constructed from the SILVA Small Subunit rRNA Database (release 96; Pruesse et al. 2007) and from the database constructed by Taylor and colleagues, which contains sponge-derived sequences along with their closest relatives (Taylor et al. 2007b). Our sequences were compared to the sequences available in public databases using BLAST (http://blast.ncbi.nlm.nih.gov/), and the nearest relative sequences obtained from different sponges, corals and non-sponge sources, were also incorporated into the ARB Database. The sequences were aligned using the ARB Integrated Aligner. The alignment was checked and corrected manually for alignment errors. The partial sequences were added to the ARB database using the ARB parsimony "quick add" tool. Initial phylogenetic trees were evaluated using the neighbor-joining algorithm (Jukes-Cantor correction) using ARB. Subsequently, the alignment was exported from the ARB database, and maximum likelihood trees were constructed using RAxML (v.7.2.5; Stamatakis 2006), 1000 bootstrap replicates and the GTR+GAMMA model of sequence evolution. The resulting trees were visualized using the program FigTree (v.1.3.1) (http://tree.bio.ed.ac.uk/software/figtree/). Monophyletic, SSC/SCC were defined based on established criteria (Hentschel et al. 2002). Sequences obtained from the sponges and corals that grouped together into one clade independent of the tree reconstruction method (neighbor-joining and maximum likelihood) were regarded as SSC and/or SCC.

1.2.6. *Estimation of microbial diversity and statistical analyses*

Based on the distance matrix generated by ARB, the sequences were assigned to operational taxonomic units (OTUs) using Mothur (Schloss et al. 2009). The clones that were only analyzed by restriction digestion were assigned to corresponding OTUs based on their restriction patterns. For the analysis of an OTU, a cut-off value of 0.03 was used (Schloss & Handelsman 2005). The rarefaction curves were also calculated using Mothur. The curves were plotted using the R software package (http://www.R-project.org). The Shannon–Wiener index (Spellerberg & Fedor 2003) was calculated to determine the abundance and richness of the bacterial community associated with *V. crypta*. The Chao1 index (Colwell & Coddington 1994) was employed to estimate total species richness. Calculations were performed using the Mothur software. In order to determine

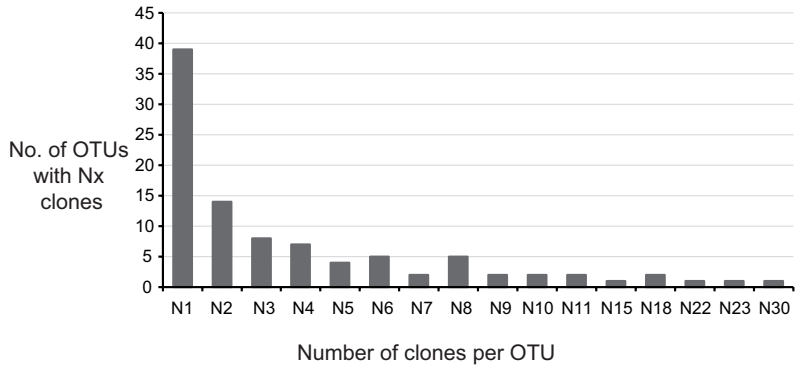


Figure 1.2. Distribution of 16S rRNA gene clones among the OTUs defined at the 97% similarity criterion. * N1, number of singletons, N2 number of doubletons, etc.

the phylogenetic composition of the clone library constructed from the microbial community associated with the *V. crypta* the percentage for each phylogenetic group was calculated based on the number of clones assigned to the particular group.

1.2.7. *Denaturing gradient gel electrophoresis*

19 samples of genus *Vaceletia* representing colonial (9 samples) and solitary (10 samples) growth forms were used for DGGE. All of the samples used are listed in Table 1.1. The bacterial 16S rRNA genes were amplified from the DNA extracts using touchdown PCR, Promega GoTaq polymerase and the universal bacterial primers 341F-GC and 907RC (Muyzer & Smalla 1998, Schäfer 2001). Cycling conditions for the Biometra thermocycler were as follows: one cycle of initial denaturation (2 min at 95°C); 15 cycles of denaturation (30 s at 94°C), primer annealing (30 s from 58°C minus 0.5°C), and elongation (2 min + 4 s at 72°C) followed by 25 cycles of denaturation (30 s at 94°C), primer annealing (30 s from 51°C minus 0.5°C), and elongation (2 min + 4 s at 72°C) and a final extension step (5 min at 72°C).

The DGGE analysis was performed with an Ingeny phorU-2 system (Ingeny International), Power Pac 300 (BioRad) as a power supplier, and a denaturing gradient of 30%–70% (urea and formamide) in a 6% polyacrylamide gel. Gels were run for 16 h at 180 V (60°C), then stained for 25 min in SYBR Gold (Molecular

Probes) and photographed using a RT Color SPOT camera and SPOT advance imaging software (Visitron Systems GmbH).
Gel images were analyzed using QuantityOne software (version 4.69, Bio-Rad). Similarities between the DGGE banding patterns were calculated using the band-matching Dice coefficient with an optimization at 0.75% and a tolerance level of 0.75%. The unweighted pair-group method with arithmetic averages (UPGMA) was used for cluster analysis to obtain similarity dendrograms.

1.2.8. *Nucleotide sequence accession numbers*

The 16S rRNA gene sequences representing all OTUs generated in this study were deposited in EMBL database under the accession numbers HE817775 to HE817870.

1.3. Results

1.3.1. *Clone library construction and OTU assignment*

427 clones were selected from the 16S rRNA clone library amplified from the solitary form of *V. crypta* from Yonge Reef, Great Barrier Reef (GBR, Australia), which possesses a high microbial abundance, in parts of the sponge outnumbering sponge cells (Fig. 1.1). From those clones, 253 were sequenced, and the remaining 174 clones were assigned to a particular OTU based on their restriction patterns. A single archaeal 16S rRNA sequence was retrieved, however the multiple genotypes cannot be entirely ruled out. Three sequences were discarded as chimeras. The remaining 250 sequences were clustered into 96 OTUs using a 97% similarity criterion. From those 96 OTUs, 39 were singletons (Fig. 1.2). Only 8 OTUs grouped more than 10 clones (two OTUs with 18 and 11 clones and single OTUs with 15, 22, 23 and with 30 clones, respectively).

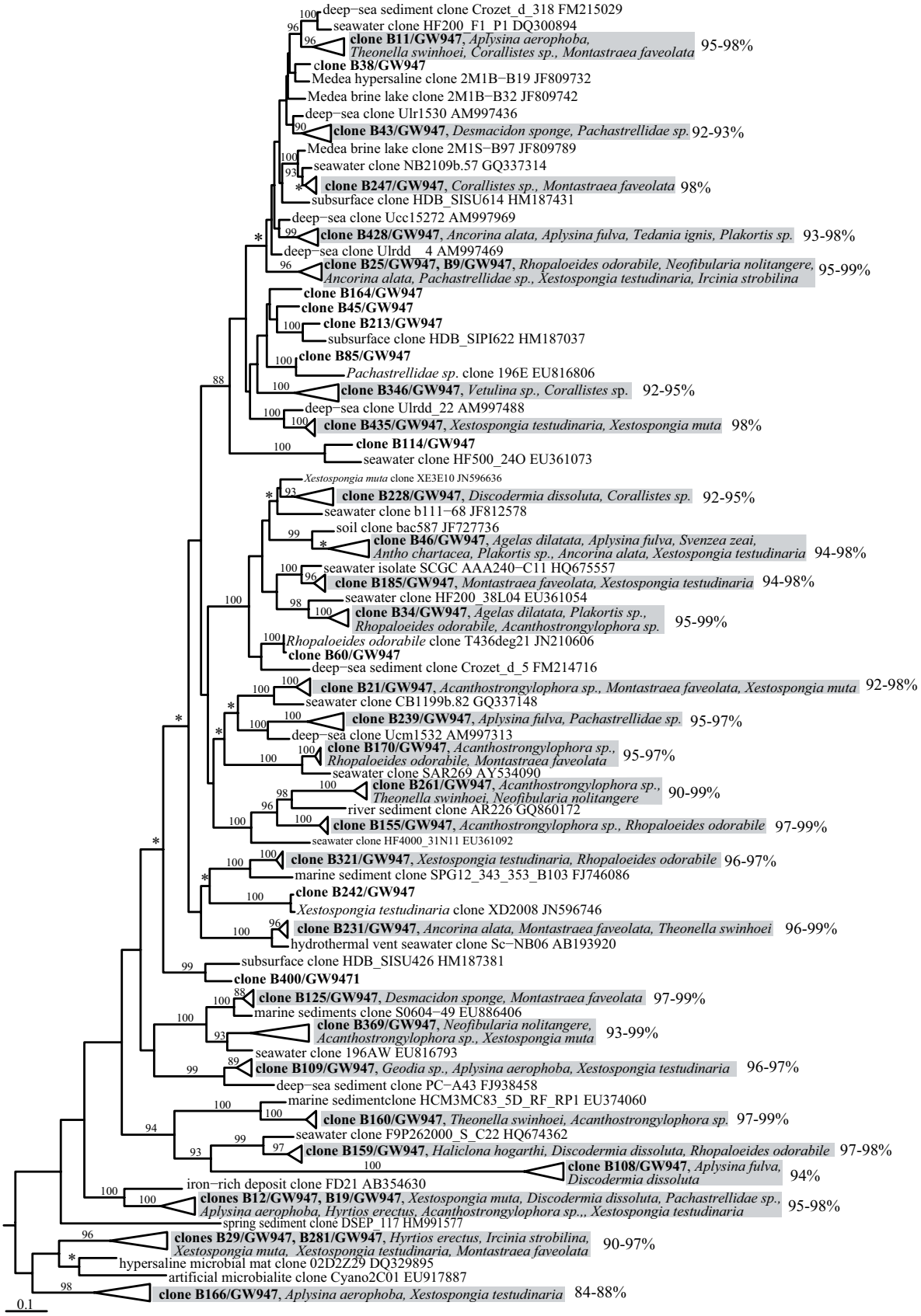
1.3.2. *Phylogenetic* analyses

Using BLAST, 88% of the 96 OTUs (84 OTUs) were found to be closely related with other previously described sponge- or coral-derived sequences. Of the OTUs, 70% (67 OTUs) were related to other sponge-derived 16S rRNA genes obtained from 22 different sponge species. A further 18% of the OTUs contained sequences obtained from four different species of corals as closest relatives. Of

those OTUs, 14 were related to 16S rRNA sequences obtained from *Montastraea faveolata*, and a single OTU was related to sequences obtained from *Oculina patagonica, Diploria strigosa* and *Erythropodium caribaeorum*. Only one OTU was closely related (99% similarity) to a validly described organism (*Delftia acidovorans*), and one was distantly related (91% similarity) to a 16S rRNA gene sequence from a chloroplast of a red alga. Further, 7% of the OTUs had sequences from marine environments as next relatives (3 OTUs from seawater, 1 OTU from basaltic glass from a seamount and 3 OTUs from sediment, with one of the 3 from deep sea). Three OTUs contained sequences from the terrestrial subsurface as next relatives. The results of the BLAST search are summarized in supplementary Table S1.1.

The phylogeny obtained with ARB showed that the majority of the sponge-derived microbial clones were assigned to the *Chloroflexi* (39 OTUs, number of clones n=144) and *Gammaproteobacteria* (13 OTUs, n=46). Clones affiliated with the *Deltaproteobacteria* (9 OTUs, n=29), *Acidobacteria* (6 OTUs, n=24), *Gemmatimonadetes* (5 OTUs, n=46), and *Alphaproteobacteria* (4 OTUs, n=11) were also observed. Numerous clones affiliated to the *Nitrospirae* and *Actinobacteria* revealed single and triple OTUs, (1 OTU, n=30) and (3 OTUs, n=34), respectively. The minor components of the clone library were clones affiliated with the *Poribacteria* (3 OTUs, n=4), *Betaproteobacteria* (2 OTUs, n=3), *Cyanobacteria* (2 OTUs, n=3), *Spirochaetes* (1 OTU, n=2), *Deinococcus-Thermus* (1 OTU, n=1), and *Bacteroidetes* (1 OTU, n=1). The single sequence obtained by PCR using universal archaeal primers was affiliated to the *Crenarchaeota*. Over 9% of the clone sequences (5 OTUs, n=30) were not classified using ARB database to any described phylum. However, based on the EMBL phylogeny the available sequences that were most similar implied an affiliation of these sequences with the phylum *Deferribacteres*. The phylogenetic trees present the OTUs with nearest similar sequences assigned to

Figure 1.3. Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to the phylum *Chloroflexi* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence →


deep−sea sediment clone Crozet_d_318 FM215029
seawater clone HF200_F1_P1 DQ300894
clone B11/GW947, Aplysina aerophoba, Theonella swinhoei, Corallistes sp., Montastraea faveolata 95-98%
clone B38/GW947
Medea hypersaline clone 2M1B−B19 JF809732
Medea brine lake clone 2M1B−B32 JF809742
deep−sea clone Ulr1530 AM997436
clone B43/GW947, Desmacidon sponge, Pachastrellidae sp. 92-93%
Medea brine lake clone 2M1S−B97 JF809789
seawater clone NB2109b.57 GQ337314
clone B247/GW947, Corallistes sp., Montastraea faveolata 98%
subsurface clone HDB_SISU614 HM187431
deep−sea clone Ucc15272 AM997969
clone B428/GW947, Ancorina alata, Aplysina fulva, Tedania ignis, Plakortis sp. 93-98%
deep−sea clone Ulrdd__4 AM997469
clone B25/GW947, B9/GW947, Rhopaloeides odorabile, Neofibularia nolitangere, Ancorina alata, Pachastrellidae sp., Xestospongia testudinaria, Ircinia strobilina 95-99%
clone B164/GW947
clone B45/GW947
clone B213/GW947
subsurface clone HDB_SIPI622 HM187037
clone B85/GW947
Pachastrellidae sp. clone 196E EU816806
clone B346/GW947, Vetulina sp., Corallistes sp. 92-95%
deep−sea clone Ulrdd_22 AM997488
clone B435/GW947, Xestospongia testudinaria, Xestospongia muta 98%
clone B114/GW947
seawater clone HF500_24O EU361073
Xestospongia muta clone XE3E10 JN596636
clone B228/GW947, Discodermia dissoluta, Corallistes sp. 92-95%
seawater clone b111−68 JF812578
soil clone bac587 JF727736
clone B46/GW947, Agelas dilatata, Aplysina fulva, Svenzea zeai, Antho chartacea, Plakortis sp., Ancorina alata, Xestospongia testudinaria 94-98%
seawater isolate SCGC AAA240−C11 HQ675557
clone B185/GW947, Montastraea faveolata, Xestospongia testudinaria 94-98%
seawater clone HF200_38L04 EU361054
clone B34/GW947, Agelas dilatata, Plakortis sp., Rhopaloeides odorabile, Acanthostrongylophora sp. 95-99%
Rhopaloeides odorabile clone T436deg21 JN210606
clone B60/GW947
deep−sea sediment clone Crozet_d_5 FM214716
clone B21/GW947, Acanthostrongylophora sp., Montastraea faveolata, Xestospongia muta 92-98%
seawater clone CB1199b.82 GQ337148
clone B239/GW947, Aplysina fulva, Pachastrellidae sp. 95-97%
deep−sea clone Ucm1532 AM997313
clone B170/GW947, Acanthostrongylophora sp., Rhopaloeides odorabile, Montastraea faveolata 95-97%
seawater clone SAR269 AY534090
clone B261/GW947, Acanthostrongylophora sp., Theonella swinhoei, Neofibularia nolitangere 90-99%
river sediment clone AR226 GQ860172
clone B155/GW947, Acanthostrongylophora sp., Rhopaloeides odorabile 97-99%
seawater clone HF4000_31N11 EU361092
clone B321/GW947, Xestospongia testudinaria, Rhopaloeides odorabile 96-97%
marine sediment clone SPG12_343_353_B103 FJ746086
clone B242/GW947
Xestospongia testudinaria clone XD2008 JN596746
clone B231/GW947, Ancorina alata, Montastraea faveolata, Theonella swinhoei 96-99%
hydrothermal vent seawater clone Sc−NB06 AB193920
subsurface clone HDB_SISU426 HM187381
clone B400/GW9471
clone B125/GW947, Desmacidon sponge, Montastraea faveolata 97-99%
marine sediments clone S0604−49 EU886406
clone B369/GW947, Neofibularia nolitangere, Acanthostrongylophora sp., Xestospongia muta 93-99%
seawater clone 196AW EU816793
clone B109/GW947, Geodia sp., Aplysina aerophoba, Xestospongia testudinaria 96-97%
deep−sea sediment clone PC−A43 FJ938458
marine sedimentclone HCM3MC83_5D_RF_RP1 EU374060
clone B160/GW947, Theonella swinhoei, Acanthostrongylophora sp. 97-99%
seawater clone F9P262000_S_C22 HQ674362
clone B159/GW947, Haliclona hogarthi, Discodermia dissoluta, Rhopaloeides odorabile 97-98%
clone B108/GW947, Aplysina fulva, Discodermia dissoluta 94%
iron−rich deposit clone FD21 AB354630
clones B12/GW947, B19/GW947, Xestospongia muta, Discodermia dissoluta, Pachastrellidae sp., Aplysina aerophoba, Hyrtios erectus, Acanthostrongylophora sp.,, Xestospongia testudinaria 95-98%
spring sediment clone DSEP_117 HM991577
clones B29/GW947, B281/GW947, Hyrtios erectus, Ircinia strobilina, Xestospongia muta, Xestospongia testudinaria, Montastraea faveolata 90-97%
hypersaline microbial mat clone 02D2Z29 DQ329895
artificial microbialite clone Cyano2C01 EU917887
clone B166/GW947, Aplysina aerophoba, Xestospongia testudinaria 84-88%
0.1

the *Chloroflexi* (Fig. 1.3), *Proteobacteria* (Fig. 1.4) and to all other phyla (Fig. 1.5).

1.3.3. *Sponge-specific and sponge-coral clusters*

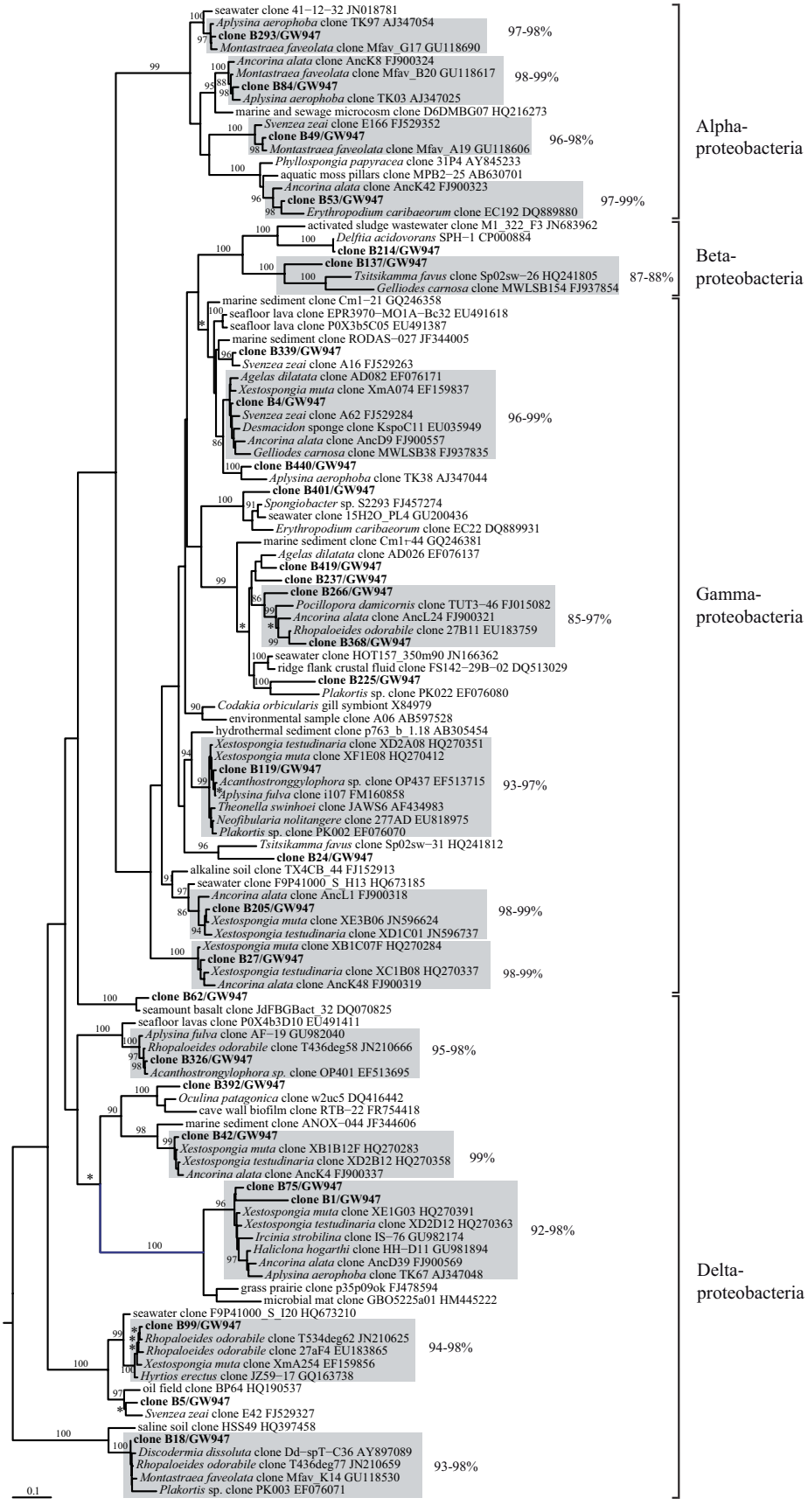
From the 84 OTUs that contained sequences similar to sequences obtained from other sponges or corals, 71 OTUs (85%) were assigned to 63 SSC or SCC. The largest number of clusters belonged to the phylum *Chloroflexi* (27 clusters with 30 OTUs). A further 15 clusters were defined among the *Proteobacteria* (15 clusters with 16 OTUs). The SSC/SCC are indicated with grey-shaded boxes in the phylogenetic trees (Figs. 1.3-1.5).

1.3.4. *Estimation of microbial diversity and statistical analyses*

The microbial community composition was calculated for all clones affiliated to each phylogenetic group and revealed a high diversity with a complex composition (Fig. 1.6). The most abundant taxa were the *Chloroflexi* (35%). Due to the complexity, variety and diversity of the phylum *Proteobacteria,* the proteobacterial classes were treated as separate phylogenetic groups (*Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*) and as separate groups for the estimations of the microbial community composition. If, for the calculation of community composition, the *Proteobacteria* were regarded as one single group (phylum), it would be the second most abundant group in the community (22%) behind the *Chloroflexi*.

A rarefaction analysis was used to assess whether the number of clones sequenced from the library represented the full diversity of the microbial community. The rarefaction curves calculated using 97% and 95% cut-off criteria for grouping OTUs at the "species" and "genus" levels as well as 90% did not reach a clear saturation (Fig. 1.7). However, according to the Chao1 index (Table 1.2),

Figure 1.4. Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to the phylum *Proteobacteria* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence →


seawater clone 41−12−32 JN018781
Aplysina aerophoba clone TK97 AJ347054
clone B293/GW947
Montastraea faveolata clone Mfav_G17 GU118690
97-98%
Ancorina alata clone AncK8 FJ900324
Montastraea faveolata clone Mfav_B20 GU118617
clone B84/GW947
Aplysina aerophoba clone TK03 AJ347025
98-99%
marine and sewage microcosm clone D6DMBG07 HQ216273
Svenzea zeai clone E166 FJ529352
clone B49/GW947
Montastraea faveolata clone Mfav_A19 GU118606
96-98%
Phyllospongia papyracea clone 31P4 AY845233
aquatic moss pillars clone MPB2−25 AB630701
Ancorina alata clone AncK42 FJ900323
clone B53/GW947
Erythropodium caribaeorum clone EC192 DQ889880
97-99%
Alpha-proteobacteria
activated sludge wastewater clone M1_322_F3 JN683962
Delftia acidovorans SPH−1 CP000884
clone B214/GW947
clone B137/GW947
Tsitsikamma favus clone Sp02sw−26 HQ241805
Gelliodes carnosa clone MWLSB154 FJ937854
87-88%
Beta-proteobacteria
marine sediment clone Cm1−21 GQ246358
seafloor lava clone EPR3970−MO1A−Bc32 EU491618
seafloor lava clone P0X3b5C05 EU491387
marine sediment clone RODAS−027 JF344005
clone B339/GW947
Svenzea zeai clone A16 FJ529263
Agelas dilatata clone AD082 EF076171
Xestospongia muta clone XmA074 EF159837
clone B4/GW947
Svenzea zeai clone A62 FJ529284
Desmacidon sponge clone KspoC11 EU035949
Ancorina alata clone AncD9 FJ900557
Gelliodes carnosa clone MWLSB38 FJ937835
96-99%
clone B440/GW947
Aplysina aerophoba clone TK38 AJ347044
clone B401/GW947
Spongiobacter sp. S2293 FJ457274
seawater clone 15H2O_PL4 GU200436
Erythropodium caribaeorum clone EC22 DQ889931
marine sediment clone Cm1+44 GQ246381
Agelas dilatata clone AD026 EF076137
clone B419/GW947
clone B237/GW947
clone B266/GW947
Pocillopora damicornis clone TUT3−46 FJ015082
Ancorina alata clone AncL24 FJ900321
Rhopaloeides odorabile clone 27B11 EU183759
clone B368/GW947
85-97%
seawater clone HOT157_350m90 JN166362
ridge flank crustal fluid clone FS142−29B−02 DQ513029
clone B225/GW947
Plakortis sp. clone PK022 EF076080
Codakia orbicularis gill symbiont X84979
environmental sample clone A06 AB597528
hydrothermal sediment clone p763_b_1.18 AB305454
Xestospongia testudinaria clone XD2A08 HQ270351
Xestospongia muta clone XF1E08 HQ270412
clone B119/GW947
Acanthostronggylophora sp. clone OP437 EF513715
Aplysina fulva clone i107 FM160858
Theonella swinhoei clone JAWS6 AF434983
Neofibularia nolitangere clone 277AD EU818975
Plakortis sp. clone PK002 EF076070
93-97%
Tsitsikamma favus clone Sp02sw−31 HQ241812
clone B24/GW947
alkaline soil clone TX4CB_44 FJ152913
seawater clone F9P41000_S_H13 HQ673185
Ancorina alata clone AncL1 FJ900318
clone B205/GW947
Xestospongia muta clone XE3B06 JN596624
Xestospongia testudinaria clone XD1C01 JN596737
98-99%
Xestospongia muta clone XB1C07F HQ270284
clone B27/GW947
Xestospongia testudinaria clone XC1B08 HQ270337
Ancorina alata clone AncK48 FJ900319
98-99%
Gamma-proteobacteria
clone B62/GW947
seamount basalt clone JdFBGBact_32 DQ070825
seafloor lavas clone P0X4b3D10 EU491411
Aplysina fulva clone AF−19 GU982040
Rhopaloeides odorabile clone T436deg58 JN210666
clone B326/GW947
Acanthostrongylophora sp. clone OP401 EF513695
95-98%
clone B392/GW947
Oculina patagonica clone w2uc5 DQ416442
cave wall biofilm clone RTB−22 FR754418
marine sediment clone ANOX−044 JF344606
clone B42/GW947
Xestospongia muta clone XB1B12F HQ270283
Xestospongia testudinaria clone XD2B12 HQ270358
Ancorina alata clone AncK4 FJ900337
99%
clone B75/GW947
clone B1/GW947
Xestospongia muta clone XE1G03 HQ270391
Xestospongia testudinaria clone XD2D12 HQ270363
Ircinia strobilina clone IS−76 GU982174
Haliclona hogarthi clone HH−D11 GU981894
Ancorina alata clone AncD39 FJ900569
Aplysina aerophoba clone TK67 AJ347048
92-98%
grass prairie clone p35p09ok FJ478594
microbial mat clone GBO5225a01 HM445222
Delta-proteobacteria
seawater clone F9P41000_S_I20 HQ673210
clone B99/GW947
Rhopaloeides odorabile clone T534deg62 JN210625
Rhopaloeides odorabile clone 27aF4 EU183865
Xestospongia muta clone XmA254 EF159856
Hyrtios erectus clone JZ59−17 GQ163738
94-98%
oil field clone BP64 HQ190537
clone B5/GW947
Svenzea zeai clone E42 FJ529327
saline soil clone HSS49 HQ397458
clone B18/GW947
Discodermia dissoluta clone Dd−spT−C36 AY897089
Rhopaloeides odorabile clone T436deg77 JN210659
Montastraea faveolata clone Mfav_K14 GU118530
Plakortis sp. clone PK003 EF076071
93-98%
0.1

we sequenced over 70% of the predicted number of microbial species, which provides a representative picture of the core microbial community of *V. crypta*. In addition, using Sanger sequencing to cover the remaining 30% of the community would be costly and time-consuming (Schmitt et al. 2012b).

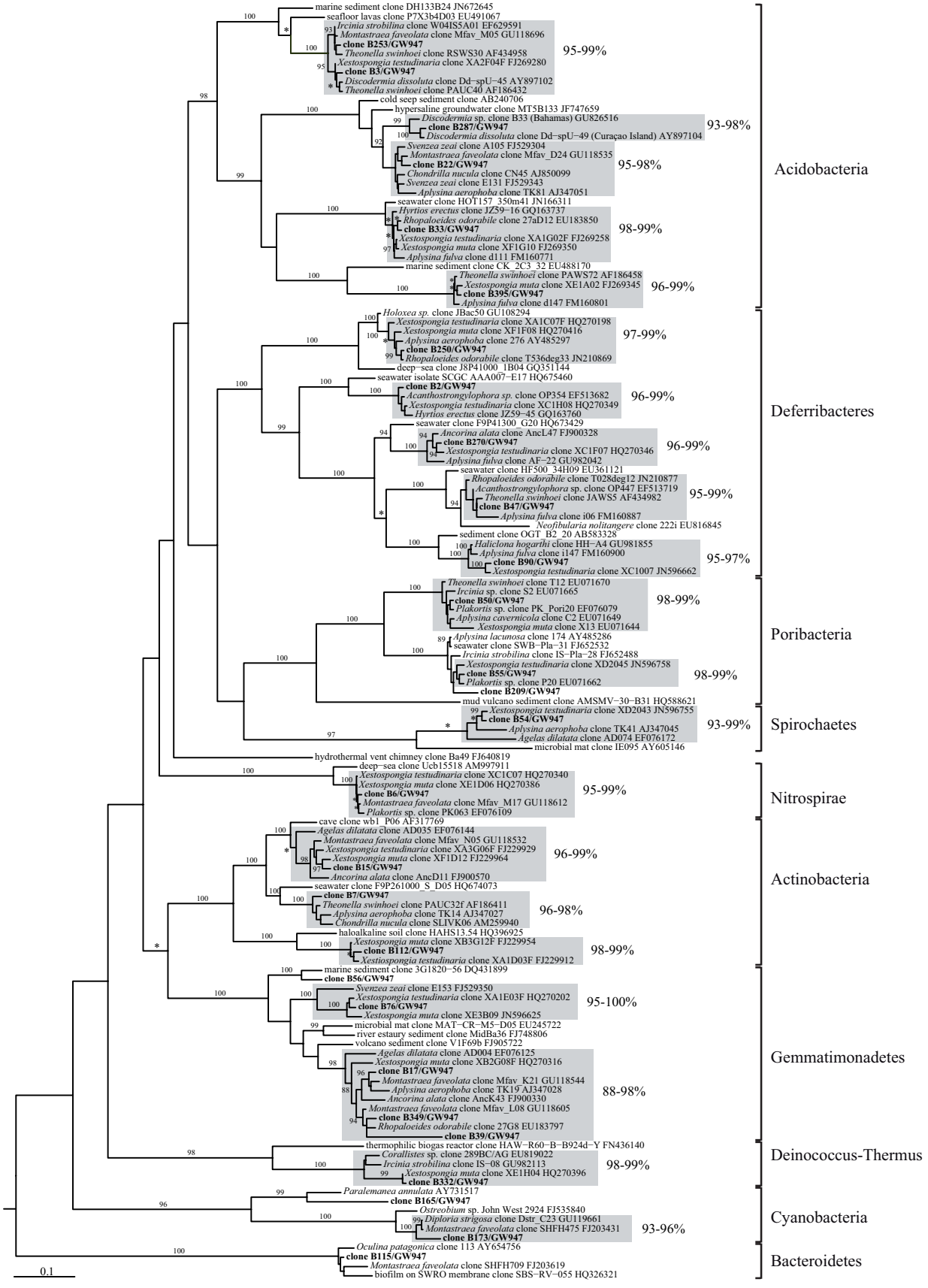
1.3.5. *Denaturing gradient gel electrophoresis*

DGGE analysis of nine colonial and ten solitary forms of individuals from genus *Vaceletia* indicated that the diversity of all the bacterial communities was very high. The number of bands ranged from 27 to 35 per sample. The lowest number of bands was obtained from the bacterial community associated with the solitary form of *Vaceletia* from Palau, Siaes Tunnel (sample #3, Fig. S1.1 of the Supplementary material), and the largest number of bands was obtained from the colonial form from Bougainville Reef (sample #2, Fig. S1.1 of the Supplementary material). The banding patterns displayed numerous co-occurring bands; however, only four bands were found in all samples from both growth forms. 18 bands were specific to the samples from the solitary *Vaceletia* form and 19 to the samples from the colonial form. A cluster analysis showed that the microbial communities appear to be growth-form specific (Fig. 1.8); however, the solitary specimens from Palau, which clustered together, displayed a higher affiliation to the cluster of colonial samples. The bacterial profiles for the samples obtained from Norfolk Ridge (colonial form) and from Solomon Island (solitary form), both from deeper sampling zones, did not cluster with the other samples.

1.4. Discussion

This is the first study assessing the phylogenetic diversity of *Bacteria* and *Archaea* in coralline sponges using molecular approaches. The 16S rRNA gene-based di-

Figure 1.5. Maximum likelihood phylogeny of *V. crypta*-derived 16S rRNA sequences affiliated to several phyla with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters; the percentage values next to the boxes indicate the similarity between the sequences belonging to the clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence →


marine sediment clone DH133B24 JN672645
seafloor lavas clone P7X3b4D03 EU491067
Ircinia strobilina clone W04IS5A01 EF629591
Montastraea faveolata clone Mfav_M05 GU118696
clone B253/GW947
Theonella swinhoei clone RSWS30 AF434958
Xestospongia testudinaria clone XA2F04F FJ269280
clone B3/GW947
Discodermia dissoluta clone Dd−spU−45 AY897102
Theonella swinhoei clone PAUC40 AF186432
95-99%
cold seep sediment clone AB240706
hypersaline groundwater clone MT5B133 JF747659
Discodermia sp. clone B33 (Bahamas) GU826516
clone B287/GW947
Discodermia dissoluta clone Dd−spU−49 (Curaçao Island) AY897104
93-98%
Svenzea zeai clone A105 FJ529304
Montastraea faveolata clone Mfav_D24 GU118535
clone B22/GW947
Chondrilla nucula clone CN45 AJ850099
Svenzea zeai clone E131 FJ529343
Aplysina aerophoba clone TK81 AJ347051
95-98%
seawater clone HOT157_350m41 JN166311
Hyrtios erectus clone JZ59−16 GQ163737
Rhopaloeides odorabile clone 27aD12 EU183850
clone B33/GW947
Xestospongia testudinaria clone XA1G02F FJ269258
Xestospongia muta clone XF1G10 FJ269350
Aplysina fulva clone d111 FM160771
98-99%
marine sediment clone CK_2C3_32 EU488170
Theonella swinhoei clone PAWS72 AF186458
Xestospongia muta clone XE1A02 FJ269345
clone B395/GW947
Aplysina fulva clone d147 FM160801
96-99%
Acidobacteria
Holoxea sp. clone JBac50 GU108294
Xestospongia testudinaria clone XA1C07F HQ270198
Xestospongia muta clone XF1F08 HQ270416
Aplysina aerophoba clone 276 AY485297
clone B250/GW947
Rhopaloeides odorabile clone T536deg33 JN210869
97-99%
deep−sea clone J8P41000_1B04 GQ351144
seawater isolate SCGC_AAA007−E17 HQ675460
clone B2/GW947
Acanthostrongylophora sp. clone OP354 EF513682
Xestospongia testudinaria clone XC1H08 HQ270349
Hyrtios erectus clone JZ59−45 GQ163760
96-99%
seawater clone F9P41300_G20 HQ673429
Ancorina alata clone AncL47 FJ900328
clone B270/GW947
Xestospongia testudinaria clone XC1F07 HQ270346
Aplysina fulva clone AF−22 GU982042
96-99%
seawater clone HF500_34H09 EU361121
Rhopaloeides odorabile clone T028deg12 JN210877
Acanthostrongylophora sp. clone OP447 EF513719
Theonella swinhoei clone JAWS5 AF434982
clone B47/GW947
Aplysina fulva clone i06 FM160887
Neofibularia nolitangere clone 222i EU816845
95-99%
sediment clone OGT_B2_20 AB583328
Haliclona hogarthi clone HH−A4 GU981855
Aplysina fulva clone i147 FM160900
clone B90/GW947
Xestospongia testudinaria clone XC1007 JN596662
95-97%
Deferribacteres
Theonella swinhoei clone T12 EU071670
Ircinia sp. clone S2 EU071665
clone B50/GW947
Plakortis sp. clone PK_Pori20 EF076079
Aplysina cavernicola clone C2 EU071649
Xestospongia muta clone X13 EU071644
98-99%
Aplysina lacunosa clone 174 AY485286
seawater clone SWB−Pla−31 FJ652532
Ircinia strobilina clone IS−Pla−28 FJ652488
Xestospongia testudinaria clone XD2045 JN596758
clone B55/GW947
Plakortis sp. clone P20 EU071662
clone B209/GW947
98-99%
Poribacteria
mud vulcano sediment clone AMSMV−30−B31 HQ588621
Xestospongia testudinaria clone XD2043 JN596755
clone B54/GW947
Aplysina aerophoba clone TK41 AJ347045
Agelas dilatata clone AD074 EF076172
93-99%
Spirochaetes
microbial mat clone IE095 AY605146
hydrothermal vent chimney clone Ba49 FJ640819
deep−sea clone Ucb15518 AM997911
Xestospongia testudinaria clone XC1C07 HQ270340
Xestospongia muta clone XE1D06 HQ270386
clone B6/GW947
Montastraea faveolata clone Mfav_M17 GU118612
Plakortis sp. clone PK063 EF076109
95-99%
Nitrospirae
cave clone wb1_P06 AF317769
Agelas dilatata clone AD035 EF076144
Montastraea faveolata clone Mfav_N05 GU118532
Xestospongia testudinaria clone XA3G06F FJ229929
Xestospongia muta clone XF1D12 FJ229964
clone B15/GW947
Ancorina alata clone AncD11 FJ900570
96-99%
seawater clone F9P261000_S_D05 HQ674073
clone B7/GW947
Theonella swinhoei clone PAUC32f AF186411
Aplysina aerophoba clone TK14 AJ347027
Chondrilla nucula clone SLIVK06 AM259940
96-98%
haloalkaline soil clone HAHS13.54 HQ396925
Xestospongia muta clone XB3G12F FJ229954
clone B112/GW947
Xestiospongia testudinaria clone XA1D03F FJ229912
98-99%
Actinobacteria
marine sediment clone 3G1820−56 DQ431899
clone B56/GW947
Svenzea zeai clone E153 FJ529350
Xestospongia testudinaria clone XA1E03F HQ270202
clone B76/GW947
Xestospongia muta clone XE3B09 JN596625
95-100%
microbial mat clone MAT−CR−M5−D05 EU245722
river estuary sediment clone MidBa36 FJ748806
volcano sediment clone V1F69b FJ905722
Agelas dilatata clone AD004 EF076125
Xestospongia muta clone XB2G08F HQ270316
clone B17/GW947
Montastraea faveolata clone Mfav_K21 GU118544
Aplysina aerophoba clone TK19 AJ347028
Ancorina alata clone AncK43 FJ900330
Montastraea faveolata clone Mfav_L08 GU118605
clone B349/GW947
Rhopaloeides odorabile clone 27G8 EU183797
clone B39/GW947
88-98%
Gemmatimonadetes
thermophilic biogas reactor clone HAW−R60−B−B924d−Y FN436140
Corallistes sp. clone 289BC/AG EU819022
Ircinia strobilina clone IS−08 GU982113
Xestospongia muta clone XE1H04 HQ270396
clone B332/GW947
98-99%
Deinococcus-Thermus
Paralemanea annulata AY731517
clone B165/GW947
Ostreobium sp. John West 2924 FJ535840
Diploria strigosa clone Dstr_C23 GU119661
Montastraea faveolata clone SHFH475 FJ203431
clone B173/GW947
93-96%
Cyanobacteria
Oculina patagonica clone 113 AY654756
clone B115/GW947
Montastraea faveolata clone SHFH709 FJ203619
biofilm on SWRO membrane clone SBS−RV−055 HQ326321
Bacteroidetes
0.1

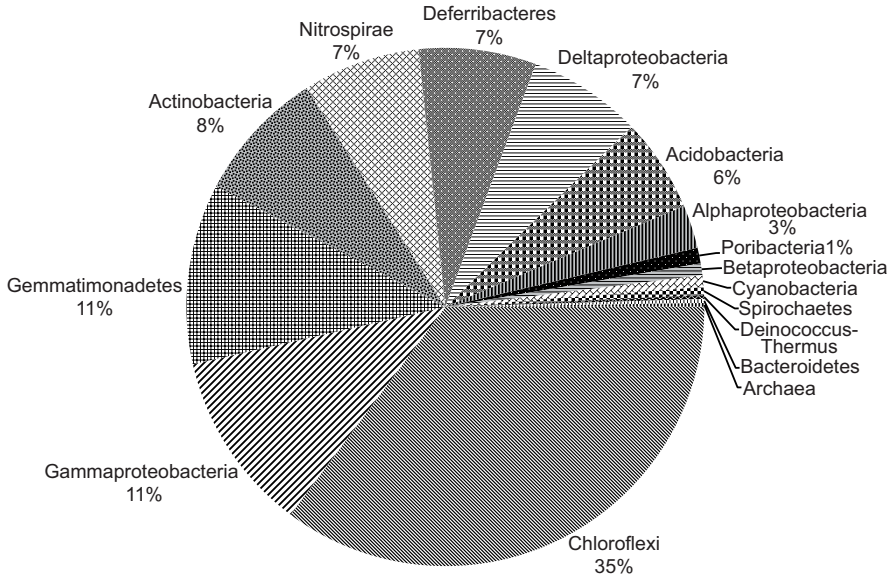


Figure 1.6. Distribution of the 16S rRNA clones among particular phylogenetic groups in the clone library obtained from the *V. crypta*

versity analysis of *V. crypta* revealed that its associated microbial community is phylogenetically complex and diverse because it is composed of representatives of the *Archaea* and 13 bacterial phyla. The phylogenetic distribution of the sequences was relatively even between phylogenetic groups, however the largest number of sequences was affiliated with the *Chloroflexi*, which have frequently been reported as members of sponge-associated microbial communities and often as the predominant group (Hentschel et al. 2002, Webster et al. 2004, Thiel et al. 2007). In a recent work Schmitt and colleagues (2011) showed that HMA sponges host more diverse, abundant, and similar *Chloroflexi* bacteria then LMA sponges. Of the sequences belonging to the *Chloroflexi*, 91% of those *V. crypta*-associated sequences fell into sponge- or sponge/coral clusters (Fig. 1.3), which is consistent with these results.

The second most abundant group of *V. crypta* symbionts belonged to the *Proteobacteria,* which are commonly found and often predominant in microbial consortia associated with different sponges from different marine sites (Friedrich et al. 1999, Schmidt et al. 2000a, Burja & Hill 2001, Friedrich et al. 2001, Webster & Hill 2001, Webster et al. 2001, Hentschel et al. 2002, Webster et al. 2004, Li et al. 2006). *Delftia acidovorans,* an obligate aerobe able to grow in 1.5% NaCl (Wen et al. 1999),

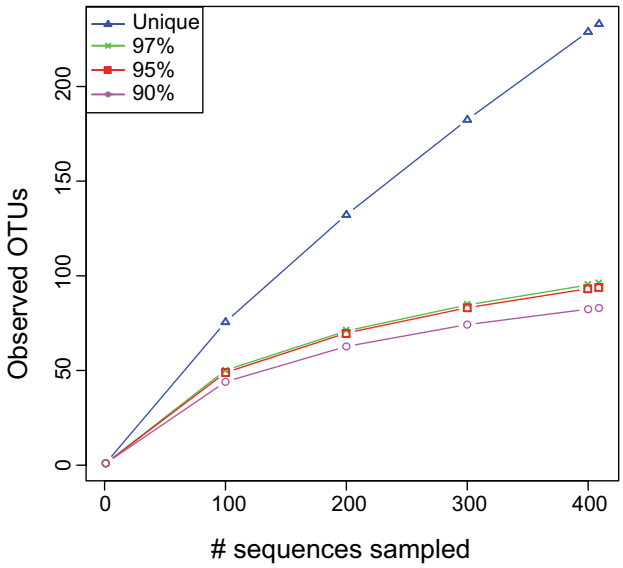


Figure 1.7. Rarefaction curves for the 16S rRNA sequences obtained from *V. crypta* Operational Taxonomic Units (OTU) were defined at the 97%, 95% and 90% similarity criterion

Table 1.2. Sample diversity

Label	OTUs	Chao estimate (95% confidence interval)	Shannon diversity index (95% confidence interval)
unique	233	957 (676-1417)	5.04 (4.93-5.15)
0.03	96	137 (114-189)	4.04 (3.94-4.14)
0.05	94	127 (108-170)	4.01 (3.91-4.11)
0.10	83	101 (90-130)	3.80 (3.69-3.91)

was the only validly described next relative for one of the *V. crypta* betaproteobacterial clones. *D. acidovorans* has been found in several habitats, such as soil, sediment, activated sludge, crude oil, fresh water and various clinical samples, and Kennedy et al. reported that it is a sponge-associated bacterium for the first time (Kennedy et al. 2008).

Actinobacteria from the microbial communities of sponges have been the focus of natural product screenings (see the review by Taylor et al. 2007b), since members of this phylum display the most promising biosynthetic potential for secondary metabolite production (Schneemann et al. 2010). Approximately half of the bioactive secondary metabolites that have been currently discovered in bacteria are attributed to the *Actinobacteria* (Lam 2006), and many new chemical entities and

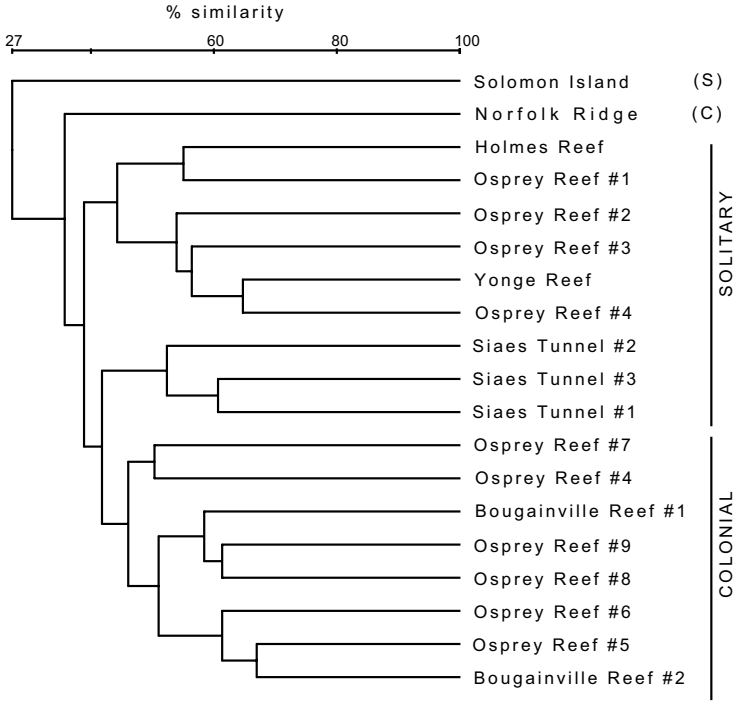


Figure 1.8. UPGMA dendrogram constructed from DGGE profiling of PCR-amplified bacterial 16S rRNA genes of microbial community associated with solitary (S) and colonial (C) forms of *Vaceletia* sponges from different locations. Sample names according to the Table 1.1 (column: Site)

bioactive metabolites have been reported from marine members of this phylum (Blunt et al. 2004, Salomon et al. 2004, Fiedler et al. 2005, Jensen et al. 2005). In this study, two OTUs belonged to the family *Acidimicrobiaceae*, which might be involved in secondary metabolite production; however, secondary metabolites are, at present, unexplored from *Vaceletia* sponges.

Investigations on prokaryotic diversity provide first hypotheses into the putative functions of the microbial communities associated with these sponges. The presence of some clades of the ammonia-oxidizing *Beta-* and *Gammaproteobacteria* or some genera of the nitrite-oxidizing *Deltaproteobacteria/Nitrospina* and *Nitrospirae* in the community, suggests that pathways for nitrogen metabolism (Bayer et al. 2008) are also present in *V. crypta*. The ammonia-oxidizing bacteria (AOB) were represented here by four OTUs. One was associated with the *Betaproteobacteria/ Nitrosospira* and several others with the *Gammaproteobacteria/Nitrososcoccus*. In addition, numerous 16S rRNA gene sequences for nitrite-oxidizing bacteria

(NOB) were found in our clone library. Three OTUs, representing 5% of the community, were affiliated with the *Deltaproteobacteria/Nitrospina*, and 30 sequences were recognized as belonging to the *Nitrospira*. All of the sequences affiliated with the phylum *Nitrospira* (9% of the community) were defined as a single OTU.

The *V. crypta* microbial community also contains microorganisms, which show high sequence homologies to known sulfur-metabolizing bacteria, indicating their possible role in sulfur cycle. One clone indicated the presence of sulfate-reducing bacteria belonging to the *Desulfurellaceae/ Deltaproteobacteria*. Hoffmann et al. (2005) provided evidence that anaerobic sulfate reduction occurs in *Geodia barretti* tissue in zones of hypoxia and anoxia, which are created by changes in sponge pumping activity. Sulfate reducing bacteria (SRB) were also detected by fluorescence *in situ* hybridization (FISH) in the Mediterranean sponges *Chondrosia reniformis* and *Petrosia ficiformis* (Schumann-Kindel et al. 1997, Manz et al. 2000).

Coralline sponges of the genus *Vaceletia* are representatives of the keratose sponges (Wörheide 2008), which form an early-branching lineage in the Demospongiae (Philippe et al. 2009, Pick et al. 2010), with their earliest fossil record most likely in the late Proterozoic era (Reitner & Wörheide 2002). Our results demonstrate that the complex microbial communities associated with *V. crypta* are very similar to the microbiota found in other sponges (Taylor et al. 2007b). An overwhelming majority of the OTUs were very closely related to other sponge- or coral-derived sequences and moreover fell into SSC/SCC, which underscores that this "living fossil" sponge shares features of its microbial community with other sponges. Such a relatively small divergence between the 16S rRNA gene sequences obtained from different sponges might suggest an environmental acquisition of symbionts (Hentschel et al. 2002, Taylor et al. 2007b). If we assume that 50 million years of evolution corresponds to an approximately 1 to 2% 16S rRNA sequence difference (Ochman et al. 1999), then a greater discrepancy should occur if these bacteria had been living separately within their host sponges for 600 million years (Taylor et al. 2007b). Moreover, small populations of endosymbiotic microorganisms enhance the fixation of mutations and are, therefore, believed to evolve more rapidly (Ochman et al. 1999). Several studies have shown that sponges from different oceans and with distant taxonomic ori-

gins harbor specific microbial consortia (Hentschel et al. 2002, Taylor et al. 2007b). Our study is consistent with that pattern because the sponges and corals that contain the microorganisms that are the closest relatives to those associated with *V. crypta* were collected from different, mostly tropical, geographic regions. To the contrary, our DGGE analysis of the 16S rRNA genes of symbionts obtained from a further nine solitary and nine colonial specimens of *Vaceletia*, reveal that solitary and colonial growth forms appear to harbor distinct communities and suggest a closer relationship between the microbial communities from the same growth form (solitary vs. colonial) than from the same geographic origin (Fig. 1.8). This observation suggests that the bacterial community might have been achieved not through an environmental acquisition, but through a different mechanism of the transmission followed by successive bacterial speciation within the sponge hosts. Erwin et al. (2012) categorized numerous factors (environmental and host related), which could affect the structure of the microbial communities and noted that factors specific to different host species might have influenced the differences between the *Ircinia*-associated symbiotic communities. Therefore, as the number of SSC/SCC (63) and the proportion of sequences within SSC or SCC (88%) by the *V. crypta* appear to be the highest ever reported, indicating a particularly tight sponge-microbe association, which might be related to the evolutionary age of the host species. In addition, some symbionts were specific for *V. crypta* because they were absent in the microbial community analyzed from another coralline sponge, *Astrosclera willeyana*, which co-occurs at, and was sampled from, the same site (Karlińska-Batres & Wörheide 2013b). A similar trend was observed in sympatric *Ircinia* species from Mediterranean Sea, which harbored different symbiont communities (Erwin et al. 2012).

This work on sclerosponges from genus *Vaceletia* enhances our knowledge about microbial communities in sponges and further provides initial insights into the diversity, structure, and composition of the microbiota of these unique sponges. Further research using deeper sequencing, FISH probes and/or specific primers designed for genes involved in denitrification, anammox or particular microbial groups (e.g. SRB and SOB) might reveal these processes in *V. crypta* providing a clearer picture of the metabolism of this sponge's microbial community. Future studies might aim to examine if other coralline sponges harbor such diverse

communities of symbionts and how much those communities differ from each other and between different geographical locations.

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Chapter 2

Phylogenetic diversity and community structure of the symbionts associated with the coralline sponge *Astrosclera willeyana* of the Great Barrier Reef

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Phylogenetic diversity and community structure of the symbionts associated with the coralline sponge *Astrosclera willeyana* of the Great Barrier Reef

Abstract

The coralline sponge *Astrosclera willeyana*, considered to be a living representative of the reef-building stromatoporoids of the Mesozoic and the Paleozoic periods, occurs widely throughout the Indo-Pacific oceans. We aimed to examine, for the first time, the phylogenetic diversity of the microbial symbionts associated with *A. willeyana* using molecular methods and to investigate the spatial variability in the sponge-derived microbial communities of *A. willeyana* from diverse sites along the Great Barrier Reef (GBR). Both, denaturing gradient gel electrophoresis (DGGE) analyses of 12 *Astrosclera* specimens and sequencing of a 16S rRNA gene clone library, constructed using a specimen of *A. willeyana* from the Yonge Reef (380 clones), revealed the presence of a complex microbial community with high diversity. An assessment of the 16S rRNA gene sequences to the particular phylogenetic groups showed domination of the *Chloroflexi* (42%) followed by the *Gammaproteobacteria* (14%), *Actinobacteria* (11%), *Acidobacteria* (8%), and the *Deferribacteres* (7%). Of the microbes that were identified, further 15% belonged to the *Deltaproteobacteria*, *Alphaproteobacteria*, and *Nitrospirae* genera. The minor phylogenetic groups *Gemmatimonadetes*, *Spirochaetes*, *Cyanobacteria*, *Poribacteria*, and the *Archaea* composed 3% of the community. Over 94% of the sequences obtained from *A. willeyana* grouped together with other sponge- or coral-derived sequences, and of these 72% formed, with nearest relatives, 46 sponge-specific or sponge-coral clusters, highlighting the uniqueness of the microbial consortia in sponges. The DGGE results showed clear divisions according to the geographical origin of the samples, indicating closer relationships between the microbial communities with respect to their geographic origin (northern vs. southern GBR).

2.1. Introduction

Sponges (Porifera) are evolutionarily ancient metazoans, with a fossil record dating back nearly 700 million years (Erwin et al. 2011). They have attracted research interest not only because of their ecological importance in aquatic ecosystems, including coral reefs, but also because of the dense and diverse microbial communities that they host in their tissues (Hentschel et al. 2006). Sponges and their associated microbes are a significant source of a wide range of bioactive compounds for pharmacological use (Wang 2006, Vogel 2008, Freeman et al. 2012). These symbiotic microbial communities, which may constitute up to 70% of the sponges' biomass (Wörheide 1998), may include bacteria, archaea, and several types of eukaryotic microbes (see review Webster & Taylor 2012). Distantly related sponges from distant geographical regions often share microbial consortia (Hentschel et al. 2002, Taylor et al. 2004); these consortia are frequently quite specific to sponges (Hentschel et al. 2002, Taylor et al. 2007b) and absent from the surrounding seawater (Lafi et al. 2009). In the ongoing discussion about the origin and maintenance of the sponge-associated microorganisms, these findings were cited as evidence supporting strict vertical transmission of the symbionts in sponges (Lee et al. 2009). However, a recent study by Webster and colleagues (2010) based on 16S rRNA gene tag pyrosequencing, reported the presence of sponge-specific microorganisms in the seawater, suggesting that environmental transmission might play a significant role in the acquisition of symbionts by juvenile sponges (Webster et al. 2010).

A unique group of the phylum Porifera, the so-called 'coralline sponges' or 'sclerosponges' (Hartman & Goreau 1970, Chombard et al. 1997), constructs a solid secondary calcareous basal skeleton that is superficially similar to the skeleton constructed by the scleractinian corals in addition to a primary, often spicular, skeleton (Reitner 1992, Chombard et al. 1997, Wörheide 2008). During long periods of the Earth's history, sclerosponges were dominant, diverse, and abundant reef-building organisms (Vacelet 1985). Today, only approximately 15 taxa exist, and these are mainly restricted to the cryptic niches of coral reefs that have reduced light and oligotrophic conditions, such as caves and deeper fore-reef areas (Reitner 1992, Wörheide 1998). Fossil records from the Silurian microbial reefs that show stromatoporoids neighboring ubiquitous microbial laminae or, less

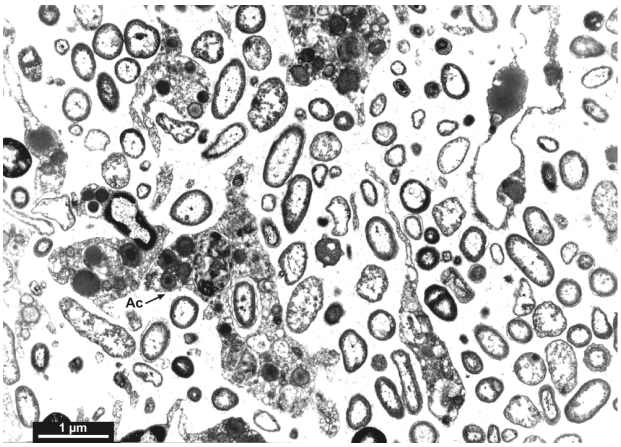


Figure 2.1. TEM micrograph of the choansosome of *A. willeyana* with numerous bacterial cells and only a few sponge cells (Ac = sponge archaeocyte). Modified from Wörheide (1998)

commonly, encrusted by cyanobacteria, might indicate that sponges and microorganisms had already formed close associations (Soja et al. 2003).

Astrosclera willeyana is regarded as a "living fossil" and considered to be a living relative of the long-extinct 'Stromatoporoidea', which formed extensive reefs during the Paleozoic and Mesozoic eras (Wood 1987, Chombard et al. 1997). *Astrosclera* was thought to be extinct until it was rediscovered in the Pacific by Lister (1900), and in today's coral reefs, *A. willeyana* is the most common coralline sponge throughout the Indo-Pacific, from the northern Red Sea to Tahiti (Wörheide 1998, 2008). Similar to all coralline sponges, *A. willeyana* grows slowly, at a rate of 0.2-1.2 mm/a (Wörheide 1998, Fallon & Guilderson 2005), is pyriform-half spherical (mushroom) in form and mostly bright orange in color (Wörheide 1998). The living tissue of *A. willeyana*, which encloses the associated microorganisms, penetrates the basal skeleton to a maximum depth of 50 % in small specimens, but this ratio decreases with increasing specimen size (Wörheide et al. 2007). Wörheide (1998) described in detail the *A. willeyana* from the Indo-Pacific and noted that they contain a large bacterial population, which is mostly rod- or coccoid-shaped, in their soft tissue, although the distribution of bacteria is not equal in all of the tissue zones. The choanosome contained a large number of symbiotic bacteria, and here, the number exceeded 70 % of the total biomass in some areas (Wörheide 1998). However, some parts of the sponges' tissues were nearly free of symbiotic bacteria (Wörheide 1998). Based on transmission electron

microscopy (TEM) studies, Wörheide (1998) distinguished four major bacterial morphotypes: rod-shaped, spherical to ovoid with a dense membrane, ellipsoid with a dense membrane and surrounded by loosely bound exopolymer secretions (EPS) sheets, and larger bacteria with a diffuse protoplasm and outer 'capsule' (supposed EPS capsule) (Fig. 2.1). Jackson et al. (2010) showed that in *A. willeyana* bacterial remains are used to seed the growth of $CaCO_3$ crystals during the process of biomineralisation. Moreover, based on fossil evidence, this study suggested that the same process of bacterially induced skeleton formation occurred in stromatoporoids during the Paleozoic and Mesozoic eras, suggesting that some ancient reef ecosystems might have been founded on this microbial–metazoan relationship (Jackson et al. 2010). Further data supporting an ancient origin of the sponge-microbial association were published recently by Jackson et al. (2011), who revealed that a gene encoding a protein that is most likely involved in skeletogenesis in *A. willeyana* was horizontally transferred from a bacterium into the *A. willeyana* genome. This horizontal gene transfer (HGT) event may have contributed to the evolution of *A. willeyana*'s bodyplan (Jackson et al. 2011). Jackson et al. (2011) demonstrated the first example of an HGT event into a sponge genome from a prokaryote and provided other evidence supporting an ancient origin for the *A. willeyana*-microbial association (Jackson et al. 2010); however, the identity of the microbial community of this coralline sponge was still unknown.

Details about the microbial communities of coralline sponges are generally not well known (Karlińska-Batres & Wörheide 2013a), but could provide insights into the evolution of this putatively ancient symbiosis. Consequently, in this study, we aimed to assess the hitherto undetermined phylogenetic diversity of the microbial symbionts that are associated with *A. willeyana* from the Great Barrier Reef (GBR). We employed the denaturing gradient gel electrophoresis (DGGE) method to investigate the spatial variability in sponge-derived microbial communities between *A. willeyana* from diverse sites along the GBR. Furthermore, a 16S rRNA gene clone library from an *A. willeyana* specimen was sequenced to perform, for the first time, a detailed characterization of its microbial community.

Table 2.1. Sample data of investigated *A. willeyana* specimens, with collection site details

Sample No.	Site (location)	Depth	Date	Latitude	Longitude
GW950	Yonge Reef	8 m	2006	14°34′20″ S	145°36′54″ E
93 (GW5431)	Mac Gillivray Reef #1	6 m	1994	14°38'56" S	145°29'30" E
92 (GW5430)	Mac Gillivray Reef #2	6 m	1994	14°38'56" S	145°29'30" E
G316237	Harrier Reef	8 m	2001	15°08′12″ S	145°41′18″ E
GW718	Ribbon Reef 7	12 m	2001	14°58'44" S	145°42'54" E
G316273	Ribbon Reef 5	9 m	2001	15°20′07″ S	145°46′33″ E
G316198	Reef No. 15-040	7 m	2001	15°22′05″ S	145°56′28″ E
G313772	Myrmidon Reef	17 m	1999	18°15′28″ S	147°22′51″ E
G313826	Hook Reef	8 m	1999	19°45′14″ S	149°10′45 E
G316066	Swain Reefs	4 m	2000	21°22′25″ S	151°14′32″ E
G316118	Merv's Reef	12 m	2001	21°53′15″ S	152°20′50″ E
GW794	Heron Island	15 m	2003	23°25'43"S	151°57'6"E

2.2. Materials and Methods

2.2.1. *Sample collection and DNA extraction*

Sampling took place during SCUBA dives at depths between 4 and 17 meters at several different sites along the GBR. Details of all samples are listed in the Table 2.1. Twelve sponges were excised with a chisel and hammer and transferred directly to plastic bags while underwater. After collection, sponge samples were preserved either in silica gel (Erpenbeck et al. 2004), DMSO buffer (20% DMSO, 0.25 M EDTA, and NaCl to saturation, pH 8.0; adapted from Seutin et al. (1991), or 95% ethanol. Living tissue was cut and crushed aseptically with a sterile scalpel on a Petri dish from samples rinsed with autoclaved Millipore water. Total DNA was extracted from 3 mg of tissue using the Qiagen DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.2.2. *Denaturing gradient gel electrophoresis*

The touchdown PCR with Promega GoTaq polymerase (Promega GmbH, Mannheim, Germany) and universal primers 341F-GC and 907RC (Muyzer & Smalla 1998, Schäfer 2001) was employed to amplify the bacterial 16S rRNA genes from all DNA extracts. The cycling conditions for the PCR reaction in a Biometra thermocycler using Promega GoTaq were as follows: one cycle of initial denaturation (2 min at 95°C), 15 cycles of denaturation (30 s at 94°C), primer annealing (30 s from 58°C minus 0.5°C), elongation (2 min + 4 s at 72°C), followed by 25 cycles of

denaturation (30 s at 94°C), primer annealing (30 s from 51°C minus 0.5°C), elongation (2 min + 4 s at 72°C), and a final extension step (5 min at 72°C). DGGE was then performed using an Ingeny phorU-2 system (Ingeny International) and Power Pac 300 (BioRad) to supply power, with a denaturing gradient of 30%–70% (urea and formamide) in a 6% polyacrylamide gel. PCR-amplified DNA (30 μl) was loaded onto the gel and run for 16 h at 180 V and at a temperature of 60°C. The gels were removed from the glass plates and stained for 25 min in SYBR Gold (Molecular Probes) and photographed with an RT Color SPOT camera and SPOT advance imaging software (Visitron Systems GmbH). The gel image data were analysed using QuantityOne, version 4.69 software (Bio-Rad). The similarities between the DGGE banding patterns were calculated using the band-matching Dice coefficient with an optimisation at 0.75% and a tolerance level of 0.75%. The unweighted pair-group method with arithmetic averages (UPGMA) was used for cluster analysis with QuantityOne (BioRad) to obtain similarity dendrograms.

2.2.3. *Construction of the 16S rRNA gene clone library*

Universal bacterial primers (616F: 5'- AGA GTT TGA TYM TGG CTC AG -3' and 1525R: 5'- AGA AAG GAG GTG ATC CAG CC -3') (Lane 1991) and GoTaq polymerase were used for the amplification of the 16S rRNA genes from the DNA extract obtained from the *A. willeyana* from the Yonge Reef (GBR, Australia, sample no. GW950). Cycling conditions for the PCR reaction in the Biometra thermocycler were as follows: initial denaturation (2 min at 95°C), followed by 35 cycles of denaturation (30 s at 94°C), primer annealing (1 min at 55°C), elongation (2 min + 4 s at 72°C), and a final extension step (5 min at 72°C). The PCR products were purified with the mi-PCR Purification Kit (metabion GmbH, Martinsried, Germany) and were cloned into the plasmid cloning vector using the Invitrogen TOPO® TA Cloning Kit for Sequencing according to the manufacturer's instructions (Life Technologies GmbH, Darmstadt, Germany). White colonies were randomly selected for the PCR re-amplification of their plasmid inserts with vector-specific primers (M13) using Promega GoTaq polymerase. PCR products of 1500 base pairs were digested in single reactions with the restriction enzyme *MspI* (Fermentas GmbH, St. Leon-Rot, Germany), following the manufacturer's instructions. From each group of clones with similar restriction patterns, one was

chosen randomly for sequencing. In addition, clones with unclear restriction pattern were sequenced. The silica-based protocol for the purification of PCR products (Boyle & Lew 1995) was modified and used to prepare the amplified inserts for sequencing.

The archaeal 16S rRNA gene was amplified from the DNA extract using touchdown PCR with the universal primers (21F: 5'- TTC CGG TTG ATC CYG CCG GA - 3' and 915R 5'- GTG CTC CCC CGC CAA TTC CT -3') (DeLong 1992, Raskin et al. 1994) and by decreasing the annealing temperature from 60 to 50.5°C (30 s each) in 0.5°C increments. The cycling conditions for the PCR reaction using the Biometra thermocycler and Promega GoTaq were as follows: one cycle of initial denaturation (2 min at 95°C), 35 cycles of denaturation (30 s at 94°C), primer annealing (30 s from 60°C minus 0.5°C), elongation (2 min + 4 s at 72°C), followed by 25 cycles of denaturation (30 s at 94°C), primer annealing (30 s at 51°C), elongation (2 min + 4 s at 72°C), and a final extension step (5 min at 72°C). The resulting strong band of approx. 900 base pairs was excised from the gel, purified with an E.Z.N.A Gel Extraction Kit (VWR International, Darmstadt, Germany) and subsequently sequenced.

2.2.4. *Sequencing*

Sequencing was performed by the Genomics Service Unit, Ludwig-Maximilians-Universität München using BigDye® Terminator v3.1 on a 48-capillary sequencer (ABI 3730, Applied Biosystems). For the cloned bacterial inserts, the primers: 610RII (5'- ACC GCG/T A/GCT GCT GGC AC -3') (Dotzauer et al. 2002), 616F (Lane 1991), and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Juretschko et al. 1998), or 614F (5'-GTG CAT GGC TGT CGT CAG CTC G -3') (this study) were used. The archaeal PCR product was sequenced with AR20F primer (5'- TTC CGG TTG ATC CYG CCRG-3') (Moyer et al. 1998). The sequences were edited and assembled using the CodonCode Aligner (http://www.codoncode.com/aligner/). The Bellerophon web application (Huber et al. 2004) was used to check for chimeras, and chimerical sequences were removed from further analysis.

2.2.5. *Phylogenetic analyses*

The sequences obtained through PCR were compared with the sequences avail-

able in the public database using the BLAST (http://blast.ncbi.nlm.nih.gov/) to find similar sequences. The sequences with the highest degree of similarity, together with our sequences, were incorporated into the ARB, which was used to run the phylogenetic analyses (Ludwig et al. 2004). The sequences were aligned using the ARB Integrated Aligner. The alignment was checked for alignment errors, and these were corrected manually. Partial sequences were added to the ARB database using the ARB parsimony "quick add" tool. The neighbor-joining method (Jukes-Cantor correction) was used to calculate the initial phylogenetic tree using ARB. Subsequently, the alignment was exported from the ARB database and maximum likelihood trees were constructed using RAxML v.7.2.5 (Stamatakis 2006) using 1000 bootstrap replicates and the GTR+GAMMA model of sequence evolution. The resulting trees were visualized using the FigTree (v.1.3.1) program.

2.2.6. *Sponge-specific and sponge-coral clusters*

To define monophyletic, sponge-specific and sponge-coral clusters, the BLAST search results were checked for similar sequences obtained from different sponges, corals, and non-sponge sources, which subsequently were incorporated into the ARB database and used to calculate phylogenies using neighbor-joining (ARB) and maximum-likelihood methods (RAxML). Based on the criteria established by Hentschel (2002), sponge-specific and sponge-coral clusters (SSC/SCC) were defined as groups of sequences from sponges and corals that cluster together in one clade, independently of the method of tree reconstruction.

2.2.7. *Estimation of microbial diversity and statistical analysis of the clone library*

The sequences were grouped as OTUs (operational taxonomic units) using a Mothur (Schloss et al. 2009), based on the distance matrix generated by ARB and a cut-off value of 0.03 (Schloss & Handelsman 2005). In addition, clones that were analyzed only by restriction digestion were assigned to corresponding OTUs based on their restriction patterns. The Mothur was used to generate rarefaction curves, Chao1 richness estimator (Colwell & Coddington 1994), and Shannon diversity indices (Spellerberg & Fedor 2003). The rarefaction curves were plotted using the R software package (http://www.R-project.org). In order to determine

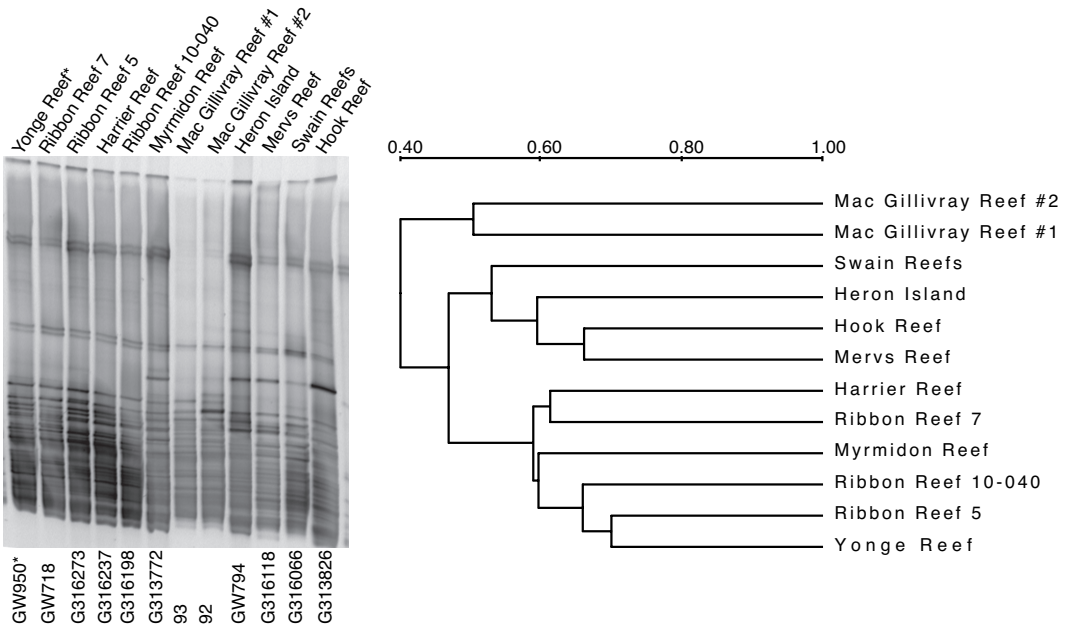


Figure 2.2. DGGE results of the PCR-amplified bacterial 16S rRNA genes of the microbial community associated with *A. willeyana* from localities along the GBR; UPGMA dendrogram (right) constructed from the DGGE banding profile (left). Samples are named according to Table 2.1 (column: Site). *Asterisk* indicates cloned sample

the phylogenetic composition of the clone library constructed from the microbial community associated with the *A. willeyana* the percentage for each phylogenetic group was calculated based on the number of clones assigned to the particular group.

2.2.8. *Nucleotide sequence accession numbers*

The final sequence data were submitted to the EMBL database under the accession numbers HE985081 to HE985159.

2.3. Results

2.3.1. Denaturing gradient gel electrophoresis

DGGE fingerprinting of the 16S rRNA gene fragments obtained from twelve individuals of *A. willeyana* from different locations along the GBR showed a complex banding pattern (Fig. 2.2). All samples revealed a very high diversity of microbes in their bacterial communities. The number of bands ranged from 28 to 37 per sample. The largest number of bands was obtained from the *A. willeyana* from

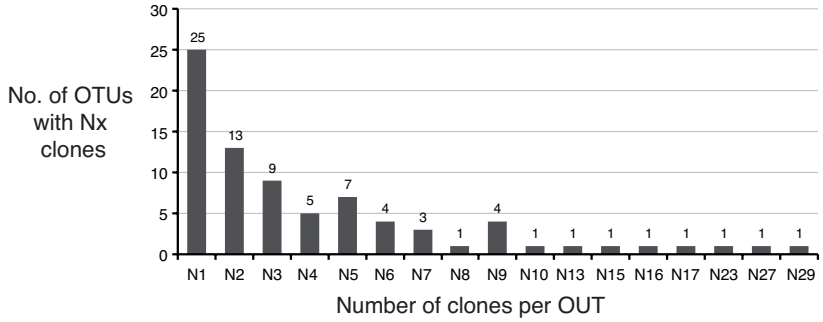


Figure 2.3. Distribution of 16S rRNA gene clones among the OTUs. * N1 represents the number of singletons, N2 the number of doubletons, etc.

Heron Island (Sample No. GW794), and the sample with the fewest bands was obtained from Swain Reefs (Sample No. G316066). The banding patterns exhibited numerous co-occurring bands; however, only four bands were found in all of the samples, and seventeen bands occurred only in one or two samples.
A cluster analysis revealed a clear division according to the geographical origin of the sample; the location of the dividing line is approximately at the latitude of Townsville. The data obtained from the microbial communities from the southern part of the GBR (Heron Island, Hook Reef, Mervs Reef and Swain Reefs), as well as the ones from the northern part (Myrmidon Reef, Ribbon Reefs, Harrier Reef, Yonge Reef), clustered together, whereas the two specimens from the Mac Gillivray Reef (a reef near Lizard Island, north of Cooktown) formed a sister group to the remaining samples (Fig. 2.2).

2.3.2. Phylogenetic analysis and sponge-specific/sponge-coral clusters

From the 16S rRNA gene clone library amplified from the *A. willeyana* from Yonge Reef (GBR, Australia), 380 clones were screened, and 298 of these clones were sequenced. The remaining 82 clones, which were not sequenced, were assigned to a particular OTU based on their restriction patterns. Nine sequences were discarded as chimeras. The remaining 289 bacterial sequences, together with a single archaeal 16S rRNA gene sequence, which was amplified directly from the DNA extract, were clustered into 79 OTUs based on a similarity criterion of 97%. Figure 2.3 shows the distribution of 16S rRNA gene clones among the OTUs. Of those 79 OTUs, 25 were singletons and 13 were doubletons. Only eight OTUs consisted of 10 or more clones (these OTUs contained 10, 13, 15-17, 23, 27, and 29 clones).

Due to the complexity and variety of the phylum *Proteobacteria*, the proteobacterial classes (*Alpha-*, *Gamma-*, and *Deltaproteobacteria*) were treated as separate phylogenetic groups. Therefore, among the 13 phylogenetic groups represented in our clone library, the *Chloroflexi* (25 OTUs, *n*=156 clones) and *Gammaproteobacteria* (12 OTUs, *n*=51) were the most abundant. Numerous clones were members of the *Actinobacteria* (6 OTUs, *n*=41), *Acidobacteria* (9 OTUs, *n*=29), *Deltaproteobacteria* (8 OTUs, *n*=22), and *Alphaproteobacteria* (8 OTUs, *n*=19). A single OTU contained 16 clones that exhibited similarity to the *Nitrospirae*. Clones that were similar to the *Gemmatimonadetes* (3 OTUs, *n*=7), *Spirochaetes* (2 OTUs, *n*=3), *Cyanobacteria* (1 OTUs, *n*=1), and *Poribacteria* (1 OTUs, *n*=1) were also observed. The single archaeal sequence that was amplified with the universal primers belonged to the *Crenarchaeota*. A group of 25 sequences (2 OTUs) were assigned in ARB to an unclassified bacterial clade, but due to BLAST search they were affiliated to the phylum *Deferribacteres*.

The BLAST results revealed that for 94% (74 OTUs) of the 79 defined OTUs, the most similar 16S rRNA gene sequences matched those that have been previously obtained from sponges or corals. 82% of the OTUs (65 OTUs) were related to sequences obtained from 12 different sponge species. 11% of the OTUs contained 16S rRNA gene sequences that were similar to those previously obtained from 2 species of corals – *Montastraea faveolata* (8 OTUs) and *Pseudopterogorgia elisabethae* (1 OTUs). The remaining 6% of the OTUs (5 OTUs) contained sequences that appeared to be distantly related to previously described environmental sequences (3 sequences from the deep-sea exhibited 96-93% similarity, 1 from sediment exhibited 95% similarity, and 1 from saline soil exhibited 88% similarity). The results of the BLAST search are summarized in a Table S2.1 in the Supplementary material.

Of the sequences from the 74 OTUs that were closely related to other sponge- or coral-derived sequences, 53 OTUs (67% of the total number of OTUs) formed 46 SSC/SCC with their nearest relatives. The largest number of OTUs that was grouped to the SSC/SCC was affiliated with the phylum *Chloroflexi* (17 OTUs formed 13 SSC/SCC), and the largest number of SSC/SCC was found in the phylum *Proteobacteria*, (15 OTUs formed 15 SSC/SCC). Grey-shaded boxes indicate all of the SSC/SCC in the phylogenetic trees (Figs. 2.4, 2.5, and 2.6). The percent-

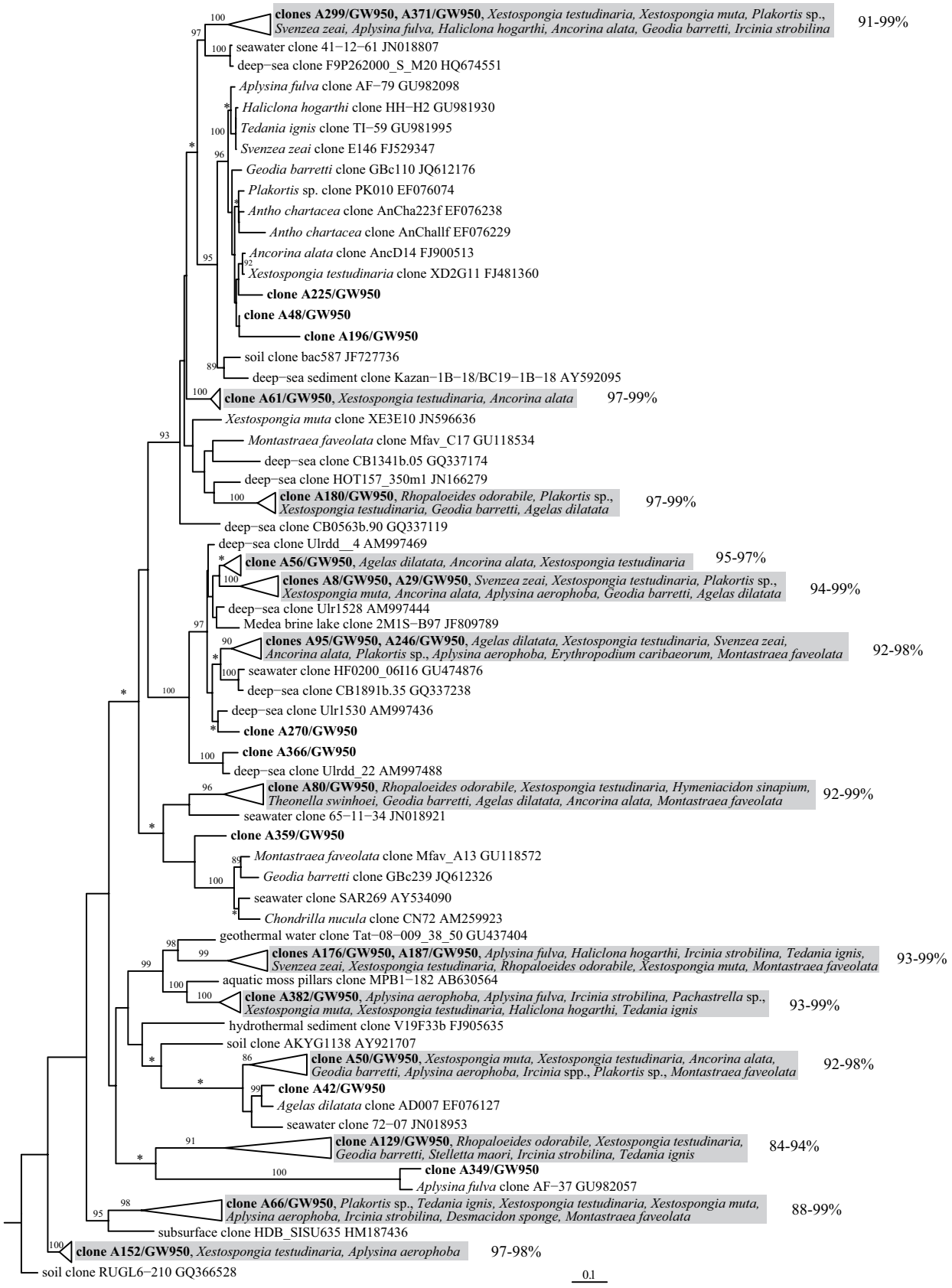
age values next to the grey-shaded boxes, ranging from 84 to 100%, indicate the degree of similarity between the sequences belonging to the clusters.

2.3.3. Microbial diversity and community structure

The microbial community of the coralline sponge *A. willeyana* was very diverse, with a complex composition (Fig. 2.7). The green non-sulphur bacteria *Chloroflexi* made up 42% of this community. Additionally, for the estimations of the microbial community composition, the proteobacterial classes (*Alpha-*, *Gamma-*, and *Deltaproteobacteria*) were treated as separate phylogenetic groups. Consequently, the next most abundant were the *Gammaproteobacteria* (14%), the *Actinobacteria* (11%), *Acidobacteria* (8%), and the *Deferribacteres* (7%). Of the identified members of the microbial community, 15% consisted of the *Deltaproteobacteria* (6%), *Alphaproteobacteria* (5%), *Nitrospirae* (4%). The minor phylogenetic groups *Gemmatimonadetes*, *Spirochaetes*, *Cyanobacteria*, *Poribacteria*, and *Archaea* composed 3% of the overall microbial community. If all *Proteobacteria* were treated as a single phylogenetic group, they accounted for 25% of the microbial community and became the second-most-abundant group behind the *Chloroflexi*.

Rarefaction curves (Fig. 2.8) indicated how well the diversity within a sample was assessed, based on the number of examined clones. The rarefaction curves were calculated for the 0.03, 0.05, and 0.1 cut-off criteria for grouping OTUs at the "species" and "genus" levels. However, the rarefaction curves didn't reach clear saturation; instead, they were only little slanted, meaning that the majority of the diversity within the clone library was detected. The rarefaction analysis suggested that the microbial diversity was not fully resolved, which is an expected finding given the high bacterial diversity associated with marine sponges (Radwan et al. 2010). Additionally, the Chao estimate suggested that the discovered OTUs accounted for 80% of the total (Tab. 2.2), which suggests these OTUs

Figure 2.4. The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA gene sequences affiliated with the phylum *Chloroflexi*, with the next most similar sequences obtained from other sponges or corals and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


clones A299/GW950, A371/GW950, *Xestospongia testudinaria, Xestospongia muta, Plakortis* sp., *Svenzea zeai, Aplysina fulva, Haliclona hogarthi, Ancorina alata, Geodia barretti, Ircinia strobilina*
91-99%
seawater clone 41−12−61 JN018807
deep−sea clone F9P262000_S_M20 HQ674551
Aplysina fulva clone AF−79 GU982098
Haliclona hogarthi clone HH−H2 GU981930
Tedania ignis clone TI−59 GU981995
Svenzea zeai clone E146 FJ529347
Geodia barretti clone GBc110 JQ612176
Plakortis sp. clone PK010 EF076074
Antho chartacea clone AnCha223f EF076238
Antho chartacea clone AnChallf EF076229
Ancorina alata clone AncD14 FJ900513
Xestospongia testudinaria clone XD2G11 FJ481360
clone A225/GW950
clone A48/GW950
clone A196/GW950
soil clone bac587 JF727736
deep−sea sediment clone Kazan−1B−18/BC19−1B−18 AY592095
clone A61/GW950, *Xestospongia testudinaria, Ancorina alata*
97-99%
Xestospongia muta clone XE3E10 JN596636
Montastraea faveolata clone Mfav_C17 GU118534
deep−sea clone CB1341b.05 GQ337174
deep−sea clone HOT157_350m1 JN166279
clone A180/GW950, *Rhopaloeides odorabile, Plakortis* sp., *Xestospongia testudinaria, Geodia barretti, Agelas dilatata*
97-99%
deep−sea clone CB0563b.90 GQ337119
deep−sea clone Ulrdd__4 AM997469
clone A56/GW950, *Agelas dilatata, Ancorina alata, Xestospongia testudinaria*
95-97%
clones A8/GW950, A29/GW950, *Svenzea zeai, Xestospongia testudinaria, Plakortis* sp., *Xestospongia muta, Ancorina alata, Aplysina aerophoba, Geodia barretti, Agelas dilatata*
94-99%
deep−sea clone Ulr1528 AM997444
Medea brine lake clone 2M1S−B97 JF809789
clones A95/GW950, A246/GW950, *Agelas dilatata, Xestospongia testudinaria, Svenzea zeai, Ancorina alata, Plakortis* sp., *Aplysina aerophoba, Erythropodium caribaeorum, Montastraea faveolata*
92-98%
seawater clone HF0200_06I16 GU474876
deep−sea clone CB1891b.35 GQ337238
deep−sea clone Ulr1530 AM997436
clone A270/GW950
clone A366/GW950
deep−sea clone Ulrdd_22 AM997488
clone A80/GW950, *Rhopaloeides odorabile, Xestospongia testudinaria, Hymeniacidon sinapium, Theonella swinhoei, Geodia barretti, Agelas dilatata, Ancorina alata, Montastraea faveolata*
92-99%
seawater clone 65−11−34 JN018921
clone A359/GW950
Montastraea faveolata clone Mfav_A13 GU118572
Geodia barretti clone GBc239 JQ612326
seawater clone SAR269 AY534090
Chondrilla nucula clone CN72 AM259923
geothermal water clone Tat−08−009_38_50 GU437404
clones A176/GW950, A187/GW950, *Aplysina fulva, Haliclona hogarthi, Ircinia strobilina, Tedania ignis, Svenzea zeai, Xestospongia testudinaria, Rhopaloeides odorabile, Xestospongia muta, Montastraea faveolata*
93-99%
aquatic moss pillars clone MPB1−182 AB630564
clone A382/GW950, *Aplysina aerophoba, Aplysina fulva, Ircinia strobilina, Pachastrella* sp., *Xestospongia muta, Xestospongia testudinaria, Haliclona hogarthi, Tedania ignis*
93-99%
hydrothermal sediment clone V19F33b FJ905635
soil clone AKYG1138 AY921707
clone A50/GW950, *Xestospongia muta, Xestospongia testudinaria, Ancorina alata, Geodia barretti, Aplysina aerophoba, Ircinia* spp., *Plakortis* sp., *Montastraea faveolata*
92-98%
clone A42/GW950
Agelas dilatata clone AD007 EF076127
seawater clone 72−07 JN018953
clone A129/GW950, *Rhopaloeides odorabile, Xestospongia testudinaria, Geodia barretti, Stelletta maori, Ircinia strobilina, Tedania ignis*
84-94%
clone A349/GW950
Aplysina fulva clone AF−37 GU982057
clone A66/GW950, *Plakortis* sp., *Tedania ignis, Xestospongia testudinaria, Xestospongia muta, Aplysina aerophoba, Ircinia strobilina, Desmacidon sponge, Montastraea faveolata*
88-99%
subsurface clone HDB_SISU635 HM187436
clone A152/GW950, *Xestospongia testudinaria, Aplysina aerophoba*
97-98%
soil clone RUGL6−210 GQ366528
0.1

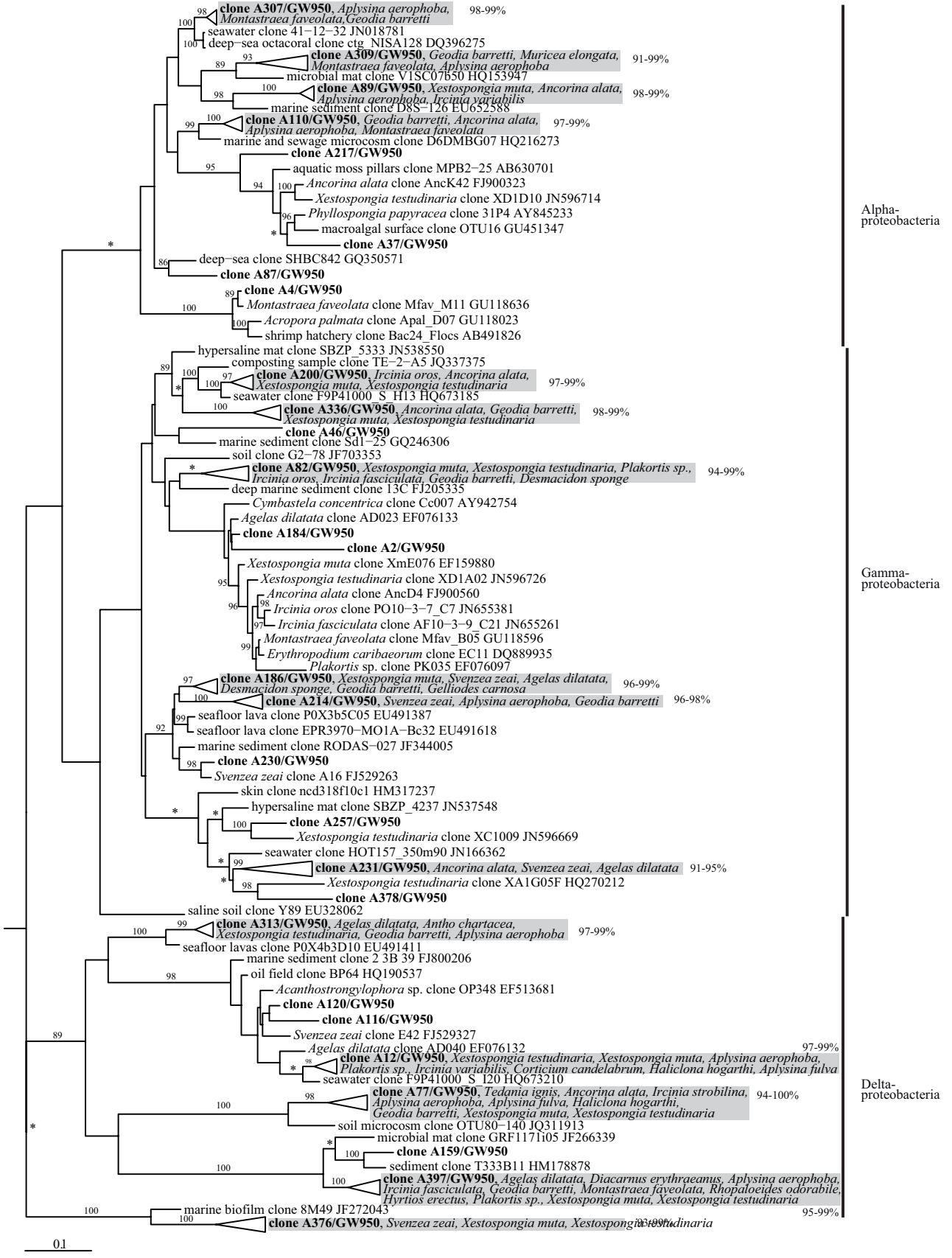
provide a comprehensive picture of the core microbial community of the *A. willeyana* (Schmitt et al. 2011). Certainly, the results obtained through the 16S rRNA gene tag pyrosequencing exposes a much higher magnitude of diversity; however, it results in higher costs and does not change the view of who the major microbial players in the sponge-associated community are (Webster & Taylor 2012).

2.4. Discussion

To our knowledge this is the first detailed assessment of microbial communities associated with the "living fossil" coralline sponge *A. willeyana* and it shows similarities with microbiota of "modern" sponges. This is also the first investigation showing spatial variability of coralline sponge microbial consortia with a phylogeographic break detected between *A. willeyana*-derived microbial communities from diverse sites along the GBR. The 16S rRNA gene-based diversity analysis revealed that the *A. willeyana* from the GBR harbors a rich and diverse microbial community, including at least one representative from one archaeal phylum and representatives from ten bacterial phyla. The *A. willeyana*-associated community appears to be typical of sponge-associated bacterial groups. The most abundant members were classified as *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, and *Acidobacteria*, which are commonly associated with sponges (Hentschel et al. 2002, Webster et al. 2004, Thiel et al. 2007, Webster & Taylor 2012), and based on the cDNA libraries were reported to be active members of the microbial communities (Kamke et al. 2010).

The *Chloroflexi* frequently dominate in the microbiota of sponges (Hentschel et al. 2002, Webster et al. 2004, Thiel et al. 2007) and are more diverse and abundant, as well as similar in high, compared with low-microbial-abundance sponges (Schmitt et al. 2011). Our results do not correspond to the results published recently by Schmitt et al. (2011), where at least 78% of the *Chloroflexi* sequences

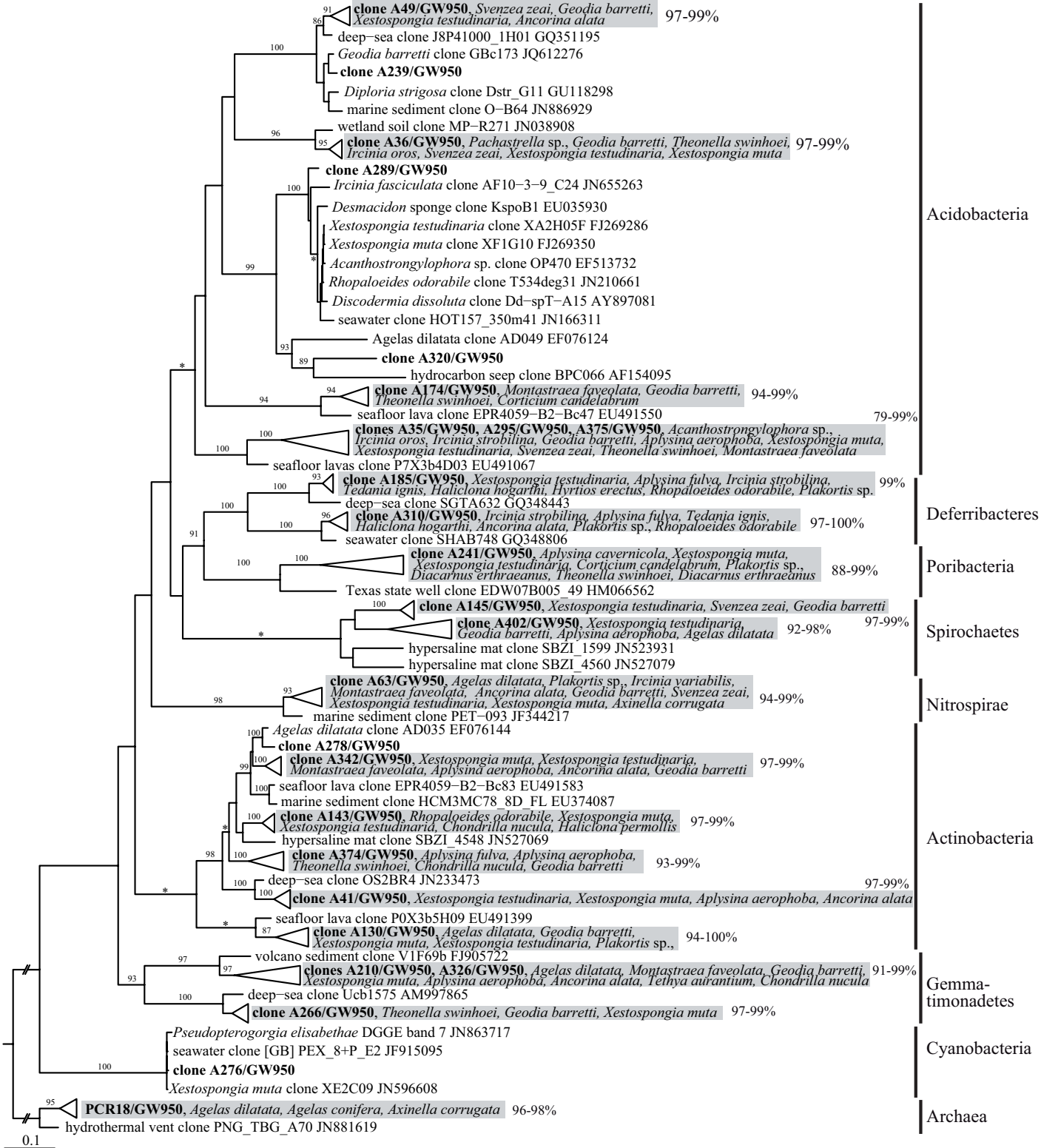
Figure 2.5 The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA gene sequences affiliated with the phylum *Proteobacteria*, with the next most similar sequences obtained from other sponges or corals and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


clone A307/GW950, Aplysina aerophoba, Montastraea faveolata,Geodia barretti
98-99%
seawater clone 41−12−32 JN018781
deep−sea octacoral clone ctg_NISA128 DQ396275
clone A309/GW950, Geodia barretti, Muricea elongata, Montastraea faveolata, Aplysina aerophoba
91-99%
microbial mat clone V1SC07B50 HQ153947
clone A89/GW950, Xestospongia muta, Ancorina alata, Aplysina aerophoba, Ircinia variabilis
98-99%
marine sediment clone D8S−126 EU652588
clone A110/GW950, Geodia barretti, Ancorina alata, Aplysina aerophoba, Montastraea faveolata
97-99%
marine and sewage microcosm clone D6DMBG07 HQ216273
clone A217/GW950
aquatic moss pillars clone MPB2−25 AB630701
Ancorina alata clone AncK42 FJ900323
Xestospongia testudinaria clone XD1D10 JN596714
Phyllospongia papyracea clone 31P4 AY845233
macroalgal surface clone OTU16 GU451347
clone A37/GW950
deep−sea clone SHBC842 GQ350571
clone A87/GW950
clone A4/GW950
Montastraea faveolata clone Mfav_M11 GU118636
Acropora palmata clone Apal_D07 GU118023
shrimp hatchery clone Bac24_Flocs AB491826
hypersaline mat clone SBZP_5333 JN538550
composting sample clone TE−2−A5 JQ337375
clone A200/GW950, Ircinia oros, Ancorina alata, Xestospongia muta, Xestospongia testudinaria
97-99%
seawater clone F9P41000_S_H13 HQ673185
clone A336/GW950, Ancorina alata, Geodia barretti, Xestospongia muta, Xestospongia testudinaria
98-99%
clone A46/GW950
marine sediment clone Sd1−25 GQ246306
soil clone G2−78 JF703353
clone A82/GW950, Xestospongia muta, Xestospongia testudinaria, Plakortis sp., Ircinia oros, Ircinia fasciculata, Geodia barretti, Desmacidon sponge
94-99%
deep marine sediment clone 13C FJ205335
Cymbastela concentrica clone Cc007 AY942754
Agelas dilatata clone AD023 EF076133
clone A184/GW950
clone A2/GW950
Xestospongia muta clone XmE076 EF159880
Xestospongia testudinaria clone XD1A02 JN596726
Ancorina alata clone AncD4 FJ900560
Ircinia oros clone PO10−3−7_C7 JN655381
Ircinia fasciculata clone AF10−3−9_C21 JN655261
Montastraea faveolata clone Mfav_B05 GU118596
Erythropodium caribaeorum clone EC11 DQ889935
Plakortis sp. clone PK035 EF076097
clone A186/GW950, Xestospongia muta, Svenzea zeai, Agelas dilatata, Desmacidon sponge, Geodia barretti, Gelliodes carnosa
96-99%
clone A214/GW950, Svenzea zeai, Aplysina aerophoba, Geodia barretti
96-98%
seafloor lava clone P0X3b5C05 EU491387
seafloor lava clone EPR3970−MO1A−Bc32 EU491618
marine sediment clone RODAS−027 JF344005
clone A230/GW950
Svenzea zeai clone A16 FJ529263
skin clone ncd318f10c1 HM317237
hypersaline mat clone SBZP_4237 JN537548
clone A257/GW950
Xestospongia testudinaria clone XC1009 JN596669
seawater clone HOT157_350m90 JN166362
clone A231/GW950, Ancorina alata, Svenzea zeai, Agelas dilatata
91-95%
Xestospongia testudinaria clone XA1G05F HQ270212
clone A378/GW950
saline soil clone Y89 EU328062
clone A313/GW950, Agelas dilatata, Antho chartacea, Xestospongia testudinaria, Geodia barretti, Aplysina aerophoba
97-99%
seafloor lavas clone P0X4b3D10 EU491411
marine sediment clone 2 3B 39 FJ800206
oil field clone BP64 HQ190537
Acanthostrongylophora sp. clone OP348 EF513681
clone A120/GW950
clone A116/GW950
Svenzea zeai clone E42 FJ529327
Agelas dilatata clone AD040 EF076132
97-99%
clone A12/GW950, Xestospongia testudinaria, Xestospongia muta, Aplysina aerophoba, Plakortis sp., Ircinia variabilis, Corticium candelabrum, Haliclona hogarthi, Aplysina fulva
seawater clone F9P41000_S_I20 HQ673210
clone A77/GW950, Tedania ignis, Ancorina alata, Ircinia strobilina, Aplysina aerophoba, Aplysina fulva, Haliclona hogarthi, Geodia barretti, Xestospongia muta, Xestospongia testudinaria
94-100%
soil microcosm clone OTU80−140 JQ311913
microbial mat clone GRF1171i05 JF266339
clone A159/GW950
sediment clone T333B11 HM178878
clone A397/GW950, Agelas dilatata, Diacarnus erythraeanus, Aplysina aerophoba, Ircinia fasciculata, Geodia barretti, Montastraea faveolata, Rhopaloeides odorabile, Hyrtios erectus, Plakortis sp., Xestospongia muta, Xestospongia testudinaria
marine biofilm clone 8M49 JF272043
95-99%
clone A376/GW950, Svenzea zeai, Xestospongia muta, Xestospongia testudinaria
93-99%
0.1
Alpha-proteobacteria
Gamma-proteobacteria
Delta-proteobacteria

from high-abundance sponges were found in sponge-specific/sponge-coral clusters SSC/SCC, whereas only 68% of the *A. willeyana*-associated sequences were attributed to *Chloroflexi* SSC/SCC. Of those, 20% of OTUs from *A. willeyana* were classified as *Dehalococcoides*, which indicates the presence of the process of anaerobic reductive dehalogenation that was reported in sponges for the first time by Ahn et al. (2003).

The second most abundant group of *A. willeyana*-associated symbionts belonged to the *Proteobacteria*, which in a recently published review on the microbial diversity of marine sponges were found to constitute nearly half of the published sequence library (Webster & Taylor 2012). In the maximum likelihood tree of the *Proteobacteria* (Fig. 2.5), two gammaproteobacterial OTUs (A46/GW947 and A2/GW947) were not placed next to the most similar sequences obtained by BLAST search (soil clone Y89 EU328062 and *Xestospongia muta* clone XF1E08 HQ270412, respectively), but rather, next to sequences to which they were probably more related. This displacement was likely caused by the low similarity of the OTUs to their closest relatives (88% and 90%, respectively). However, the positions of the branches were not supported (support values below 50). The presence of the ammonia-oxidizing *Gammaproteobacteria* and some genera of the nitrite-oxidizing *Gammaproteobacteria* and *Deltaproteobacteria/Nitrospina*, together with the presence of representatives of the phylum *Nitrospirae* (Fig. 2.6), which are responsible for the two steps of the nitrification process, suggest pathways for nitrogen metabolism in the sponge tissues (Bayer et al. 2007). The ammonia-oxidizing bacteria (AOB) were represented here by three OTUs associated with the *Gammaproteobacteria/Nitrosococcus*. The nitrite-oxidizing bacteria (NOB), which are responsible for the second step of nitrification and play a major role in removing toxic nitrite from the environment for living organisms (Philips et al. 2002), were represented by three single OTUs affiliated with the *Gammaproteobacteria/*

Figure 2.6 The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA gene sequences affiliated with several phyla, with the next most similar sequences obtained from other sponges or corals and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study. *Shaded boxes* represent sponge-specific clusters. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


clone A49/GW950, Svenzea zeai, Geodia barretti, Xestospongia testudinaria, Ancorina alata 97-99%
deep−sea clone J8P41000_1H01 GQ351195
Geodia barretti clone GBc173 JQ612276
clone A239/GW950
Diploria strigosa clone Dstr_G11 GU118298
marine sediment clone O−B64 JN886929
wetland soil clone MP−R271 JN038908
clone A36/GW950, Pachastrella sp., Geodia barretti, Theonella swinhoei, Ircinia oros, Svenzea zeai, Xestospongia testudinaria, Xestospongia muta 97-99%
clone A289/GW950
Ircinia fasciculata clone AF10−3−9_C24 JN655263
Desmacidon sponge clone KspoB1 EU035930
Xestospongia testudinaria clone XA2H05F FJ269286
Xestospongia muta clone XF1G10 FJ269350
Acanthostrongylophora sp. clone OP470 EF513732
Rhopaloeides odorabile clone T534deg31 JN210661
Discodermia dissoluta clone Dd−spT−A15 AY897081
seawater clone HOT157_350m41 JN166311
Agelas dilatata clone AD049 EF076124
clone A320/GW950
hydrocarbon seep clone BPC066 AF154095
clone A174/GW950, Montastraea faveolata, Geodia barretti, Theonella swinhoei, Corticium candelabrum 94-99%
seafloor lava clone EPR4059−B2−Bc47 EU491550
79-99%
clones A35/GW950, A295/GW950, A375/GW950, Acanthostrongylophora sp., Ircinia oros, Ircinia strobilina, Geodia barretti, Aplysina aerophoba, Xestospongia muta, Xestospongia testudinaria, Svenzea zeai, Theonella swinhoei, Montastraea faveolata
seafloor lavas clone P7X3b4D03 EU491067
Acidobacteria
clone A185/GW950, Xestospongia testudinaria, Aplysina fulva, Ircinia strobilina, Tedania ignis, Haliclona hogarthi, Hyrtios erectus, Rhopaloeides odorabile, Plakortis sp. 99%
deep−sea clone SGTA632 GQ348443
clone A310/GW950, Ircinia strobilina, Aplysina fulva, Tedania ignis, Haliclona hogarthi, Ancorina alata, Plakortis sp., Rhopaloeides odorabile 97-100%
seawater clone SHAB748 GQ348806
Deferribacteres
clone A241/GW950, Aplysina cavernicola, Xestospongia muta, Xestospongia testudinaria, Corticium candelabrum, Plakortis sp., Diacarnus erthraeanus, Theonella swinhoei, Diacarnus erthraeanus 88-99%
Texas state well clone EDW07B005_49 HM066562
Poribacteria
clone A145/GW950, Xestospongia testudinaria, Svenzea zeai, Geodia barretti
clone A402/GW950, Xestospongia testudinaria, Geodia barretti, Aplysina aerophoba, Agelas dilatata 92-98%
97-99%
hypersaline mat clone SBZI_1599 JN523931
hypersaline mat clone SBZI_4560 JN527079
Spirochaetes
clone A63/GW950, Agelas dilatata, Plakortis sp., Ircinia variabilis, Montastraea faveolata, Ancorina alata, Geodia barretti, Svenzea zeai, Xestospongia testudinaria, Xestospongia muta, Axinella corrugata 94-99%
marine sediment clone PET−093 JF344217
Nitrospirae
Agelas dilatata clone AD035 EF076144
clone A278/GW950
clone A342/GW950, Xestospongia muta, Xestospongia testudinaria, Montastraea faveolata, Aplysina aerophoba, Ancorina alata, Geodia barretti 97-99%
seafloor lava clone EPR4059−B2−Bc83 EU491583
marine sediment clone HCM3MC78_8D_FL EU374087
clone A143/GW950, Rhopaloeides odorabile, Xestospongia muta, Xestospongia testudinaria, Chondrilla mucula, Haliclona permollis 97-99%
hypersaline mat clone SBZI_4548 JN527069
clone A374/GW950, Aplysina fulva, Aplysina aerophoba, Theonella swinhoei, Chondrilla nucula, Geodia barretti 93-99%
deep−sea clone OS2BR4 JN233473
97-99%
clone A41/GW950, Xestospongia testudinaria, Xestospongia muta, Aplysina aerophoba, Ancorina alata
seafloor lava clone P0X3b5H09 EU491399
clone A130/GW950, Agelas dilatata, Geodia barretti, Xestospongia muta, Xestospongia testudinaria, Plakortis sp., 94-100%
Actinobacteria
volcano sediment clone V1F69b FJ905722
clones A210/GW950, A326/GW950, Agelas dilatata, Montastraea faveolata, Geodia barretti, Xestospongia muta, Aplysina aerophoba, Ancorina alata, Tethya aurantium, Chondrilla nucula 91-99%
deep−sea clone Ucb1575 AM997865
clone A266/GW950, Theonella swinhoei, Geodia barretti, Xestospongia muta 97-99%
Gemma-timonadetes
Pseudopterogorgia elisabethae DGGE band 7 JN863717
seawater clone [GB] PEX_8+P_E2 JF915095
clone A276/GW950
Xestospongia muta clone XE2C09 JN596608
Cyanobacteria
PCR18/GW950, Agelas dilatata, Agelas conifera, Axinella corrugata 96-98%
hydrothermal vent clone PNG_TBG_A70 JN881619
Archaea
0.1

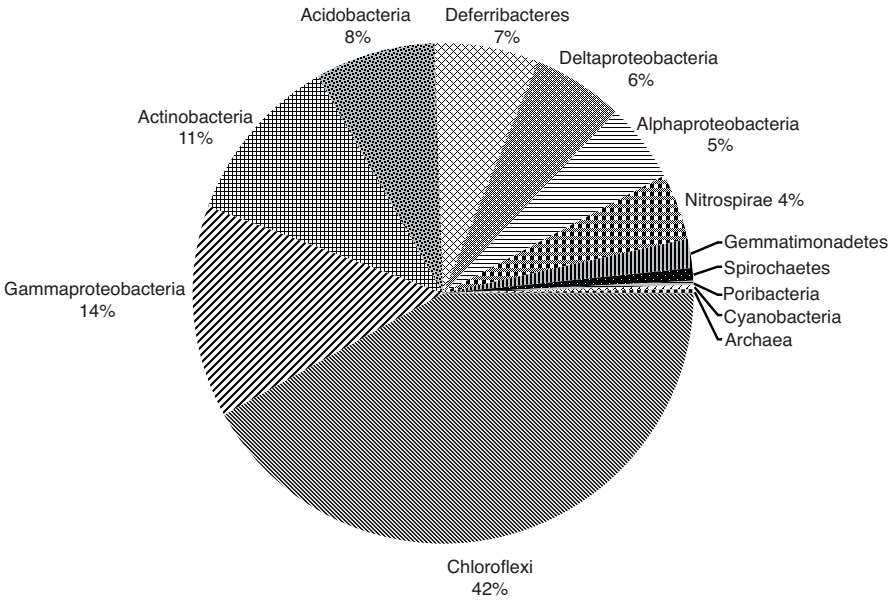


Figure 2.7. Distribution of the 16S rRNA gene clones among particular phylogenetic groups in the clone library obtained from the *A. willeyana* from Yonge Reef, GBR

Nitrococcus, *Deltaproteobacteria*/*Nitrospina*, and with the phylum *Nitrospirae*. Nitrifying bacteria have been reported from numerous sponges (Bayer et al. 2007, Mohamed et al. 2010, Schläppy et al. 2010). Recently, Off et al. (2010) cultivated for the first time nitrifying bacteria from a marine sponge. They obtained a culture of a novel *Nitrospira*-like bacterium (Aa01) from the mesohyl of the *Aplysina aerophoba*, characterized it phylogenetically, and analyzed its most important physiological features (Off et al. 2010). An additional step of nitrogen metabolism in *A. willeyana* was indicated by the presence of *Cyanobacteria,* which in many sponges are responsible for nitrogen fixation, particularly in the shallow areas of coral reefs (Wilkinson & Fay 1979, Mohamed et al. 2008a). Phylogenetic analysis revealed that the single cyanobacterial OTU was not closely affiliated with the *Candidatus "Synechococcus spongiarum,"* which formed a sponge-specific cluster based on 18 sponges collected from various geographic locations (Hentschel et al. 2006). The closest relative was a 16S rRNA gene sequence obtained from a coral *Pseudopterogorgia elisabethae* (JN863717, 99% similarity), and the free-living marine *Synechococcus* sp. exhibited the same sequence similarity (Fig. 2.6).

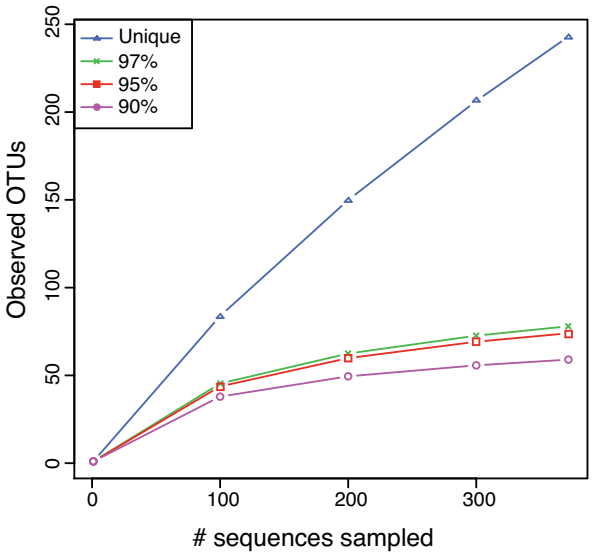


Figure 2.8. Rarefaction curves for the 16S rRNA gene sequences obtained from *A. willeyana*. Operational Taxonomic Units (OTUs) were defined at the 97%, 95% and 90% similarity criteria

The presence of sulfate-reducing bacteria (SRB) was indicated by three OTUs affiliated to the *Desulfurellaceae/ Deltaproteobacteria*. Recently, Meyer and Kuever (2008) provided evidence for a sponge-specific sulfur cycle in the deep-water sponge *Polymastia cf. corticata* based on the activities of sulfate-reducing and sulfide-oxidizing symbionts caused by changes in the pumping activity of sponges (Hoffmann et al. 2005). However, the microbial community of *A. willeyana* as documented here provided no indication of sulfide-oxidizing bacteria (SOB).Among the sponge-associated bacteria, the *Actinobacteria* are of great interest as producers of commercially useful enzymes and therapeutically useful bioactive molecules (Cook & Meyers 2003, Takahashi & Omura 2003), which have obvious implications for natural products and drug discovery. The actinobacterial OTUs obtained from the *A. willeyana* fell into two groups within the family *Acidimicrobiaceae*, which contains several large SSC (Taylor et al. 2007b). Correspondingly, 83% of the *A. willeyana* OTUs merged with the SSC and were mostly similar to the group, with the nearest (but still distantly related) culturable representative being the wastewater bacterium *Microthrix parvicella* (Taylor et al. 2007b).

Further abundant members of the *A. willeyana*-associated microbiota were *Acidobacteria*, whose functional role in the sponge microbial community is still uncer-

Table 2.2. Sample diversity

Label	OTUs	Chao estimate (95% confidence interval)	Shannon diversity index (95% confidence interval)
unique	242	726 (997-552)	5.26 (5.35-5.16)
0.03	79	100 (136-86)	3.88 (3.98-3.77)
0.05	74	99 (142-83)	3.77 (3.88-3.66)
0.10	57	64 (85-58)	3.54 (3.64-3.44)

tain (Meyer & Kuever 2008), although it is one of the most common phyla recovered from marine sponges (Webster & Taylor 2012). Recently, the vertical transmission of the *Acidobacteria* from an adult sponge of the species *Svenzea zeai* to its embryo was discovered (Lee et al. 2009). Furthermore, Mohamed et al. (2008b) successfully isolated an Acidobacterium strain (N2yML4) from the sponge species *Mycale laxissima* after the maintenance of this sponge in aquaculture. A detailed investigation of these novel cultured bacteria may provide insights into its metabolic capabilities and importance to the sponge host (Mohamed et al. 2008b). Significant fractions of *A. willeyana*-associated symbionts were indirectly (through next similar sequences) assigned to the phylum *Deferribacteres*, which enclose chemoorganotrophic heterotrophs that respire anaerobically (Garrity & Holt 2001). Those results would be comparable with a recently published study by Montalvo and Hill (2011), where the community associated with a giant barrel sponge *X. testudinaria* exhibited the *Deferribacteres* in similar abundance.

Our results are consistent with several studies, which have shown that sponges from different oceans and with distant taxonomic origins harbor specific microbial consortia (Taylor et al. 2004, Hentschel et al. 2006). The overwhelming majority of the closest relatives for the *A. willeyana*-associated OTUs were microorganisms from sponges and corals collected from different, mostly tropical, geographic regions. Moreover, an overwhelming number of those could be affiliated with larger SSC/SCC, with very high similarity. This underlines that the microbial community of the *A. willeyana* shares microbiota with recently analyzed sponges. Our results revealed the presence of some specific bacterial groups in the microbial community of *A. willeyana* that were absent in the microbiota obtained from another coralline sponge, *Vaceletia crypta*, which co-occurs and was

sampled from the same site during the same dive (Karlińska-Batres & Wörheide 2013a). The differences in the microbial communities of the two closely neighboring sponges may indicate the existence of some mechanisms for the selection of symbionts. Further studies are necessary for the comprehensive comparison of the microbial communities of both coralline sponges to demonstrate an exact relationship.

The community structure of the "living fossil" coralline sponge *A. willeyana* was very complex, with no clear domination of any of the phylogenetic groups found. The DGGE results of the 16S rRNA gene sequences of bacteria associated with twelve samples of *A. willeyana* support the above conclusions but suggest a closer relationship between the microbial communities regarding their geographic origin (northern vs. southern GBR). The microbial communities of the *A. willeyana* could differ due to environmental differences, though the lack of measurements precludes further conclusions. However these observed geographical differences in bacterial community composition could also be caused by the genetic variability of the host sponge (Taylor et al. 2005). Furthermore, in the area of the GBR, a similar deep phylogeographic break with distinct northern and southern clades was revealed for the calcareous sponges *Leucetta chagosensis* and *Pericharax heteroraphis* (Leucettidae) (Wörheide et al. 2002b, Wörheide et al. 2008). Based on spicule morphology in Indo-Pacific populations, Wörheide (1998) distinguished geographic sub-species, and subsequent investigations of nuclear internally transcribed spacer rDNA (ITS) seemed to support the presence of at least two distinct cryptic species (Wörheide et al. 2002a). Nevertheless, these findings were not confirmed by mitochondrial marker analysis, and the northern and southern GBR populations of *A. willeyana* could not be distinguished as sibling species (Wörheide 2006). However, the mitochondrial markers may not fully resolve the genetic divergence of sponge populations, as in the case of the Mediterranean sponge *Crambe crambe*, which revealed strong population structure through microsatellite investigations (Duran et al. 2004a) but not in mitochondrial DNA (Duran et al. 2004b). Therefore, the split of the southern and northern GBR microbial communities of *A. willeyana* might be an additional indicator of the existence of cryptic species. It would be interesting to explore in future studies the differences in the microbial communities of *A. willeyana* over a wide geographic

range from the Red Sea to the central Pacific and to clarify the overlap in microbiota with the distribution of *Astrosclera* cryptic species.

Acknowledgments

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Chapter 3

Spatial variability of microbial communities of the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific

This chapter is currently in preparation for standalone publication:

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Spatial variability of microbial communities of the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific

Abstract

The coralline sponge *Astrosclera willeyana*, considered to be a living representative of the reef-building stromatoporoids of the Mesozoic and Paleozoic, is the most common coralline sponge to be found throughout Indo-Pacific coral reefs. Here we used molecular methods to examine the microbiota of *A. willeyana* over its almost whole geographic range, from the Red Sea to the central Pacific. Denaturing gradient gel electrophoresis analyses of 42 *Astrosclera* specimens revealed a high microbial diversity and a complex composition in all of the investigated samples. Clearly distinct banding patterns indicated closer associations of the microbiota according to their geographic origin. Moreover, we provide the first insights into the hitherto undetermined diversity and composition of microbial communities associated with coralline sponges from the Red Sea. Random sequencing of a 16S rDNA clone library constructed from a single specimen of *A. willeyana* from the northern Red Sea exposed a very complex consortia, with the most abundant being *Chloroflexi*, followed by *Gammaproteobacteria*, and *Deltaproteobacteria*. Further members of the community belonged to *Actinobacteria*, *Alphaproteobacteria* and *Acidobacteria*, *Deferribacteria*, *Nitrospirae*, *Gemmatimonadetes*, *Spirochaetes* as well as one uncertain bacterial group. A comparison with a 16S rRNA clone library obtained previously from *A. willeyana* from the Great Barrier Reef revealed both similarities and substantial differences in the composition of the microbiota. This study provides novel information on microbiota in coralline sponges, a diversity that has not been sufficiently investigated. Furthermore, it implies that the differences in symbiotic community composition may be an additional indicator of previously postulated cryptic host species.

3.1. Introduction

Marine sponges harbor abundant and diverse microbial communities (Taylor et al. 2007, Webster & Taylor 2012); and those of the most ancient symbiotic associations between microorganisms and metazoa are estimated to have been in existence for 600 million years (Wilkinson 1984). Microbial communities may contribute up to 70% of the sponges' biomass (Wörheide 1998) and impact host metabolism, health and evolution (see Taylor et al. 2007, Webster & Taylor 2012 for a review). Recent comprehensive phylogenetic analyses on 7546 sponge-derived 16S and 18S rRNA sequences confirmed the existence of sponge-specific microbes, and, in total, 27% of the sequences in this study fell into monophyletic, sponge-specific sequence clusters (Simister et al. 2012). However, next-generation sequencing analysis revealed that putatively sponge-specific bacteria also occur in other marine environments and are probably capable of surviving outside the host, although generally at extremely low abundances (Webster et al. 2010, Taylor et al. 2013). Microbial communities in sponges are regarded as highly specific to the host species and generally stable across time and space (Taylor et al. 2007). In a recent pyrosequencing analysis of 32 marine sponge species from eight worldwide locations, Schmitt et al. (2012b) hypothesized that different sponges share a very small 'core community', and that they host mainly species-specific communities. These results suggest that broader investigations of microbial diversity in different sponge species may contribute to a clarification of sponge-specific microbiota, which may play a key role in the evolution of this putatively ancient symbiosis and in sponge response to climate change and environmental stress (Webster et al. 2011, Webster & Taylor 2012, Webster et al. 2013).

Astrosclera willeyana belongs to a group of coralline sponges which build a solid secondary calcareous skeleton (Reitner 1992, Chombard et al. 1997) in addition to a primary, often spicular, one. Coralline sponges (also called sclerosponges) contributed to the construction of reefs, where they dominated in the late Paleozoic and Mesozoic (Vacelet 1985). Regarded as a "living fossil", *A. willeyana* occurs in cryptic and light-reduced environments (e.g. reef caves) (Reitner et al. 1996), from the northern Red Sea to Tahiti (Wörheide 1998), and is the most common coralline sponge throughout the Indo-Pacific coral reefs (Reitner et al. 1996). Vacelet (1981) was the first to observe that different regional populations of *A. willeyana*

vary in spicule morphology, which is used as a criterion for taxonomic identification of the sponges. Based on a detailed morphological study of *A. willeyana* spicules distinguishing several regional populations (Wörheide 1998), and based on molecular investigations of nuclear internal transcribed spacer rDNA (ITS), Wörheide et al. (2002a) proposed the presence of at least three distinct cryptic species. However, this hypothesis was not consistent with the results of a subsequent mitochondrial marker analysis – probably due to very low mtDNA substitution rates in this taxon (Wörheide 2006).

Wörheide (1998) was the first to report large microbial communities in the living tissue of *A. willeyana* from the Indo-Pacific and noted that bacteria may make up more than 70% of the total biomass of some of the sponge's areas; on the contrary, other parts of the sponge's tissues lack bacteria almost entirely (Wörheide 1998). Recently, Karlińska-Batres and Wörheide (2013a) were the first to use molecular methods to explore the microbial diversity of coralline sponges, and they also gave the first insight into the composition of the symbiotic community of *A. willeyana* from the Great Barrier Reef (GBR) (Karlińska-Batres & Wörheide 2013b). Denaturing gradient gel electrophoresis (DGGE) revealed a clear split of the microbiota of *A. willeyana* specimens from the southern and northern parts of the GBR, thus further corroboration was provided for the existence of *A. willeyana* cryptic species (Karlińska-Batres & Wörheide 2013b). An investigation of the symbiotic communities of *A. willeyana* from other geographical locations will not only bring insight into the under-investigated microbial diversity in coralline sponges, but might also provide additional data to test the presence of cryptic species in *Astrosclera*.

Hence, we aimed to explore the differences in the microbial communities of *A. willeyana* over a wide geographic range, from the Red Sea to the central Pacific, and to test whether distinct microbiota correlate with the distribution of putative *Astrosclera* cryptic species. Therefore, we created a clone library from a microbial community of *A. willeyana* from the popular "Canyon" dive site in the Gulf of Aqaba (Dahab, Red Sea) to compare it with a previously assessed clone library obtained from *A. willeyana* from the Yonge Reef, GBR, Australia. Furthermore, we performed DGGE analysis to investigate any resemblance between the microbial communities covering nearly the total area of occurrence of *A. willeyana*, i.e. more than 20,000 km.

3.2. Materials and Methods

3.2.1. *Samples collection*

Samples of *A. willeyana* were collected during SCUBA dives at depths of between 4 and 23 meters at several sites located in the western and southern Pacific Ocean, Coral Sea and Red Sea (Table 3.1). Forty-two sponges were excised with chisel and hammer and transferred directly to plastic bags while underwater. Sponge samples were preserved either in silica gel (Erpenbeck et al. 2004), DMSO buffer (adapted from Seutin et al. (1991), or 95% ethanol. Karlińska-Batres and Wörheide (2013a) previously described the processing of sponge samples and DNA extractions in detail.

3.2.2. *Construction of 16S rRNA gene clone libraries and phylogenetic analyses*

The clone library from *A. willeyana* sample no. GW950 from Yonge Reef, GBR, Australia was described in detail by Karlińska-Batres and Wörheide (2013b). A second clone library was constructed from a sample of *A. willeyana* from the Red Sea (sample no. GW1046) using the same procedure, including PCR amplification and sequencing of individual clones but without the restriction digestion step (Karlińska-Batres & Wörheide 2013b). Sequences obtained from both samples, together with the most similar sequences determined by BLAST, were imported into the ARB program (Ludwig et al. 2004) and subsequently aligned using the ARB Integrated Aligner. The resulting alignment was checked and corrected manually for alignment errors. The neighbor-joining method (Jukes-Cantor correction) was used to calculate the initial phylogenetic tree using ARB. Subsequently, the alignment was exported from the ARB database and maximum likelihood trees were constructed using RAxML v.7.2.5 (Stamatakis 2006), using 1000 bootstrap replicates and the GTR+GAMMA model of sequence evolution. The resulting trees were visualized with the use of the FigTree v.1.3.1 program.

Table 3.1. Sample data of investigated *A. willeyana* specimens, with collection site details. [a] As the exact coordinates for the marked sampling sites were not available, the given coordinates are based on the Gazetteer of Conventional Names, Third Edition, August 1988, US Board on Geographic Names. [b] As the exact coordinates for the Red Sea, Canyon were not available, the given coordinates are based on the Google Earth

Sample No.	Location	Site (location)	Depth	Date	Latitude	Longitude
RS1	Red Sea	Canyon #1	15 m	1992	28°30'20'' N[b]	34°31'25'' E[b]
RS2	Red Sea	Canyon #2	15 m	1992	28°30'20'' N[b]	34°31'25'' E[b]
RS3	Red Sea	Canyon #3	15 m	1992	28°30'20'' N[b]	34°31'25'' E[b]
RS4	Red Sea	Canyon #4	15 m	1992	28°30'20'' N[b]	34°31'25'' E[b]
GW1046	Red Sea	Canyon #5	15 m	2006	28°30'20'' N[b]	34°31'25'' E[b]
GW950	GBR	Yonge Reef	8 m	2006	14°34'20'' S	145°36'54'' E
93 (GW5431)	GBR	Mac Gillivray Reef #1	6 m	1994	14°38'56'' S	145°29'30'' E
92 (GW5430)	GBR	Mac Gillivray Reef #2	6 m	1994	14°38'56'' S	145°29'30'' E
G316237	GBR	Harrier Reef	8 m	2001	15°08'12'' S	145°41'18'' E
GW718	GBR	Ribbon Reef 7	12 m	2001	14°58'44'' S	145°42'54'' E
G316273	GBR	Ribbon Reef 5	9 m	2001	15°20'07'' S	145°46'33'' E
G316198	GBR	Reef No. 15-040	7 m	2001	15°22'05'' S	145°56'28'' E
G313772	GBR	Myrmidon Reef	17 m	1999	18°15'28'' S	147°22'51'' E
G313826	GBR	Hook Reef	8 m	1999	19º45'14'' S	149º10'45'' E
G316066	GBR	Swain Reefs	4 m	2000	21º22'25'' S	151º14'32'' E
G316118	GBR	Merv's Reef	12 m	2001	21º53'15'' S	152º20'50'' E
GW794	GBR	Heron Island	15 m	2003	23°25'43'' S	151°57'6'' E
GW972	GBR, Coral Sea	South Island #1	6 m	2010	14°42'10'' S	145°27'3'' E
GW977	GBR, Coral Sea	South Island #2	6 m	2010	14°42'10'' S	145°27'3'' E
G316283	Coral Sea	Osprey Reef	14 m	2006	13°53'30'' S	146°33'6'' E
UF6	French Polynesia	Tuamotus	10 m	2005	14°58'60'' S	147°37'0'' W
UF8	French Polynesia	Moorea	12-16 m	2005	15°00' S[a]	140°00' W[a]
G316176	Guam	Haputo #1	5-18 m	2001	13° 28' N[a]	144° 47' E[a]
G316179	Guam	Haputo #2	5-18 m	2001	13° 28' N[a]	144° 47' E[a]
GW769.7	Palau	Siaes Tunnel #1	5-18 m	2002	7° 30' N[a]	134° 30' E[a]
GW769.6	Palau	Siaes Tunnel #2	5-18 m	2002	7° 30' N[a]	134° 30' E[a]
GW769.5	Palau	Siaes Tunnel #3	5-18 m	2002	7° 30' N[a]	134° 30' E[a]
GW769.4	Palau	Siaes Tunnel #4	5-18 m	2002	7° 30' N[a]	134° 30' E[a]
GW769.1	Palau	Siaes Tunnel #5	5-18 m	2002	7° 30' N[a]	134° 30' E[a]
G313888	Vanuatu	Vanu Lava	18-23 m	1999	13º56'48'' S	167º26'28'' E
G313906	Vanuatu	Mota Lava	15 m	1999	13º39'3'' S	167º39'14'' E
G313935	Vanuatu		5-18 m	1999	16° 00' S[a]	167° 00' E[a]
JH47	Vanuatu	Espiritu Santo #1	5-18 m		16° 00' S[a]	167° 00' E[a]
JH23	Vanuatu	Espiritu Santo #2	5-18 m		16° 00' S[a]	167° 00' E[a]
JH3	Vanuatu	Espiritu Santo #3	5-18 m		16° 00' S[a]	167° 00' E[a]
102 (GW5440)	Fiji	Waya Island #1	15 m	1999	18° 00' S[a]	175° 00' E[a]
101 (GW5439)	Fiji	Waya Island #2	15 m	1999	18° 00' S[a]	175° 00' E[a]
98 (GW5436)	Fiji	Astrolabe Reef #1	8 m	1998	18° 00' S[a]	175° 00' E[a]
97 (GW5435)	Fiji	Astrolabe Reef #2	6 m	1998	18° 00' S[a]	175° 00' E[a]
96 (GW5434)	Fiji	Astrolabe Reef #3	6 m	1998	18° 00' S[a]	175° 00' E[a]
95 (GW5433)	Fiji	Astrolabe Reef #4	15 m	1998	18° 00' S[a]	175° 00' E[a]
94 (GW5432)	Fiji	Astrolabe Reef #5	15 m	1998	18° 00' S[a]	175° 00' E[a]

3.2.3. *Sponge-specific and sponge-coral clusters*

Monophyletic, sponge-specific and sponge-coral clusters (SSC/SCC) were defined based on criteria established by Hentschel et al. (2002). The BLAST search results were checked for similar sequences obtained from different sponges, corals and non-sponge sources, which were subsequently incorporated into the ARB database and used to calculate phylogenies using neighbor-joining (ARB) and maximum likelihood methods (RAxML).

3.2.4. *Estimation of microbial diversity and statistical analysis of the clone libraries*

The distance matrix generated by ARB was used to assign sequences obtained from the samples of *A. willeyana*, from Yonge Reef, GBR and from the Red Sea, to operational taxonomic units (OTUs) using Mothur (Schloss et al. 2009) and with a cut-off value of 0.03 (Schloss & Handelsman 2005). The clones from the GBR sample that were analyzed only by restriction digestion were also assigned to a corresponding OTU based on their restriction pattern. To determine the abundance and richness of the bacterial communities associated with each sponge, the Shannon and Simpson diversity indices (Spellerberg & Fedor 2003) were calculated to describe species diversity. The Chao1 and ACE (abundance-based coverage estimator) richness index (Colwell & Coddington 1994) was used to estimate total species richness. The LIBSHUFF method was applied in order to determine the significance of differences between the clone libraries (Schloss et al. 2004). The method compares more than two libraries at once with the same distance matrix in order to determine whether two libraries were drawn from the same population. Mothur was used to perform the calculations and to generate a Venn diagram to compare the richness shared between the microbial communities of both sponges. The rarefaction curves calculated with Mothur were plotted using the R software package (http://www.R-project.org).

3.2.5. *Nucleotide sequence accession numbers*

Clone sequences obtained from *A. willeyana* from the Yonge Reef, GBR were previously deposited in an EMBL database under the accession numbers HE985081-HE985159. The sequences obtained during this study from the specimen from the

Red Sea were deposited in the EMBL database under the accession numbers HG423455-HG423535.

3.2.6. *Denaturing gradient gel electrophoresis*

The bacterial 16S rRNA genes from all 42 DNA extracts were amplified by touchdown PCR with GoTaq polymerase (Promega GmbH, Mannheim, Germany) and universal primers 341F-GC and 907RC (Muyzer & Smalla 1998, Schäfer 2001) in a Biometra (Göttingen, Germany) thermocycler. PCR reactions were performed as described by Karlińska-Batres and Wörheide (2013b). A phorU-2 system (Ingeny, Goes, Netherlands) and Power Pac 300 (BioRad, Munich, Germany) to supply the power were used for the DGGE with a denaturing gradient of 30%–70% (urea and formamide) in a 6% polyacrylamide gel. PCR-amplified DNA (30 μl) was loaded onto the gel and run for 16 h at 180 V and at a temperature of 60 °C. Due to the large number of samples we processed them on two different DGGE gels; the horizontal line in Table 1 indicates a separation of the samples between the gels. Gel 1 included samples from the GBR and the Red Sea; gel 2 included samples from the Coral Sea (also GBR), French Polynesia, Guam, Fiji, Palau, and Vanuatu. After DGGE the gels were soaked for 25 min in SYBR Gold (Molecular Probes, Darmstadt, Germany) and photographed with an RT Color SPOT camera and SPOT advanced imaging software (Visitron Systems, Puchheim, Germany). QuantityOne version 4.69 software (Bio-Rad) was used for gel image data analysis. Automatic assignment of band positions was checked and corrected manually. The band-matching Dice coefficient with optimization at 0.75% and a tolerance level of 0.75% was used for the similarity calculations between the DGGE banding patterns. Cluster analyses were performed using the unweighted pair group method with arithmetic averages (UPGMA) to obtain similarity dendrograms.

3.3. Results

3.3.1. Clone library construction, OTU assignment and phylogenetic analyses

A total of 380 clones were selected from the 16S rRNA clone library as amplified from *A. willeyana* from the Yonge Reef (GW950). From that number, 298 clones were sequenced and 9 sequences were discarded as chimeras. Through clustering

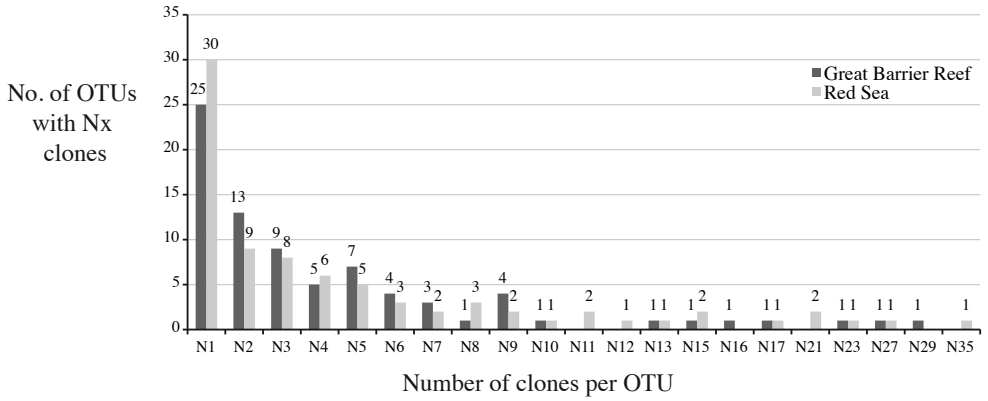


Figure 3.1. Distribution of 16S rRNA gene clones among the OTUs. * N1 represents the number of singletons, N2 the number of doubletons, etc.

of the remaining 289 clone sequences together with a single archaeal 16S rRNA sequence in Mothur, 79 OTUs were retrieved using a 97% similarity criterion. A further 82 clones were assigned to a particular OTU based on their restriction patterns. From the 16S rRNA clone library amplified from *A. willeyana* from the Red Sea (GW1046), 427 clones were selected and sequenced and from those one chimerical sequence was discarded. The remaining 426 clones were clustered into 81 OTUs using Mothur (97% similarity criterion). The singletons constituted 32% of the clone library of *A. willeyana* from the GBR and about 37% of the specimen from the Red Sea (Fig. 3.1). Both specimens of *A. willeyana* revealed 16S rRNA gene sequences classified as *Chloroflexi, Gammaproteobacteria, Actinobacteria*, *Acidobacteria*, *Deferribacteres*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Nitrospirae,* and *Spirochaetes* (Tab. 3.2). Additionally, the GBR specimen exposed sequences belonging to *Poribacteria*, *Cyanobacteria*, and *Crenarchaeota*; the specimen from the Red Sea also revealed a single sequence of uncertain affiliation, which differed from bacteria from any described phylum. The phylogenetic trees present the OTUs from both *A. willeyana* with the nearest similar sequences assigned to *Chloroflexi* (Fig. 3.2) and *Proteobacteria* (Fig. 3.3A and B), and to all other phyla (Fig. 3.4).

3.3.2. Closest relatives

Based on the BLAST results, *A. willeyana* from the GBR had a slightly higher fraction of OTUs that were closely related to other previously described sponge- or coral-derived microbial sequences, i.e. 94% (74 OTUs out of 79 defined OTUs) in

Table 3.2. Distribution of the 16S rRNA clones and OTUs defined at distance 0.03 among particular phylogenetic groups in the clone libraries obtained from the *A. willeyana* samples

Phylogenetic group	Great Barrier Reef		Red Sea	
	No. of clones	No. of OTUs	No. of clones	No. of OTUs
Chloroflexi	156	25	174	27
Gammaproteobacteria	51	12	95	17
Actinobacteria	41	6	28	5
Acidobacteria	29	9	21	5
Deferribacteres	25	2	18	3
Deltaproteobacteria	22	8	46	9
Alphaproteobacteria	19	8	27	9
Nitrospirae	16	1	9	2
Gemmatimonadetes	7	3	5	2
Spirochaetes	3	2	2	1
Poribacteria	1	1	0	0
Cyanobacteria	1	1	0	0
uncertain affiliation	0	0	1	1
Archaea	1	1	0	0
	372	79	426	81

comparison with the Red Sea specimen of 88% (71 OTUs out of 81 defined OTUs). The closest Red Sea-associated relatives were obtained from 18 different sponge species, and those from the GBR were obtained only from 13 species. Both *Astrosclera* specimens shared closest relatives hosted by 10 sponge species (*Ancorina alata*, *Agelas dilatata*, *Aplysina fulva*, *Axinella corrugata*, *Geodia baretti*, *Plakortis* sp., *Rhopaloeides odorabile*, *Svenzea zeai*, *Xestospongia muta*, *Xestospongia testudinaria*). The GBR specimen revealed closest relatives from a further 3 sponges (*Acanthostrongylophora* sp., *Phyllospongia papyracea*, *Theonella swinhoei*), and the Red Sea specimen from a further 8 sponges (*Desmacidon* sp., *Haliclona hogarthi*, *Haliclona simulans*, *Ircinia oros*, *Ircinia strobilina*, *Ircinia variabilis*, *Pachastrella* sp., *Sigmadocia fibulata*). Both sponges revealed similar fractions of OTUs with closest relatives obtained from corals (GBR 11%, Red Sea 10%); the most numerous sequences were obtained from *Montastraea faveolata* (8 OTUs from the GBR

sponge, and 7 OTUs from the Red Sea sponge), and single OTUs from two different corals (GBR – *Pseudopterogorgia elisabethae* and Red Sea – *Porites astreoides*). *A. willeyana* from the GBR had a distinctly lower ratio (6%) of OTUs, with closest relatives derived from the environment (Red Sea specimen 12%). Only *A. willeyana* from the Red Sea exposed two OTUs with a closest sequence derived from a validly described organism (94% similarity with a sponge isolate *Pseudovibrio denitrificans* and 99% similarity with a copepod isolate *Pseudoalteromonas piscicida*), as well as one OTU with a closely related (99%) 16S rRNA sequence of *Pseudomonas* sp. isolated from costal sediment water. The results of the BLAST search are summarized in a table in Supplementary Material (Tab. S3.1).

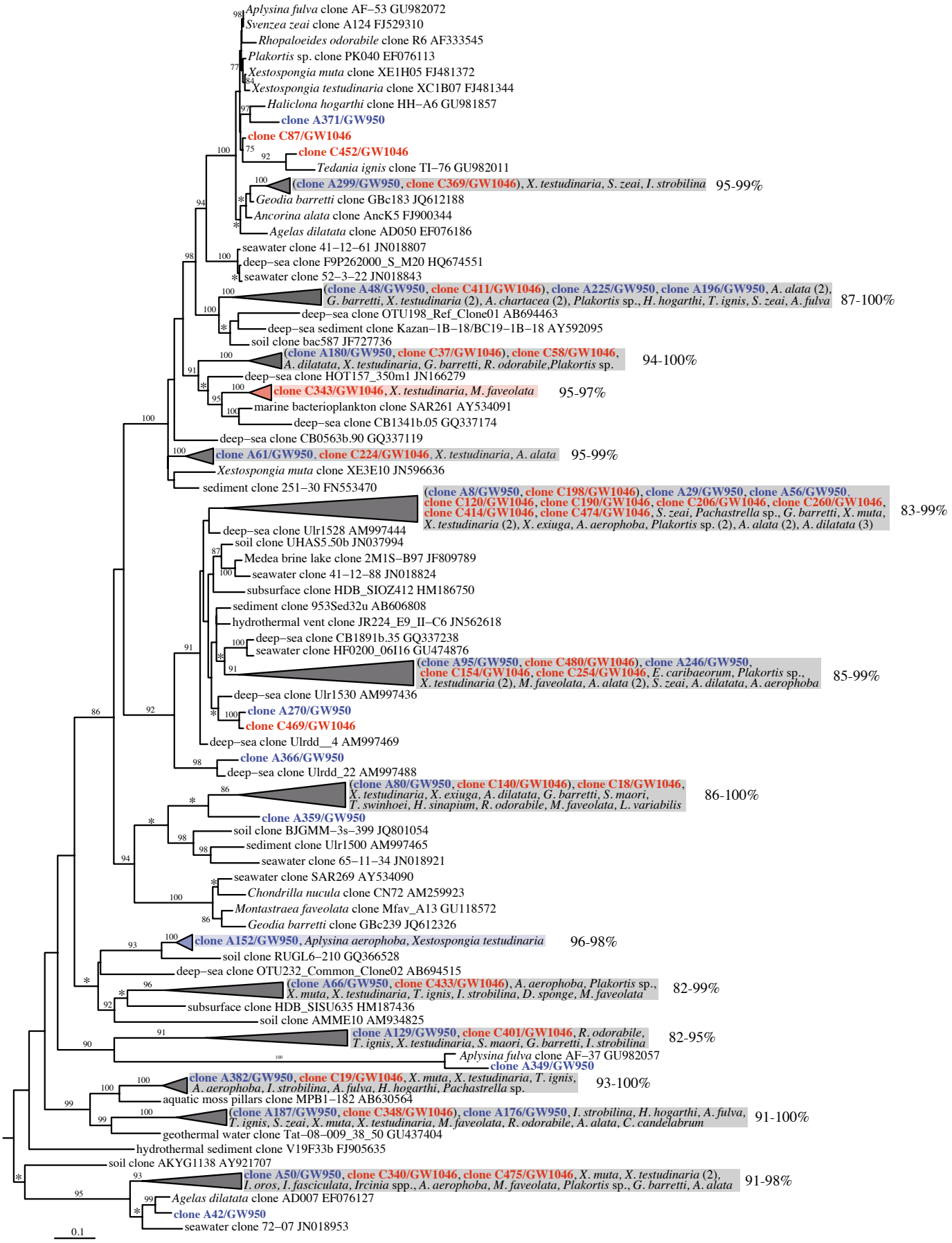
3.3.3. Shared OTUs

Cloned *A. willeyana* samples shared 31 OTUs, which represented 61% of the GBR clones (n=227) and 55% of the Red Sea clones (n=236). The largest number of shared OTUs belonged to the phylum *Proteobacteria* (12 OTUs), which grouped 43 clones from the GBR specimen and 70 clones from the Red Sea specimen. Ten shared OTUs belonged to the *Chloroflexi* and grouped 100 clones from the GBR specimen and 102 clones from the Red Sea specimen. *A. willeyana* from the GBR shared seven OTUs with 10–29 clones, and Red Sea *A. willeyana* shared 9 OTUs with 10–27 clones. In the phylogenetic trees (Figs. 3.2–3.4) the shared OTUs are indicated as clone names in brackets.

3.2.4. *Sponge-specific and sponge-coral clusters*

From both coralline sponges, 67% of the OTUs closely related to other sponge- or coral-derived sequences fell into 49 SSCs/SCCs (GBR – 53 OTUs, Red Sea – 54

Figure 3.2. The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA sequences affiliated to the phylum *Chloroflexi*, with the next most similar sequences obtained from other sponges or corals and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study from *A. willeyana* samples (*blue* from the GBR; *red* from the Red Sea); *clone names in brackets* indicate shared OTUs. *Shaded boxes* represent sponge-specific clusters: *grey* - clusters shared, *blue* with the clones from the GBR, *red* with the clones from the Red Sea; *numbers in parenthesis next to sponge names* indicate the number of sequences per sponge. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


Aplysina fulva clone AF–53 GU982072
Svenzea zeai clone A124 FJ529310
Rhopaloeides odorabile clone R6 AF333545
Plakortis sp. clone PK040 EF076113
Xestospongia muta clone XE1H05 FJ481372
Xestospongia testudinaria clone XC1B07 FJ481344
Haliclona hogarthi clone HH–A6 GU981857
clone A371/GW950
clone C87/GW1046
clone C452/GW1046
Tedania ignis clone TI–76 GU982011
(clone A299/GW950, clone C369/GW1046), X. testudinaria, S. zeai, I. strobilina 95-99%
Geodia barretti clone GBc183 JQ612188
Ancorina alata clone AncK5 FJ900344
Agelas dilatata clone AD050 EF076186
seawater clone 41–12–61 JN018807
deep–sea clone F9P262000_S_M20 HQ674551
seawater clone 52–3–22 JN018843
(clone A48/GW950, clone C411/GW1046), clone A225/GW950, clone A196/GW950, A. alata (2), G. barretti, X. testudinaria (2), A. chartacea (2), Plakortis sp., H. hogarthi, T. ignis, S. zeai, A. fulva 87-100%
deep–sea clone OTU198_Ref_Clone01 AB694463
deep–sea sediment clone Kazan–1B–18/BC19–1B–18 AY592095
soil clone bac587 JF727736
(clone A180/GW950, clone C37/GW1046), clone C58/GW1046, A. dilatata, X. testudinaria, G. barretti, R. odorabile,Plakortis sp. 94-100%
deep–sea clone HOT157_350m1 JN166279
clone C343/GW1046, X. testudinaria, M. faveolata 95-97%
marine bacterioplankton clone SAR261 AY534091
deep–sea clone CB1341b.05 GQ337174
deep–sea clone CB0563b.90 GQ337119
clone A61/GW950, clone C224/GW1046, X. testudinaria, A. alata 95-99%
Xestospongia muta clone XE3E10 JN596636
sediment clone 251–30 FN553470
(clone A8/GW950, clone C198/GW1046), clone A29/GW950, clone A56/GW950, clone C120/GW1046, clone C190/GW1046, clone C206/GW1046, clone C260/GW1046, clone C414/GW1046, clone C474/GW1046, S. zeai, Pachastrella sp., G. barretti, X. muta, X. testudinaria (2), X. exiuga, A. aerophoba, Plakortis sp. (2), A. alata (2), A. dilatata (3) 83-99%
deep–sea clone Ulr1528 AM997444
soil clone UHAS5.50b JN037994
Medea brine lake clone 2M1S–B97 JF809789
seawater clone 41–12–88 JN018824
subsurface clone HDB_SIOZ412 HM186750
sediment clone 953Sed32u AB606808
hydrothermal vent clone JR224_E9_II–C6 JN562618
deep–sea clone CB1891b.35 GQ337238
seawater clone HF0200_06I16 GU474876
(clone A95/GW950, clone C480/GW1046), clone A246/GW950, clone C154/GW1046, clone C254/GW1046, E. caribaeorum, Plakortis sp., X. testudinaria (2), M. faveolata, A. alata (2), S. zeai, A. dilatata, A. aerophoba 85-99%
deep–sea clone Ulr1530 AM997436
clone A270/GW950
clone C469/GW1046
deep–sea clone Ulrdd__4 AM997469
clone A366/GW950
deep–sea clone Ulrdd_22 AM997488
(clone A80/GW950, clone C140/GW1046), clone C18/GW1046, X. testudinaria, X. exiuga, A. dilatata, G. barretti, S. maori, T. swinhoei, H. sinapium, R. odorabile, M. faveolata, L. variabilis 86-100%
clone A359/GW950
soil clone BJGMM–3s–399 JQ801054
sediment clone Ulr1500 AM997465
seawater clone 65–11–34 JN018921
seawater clone SAR269 AY534090
Chondrilla nucula clone CN72 AM259923
Montastraea faveolata clone Mfav_A13 GU118572
Geodia barretti clone GBc239 JQ612326
clone A152/GW950, Aplysina aerophoba, Xestospongia testudinaria 96-98%
soil clone RUGL6–210 GQ366528
deep–sea clone OTU232_Common_Clone02 AB694515
(clone A66/GW950, clone C433/GW1046), A. aerophoba, Plakortis sp., X. muta, X. testudinaria, T. ignis, I. strobilina, D. sponge, M. faveolata 82-99%
subsurface clone HDB_SISU635 HM187436
soil clone AMME10 AM934825
clone A129/GW950, clone C401/GW1046, R. odorabile, T. ignis, X. testudinaria, S. maori, G. barretti, I. strobilina 82-95%
Aplysina fulva clone AF–37 GU982057
clone A349/GW950
clone A382/GW950, clone C19/GW1046, X. muta, X. testudinaria, T. ignis, A. aerophoba, I. strobilina, A. fulva, H. hogarthi, Pachastrella sp. 93-100%
aquatic moss pillars clone MPB1–182 AB630564
(clone A187/GW950, clone C348/GW1046), clone A176/GW950, I. strobilina, H. hogarthi, A. fulva, T. ignis, S. zeai, X. muta, X. testudinaria, M. faveolata, R. odorabile, A. alata, C. candelabrum 91-100%
geothermal water clone Tat–08–009_38_50 GU437404
hydrothermal sediment clone V19F33b FJ905635
soil clone AKYG1138 AY921707
clone A50/GW950, clone C340/GW1046, clone C475/GW1046, X. muta, X. testudinaria (2), I. oros, I. fasciculata, Ircinia spp., A. aerophoba, M. faveolata, Plakortis sp., G. barretti, A. alata 91-98%
Agelas dilatata clone AD007 EF076127
clone A42/GW950
seawater clone 72–07 JN018953
0.1

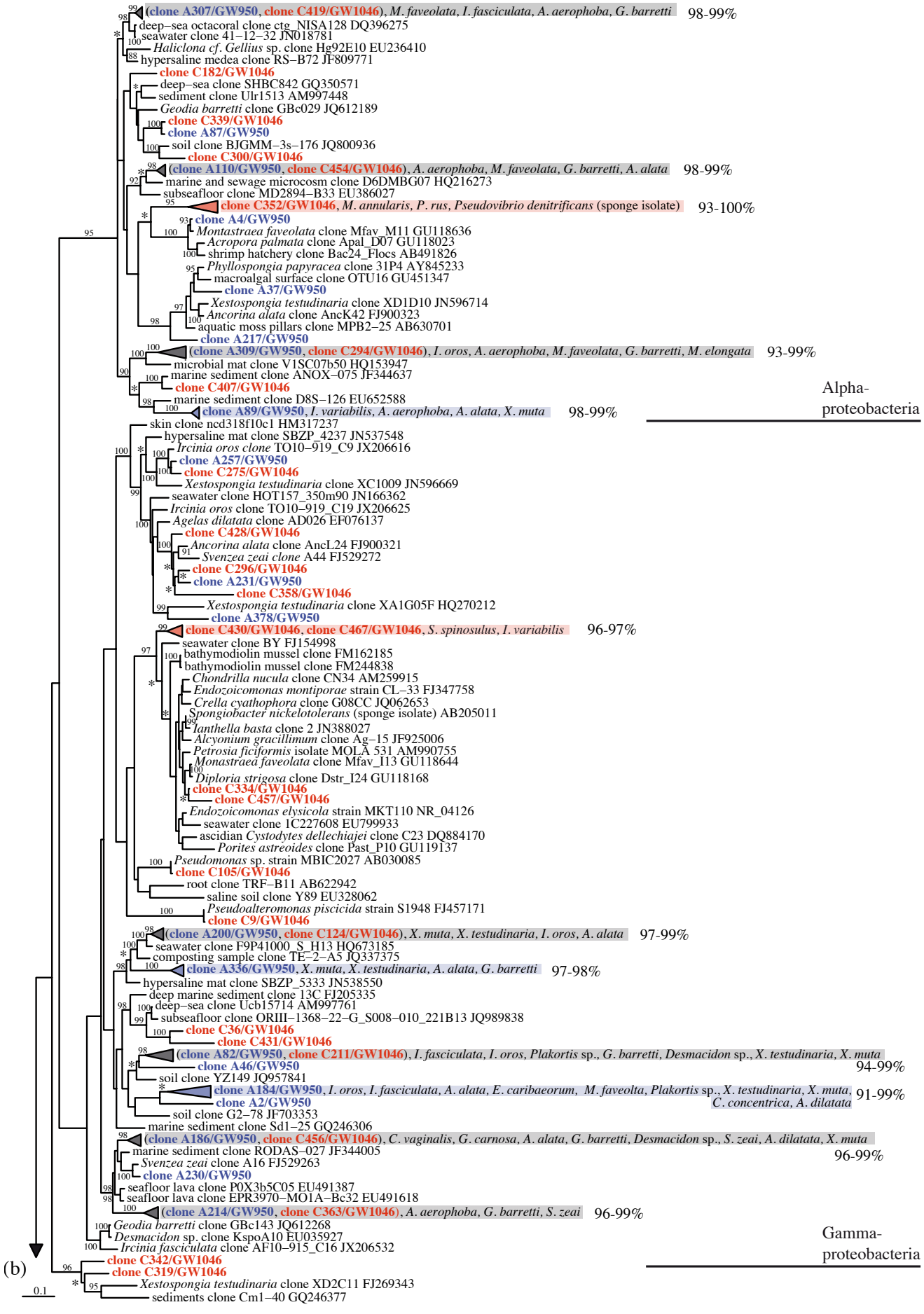
OTUs). The *Astrosclera* specimens shared 33 SSCs/SCCs and, additionally, 10 OTUs of the GBR specimen were assigned to 10 individual SSCs/SCCs, and 10 OTUs of the Red Sea specimen were assigned to 5 individual SSCs/SCCs.

3.2.5. *Microbial diversity and community structure*

An analysis of the clone library showed that the microbial community of *A. willeyana* from the GBR was slightly more diverse due to the presence of *Poribacteria, Cyanobacteria*, and *Archaea*; however, members of an unclassified clade were found only by the *A. willeyana* from the Red Sea (Fig. 3.5). Furthermore, both communities varied notably in the abundance of *Gammaproteobacteria* (14% for the GBR and 22% for the Red Sea) and *Deltaproteobacteria* (6% for the GBR and 11% for the Red Sea) as well as slightly in the abundance of *Actinobacteria* and *Acidobacteria* (11% and 8% for the GBR and 7% and 5% for the Red Sea, respectively). In both communities the most abundant taxa were *Chloroflexi* (43% for the GBR and 41% for the Red Sea). Libhuff statistical analysis of the libraries (Schloss et al. 2004) confirmed a highly significant difference between the microbial communities of the two *A. willeyana* samples ($P<0.0001$). A Venn diagram of the OTU distributions at a distance of 0.03 revealed that of the 129 defined and different OTUs, 24% were shared between the communities of *A. willeyana* from the GBR and Red Sea.

A slightly higher Shannon-Wiener index for *A. willeyana* from the GBR confirmed a greater complexity of its microbiota. However, the Simpson index, which gives a strong weighting to the dominants, showed no differences between the two in-

Figure 3.3A. The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA sequences affiliated to the phylum *Proteobacteria*, with the next most similar sequences obtained from other sponges or corals and from the environment. The tree is displayed as two subtrees (a, b), arrows go to the remaining tree parts. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study from *A. willeyana* samples (*blu*e from the GBR; *red* from the Red Sea); *clone names in brackets* indicate shared OTUs. *Shaded boxes* represent sponge-specific clusters: *grey* - clusters shared, *blue* with the clones from the GBR, *red* with the clones from the Red Sea; *numbers in parenthesis next to sponge names* indicate the number of sequences per sponge. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


(clone A307/GW950, clone C419/GW1046), M. faveolata, I. fasciculata, A. aerophoba, G. barretti
98-99%
deep–sea octacoral clone ctg_NISA128 DQ396275
seawater clone 41–12–32 JN018781
Haliclona cf. Gellius sp. clone Hg92E10 EU236410
hypersaline medea clone RS–B72 JF809771
clone C182/GW1046
deep–sea clone SHBC842 GQ350571
sediment clone Ulr1513 AM997448
Geodia barretti clone GBc029 JQ612189
clone C339/GW1046
clone A87/GW950
soil clone BJGMM–3s–176 JQ800936
clone C300/GW1046
(clone A110/GW950, clone C454/GW1046), A. aerophoba, M. faveolata, G. barretti, A. alata
98-99%
marine and sewage microcosm clone D6DMBG07 HQ216273
subseafloor clone MD2894–B33 EU386027
clone C352/GW1046, M. annularis, P. rus, Pseudovibrio denitrificans (sponge isolate)
93-100%
clone A4/GW950
Montastraea faveolata clone Mfav_M11 GU118636
Acropora palmata clone Apal_D07 GU118023
shrimp hatchery clone Bac24_Flocs AB491826
Phyllospongia papyracea clone 31P4 AY845233
macroalgal surface clone OTU16 GU451347
clone A37/GW950
Xestospongia testudinaria clone XD1D10 JN596714
Ancorina alata clone AncK42 FJ900323
aquatic moss pillars clone MPB2–25 AB630701
clone A217/GW950
(clone A309/GW950, clone C294/GW1046), I. oros, A. aerophoba, M. faveolata, G. barretti, M. elongata
93-99%
microbial mat clone V1SC07b50 HQ153947
marine sediment clone ANOX–075 JF344637
clone C407/GW1046
marine sediment clone D8S–126 EU652588
clone A89/GW950, I. variabilis, A. aerophoba, A. alata, X. muta
98-99%
Alpha-proteobacteria
skin clone ncd318f10c1 HM317237
hypersaline mat clone SBZP_4237 JN537548
Ircinia oros clone TO10–919_C9 JX206616
clone A257/GW950
clone C275/GW1046
Xestospongia testudinaria clone XC1009 JN596669
seawater clone HOT157_350m90 JN166362
Ircinia oros clone TO10–919_C19 JX206625
Agelas dilatata clone AD026 EF076137
clone C428/GW1046
Ancorina alata clone AncL24 FJ900321
Svenzea zeai clone A44 FJ529272
clone C296/GW1046
clone A231/GW950
clone C358/GW1046
Xestospongia testudinaria clone XA1G05F HQ270212
clone A378/GW950
clone C430/GW1046, clone C467/GW1046, S. spinosulus, I. variabilis
96-97%
seawater clone BY FJ154998
bathymodiolin mussel clone FM162185
bathymodiolin mussel clone FM244838
Chondrilla nucula clone CN34 AM259915
Endozoicomonas montiporae strain CL–33 FJ347758
Crella cyathophora clone G08CC JQ062653
Spongiobacter nickelotolerans (sponge isolate) AB205011
Ianthella basta clone 2 JN388027
Alcyonium gracillimum clone Ag–15 JF925006
Petrosia ficiformis isolate MOLA 531 AM990755
Monastraea faveolata clone Mfav_I13 GU118644
Diploria strigosa clone Dstr_I24 GU118168
clone C334/GW1046
clone C457/GW1046
Endozoicomonas elysicola strain MKT110 NR_04126
seawater clone 1C227608 EU799933
ascidian Cystodytes dellechiajei clone C23 DQ884170
Porites astreoides clone Past_P10 GU119137
Pseudomonas sp. strain MBIC2027 AB030085
clone C105/GW1046
root clone TRF–B11 AB622942
saline soil clone Y89 EU328062
Pseudoalteromonas piscicida strain S1948 FJ457171
clone C9/GW1046
(clone A200/GW950, clone C124/GW1046), X. muta, X. testudinaria, I. oros, A. alata
97-99%
seawater clone F9P41000_S_H13 HQ673185
composting sample clone TE–2–A5 JQ337375
clone A336/GW950, X. muta, X. testudinaria, A. alata, G. barretti
97-98%
hypersaline mat clone SBZP_5333 JN538550
deep marine sediment clone 13C FJ205335
deep–sea clone Ucb15714 AM997761
subseafloor clone ORIII–1368–22–G_S008–010_221B13 JQ989838
clone C36/GW1046
clone C431/GW1046
(clone A82/GW950, clone C211/GW1046), I. fasciculata, I. oros, Plakortis sp., G. barretti, Desmacidon sp., X. testudinaria, X. muta
clone A46/GW950
94-99%
soil clone YZ149 JQ957841
clone A184/GW950, I. oros, I. fasciculata, A. alata, E. caribaeorum, M. faveolta, Plakortis sp., X. testudinaria, X. muta, C. concentrica, A. dilatata
clone A2/GW950
91-99%
soil clone G2–78 JF703353
marine sediment clone Sd1–25 GQ246306
(clone A186/GW950, clone C456/GW1046), C. vaginalis, G. carnosa, A. alata, G. barretti, Desmacidon sp., S. zeai, A. dilatata, X. muta
marine sediment clone RODAS–027 JF344005
Svenzea zeai clone A16 FJ529263
clone A230/GW950
96-99%
seafloor lava clone P0X3b5C05 EU491387
seafloor lava clone EPR3970–MO1A–Bc32 EU491618
(clone A214/GW950, clone C363/GW1046), A. aerophoba, G. barretti, S. zeai
96-99%
Geodia barretti clone GBc143 JQ612268
Desmacidon sp. clone KspoA10 EU035927
Ircinia fasciculata clone AF10–915_C16 JX206532
Gamma-proteobacteria
clone C342/GW1046
clone C319/GW1046
Xestospongia testudinaria clone XD2C11 FJ269343
sediments clone Cm1–40 GQ246377
(b)
0.1

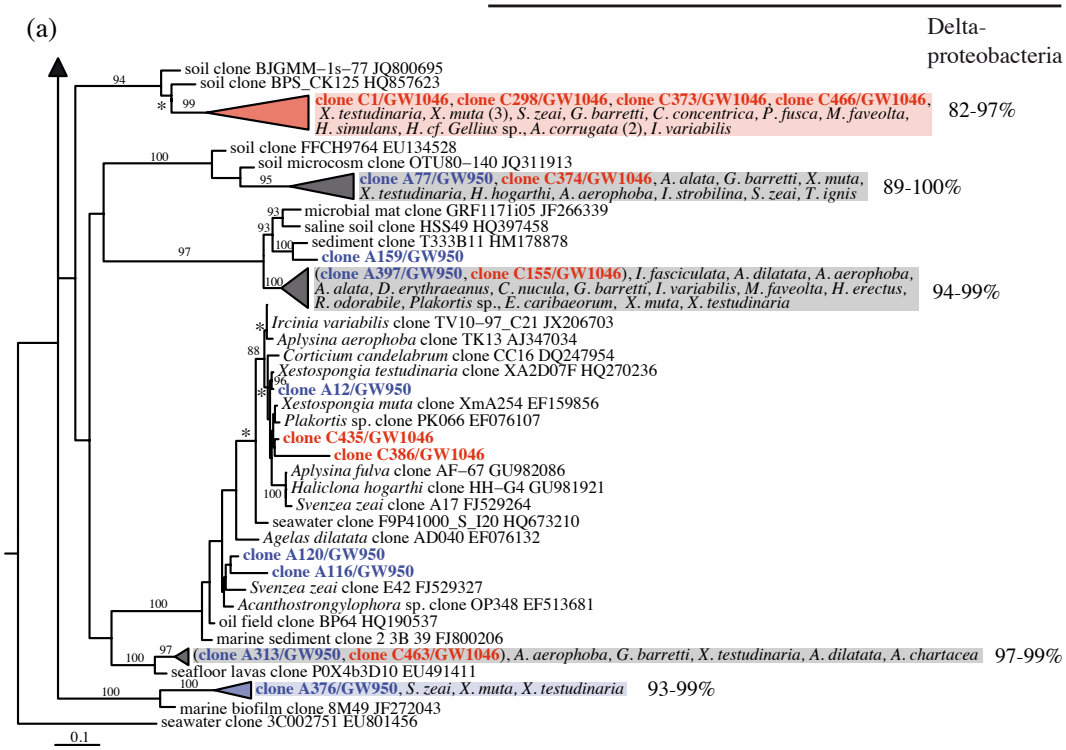


Figure 3.3B. Continued

vestigated communities. According to the Chao1 index and the abundance-based coverage estimator (ACE; Tab. 3.3), we sequenced 80% of the predicted number of microbial species in the community associated with *A. willeyana* from the GBR; for *A. willeyana* from the Red Sea the values given by the estimators differed (65% according to the Chao1 index and 72% for ACE) and indicated a less effective sampling. In contrast, the rarefaction curves for the Red Sea sample calculated for the 0.03, 0.05, and 0.1 cut-off criteria indicated a more successful sampling than was denoted by the Chao1 and ACE estimators; however, they did not reach clear saturation, but were rather very flat (Fig 3.6). Despite these differences, the sampled diversity provides a comprehensive picture of the core microbial communities of both coralline sponges (Schmitt et al. 2011).

3.2.6. *Denaturing gradient gel electrophoresis*

We found very complex DGGE banding profiles in all of the samples with numerous bands: the samples from Fiji and the Red Sea showed the lowest average number of bands (26 bands from the Red Sea and Waya Island, Fiji, and 27 bands

by the Astrolabe Reef, Fiji); the Haputo, Guam, samples showed the highest average of bands (34). On Gel 1 we detected 73 different band types, with only two predominant bands, which were present in all of the *Astrosclera* samples, and another 4 bands, which were missing from four individual samples. On Gel 2 we found 84 different band types, and here also only 2 bands were present in all 25 samples, and a further 2 in 24 samples (the banding profiles of both gels are available in the supplementary material – Fig. S3.1 and Fig. S3.2). Due to the difficulty of making an accurate comparison between the gels, we performed the cluster analysis separately. However, on both gels the samples were clustered together according to their geographical origin (Fig 3.7). Analysis of Gel 1 demonstrated a clear division between North GBR (N'GBR), South GBR (S'GBR) and GBR inshore samples with separation of the Red Sea. Analysis of Gel 2 demonstrated a definite division between the microbial communities associated with *A. willeyana* from Palau, the Coral Sea, and the South Pacific (French Polynesia); whereas within the Western Pacific the microbial communities of *A. willeyana* sampled to the north of the largest Fijian island, Viti Levu, (Guam, Vanuatu and the Waya Island) clustered together and formed a sister group to the specimens from the Astrolabe Reef (to the south of Viti Levu) (Fig 3.7).

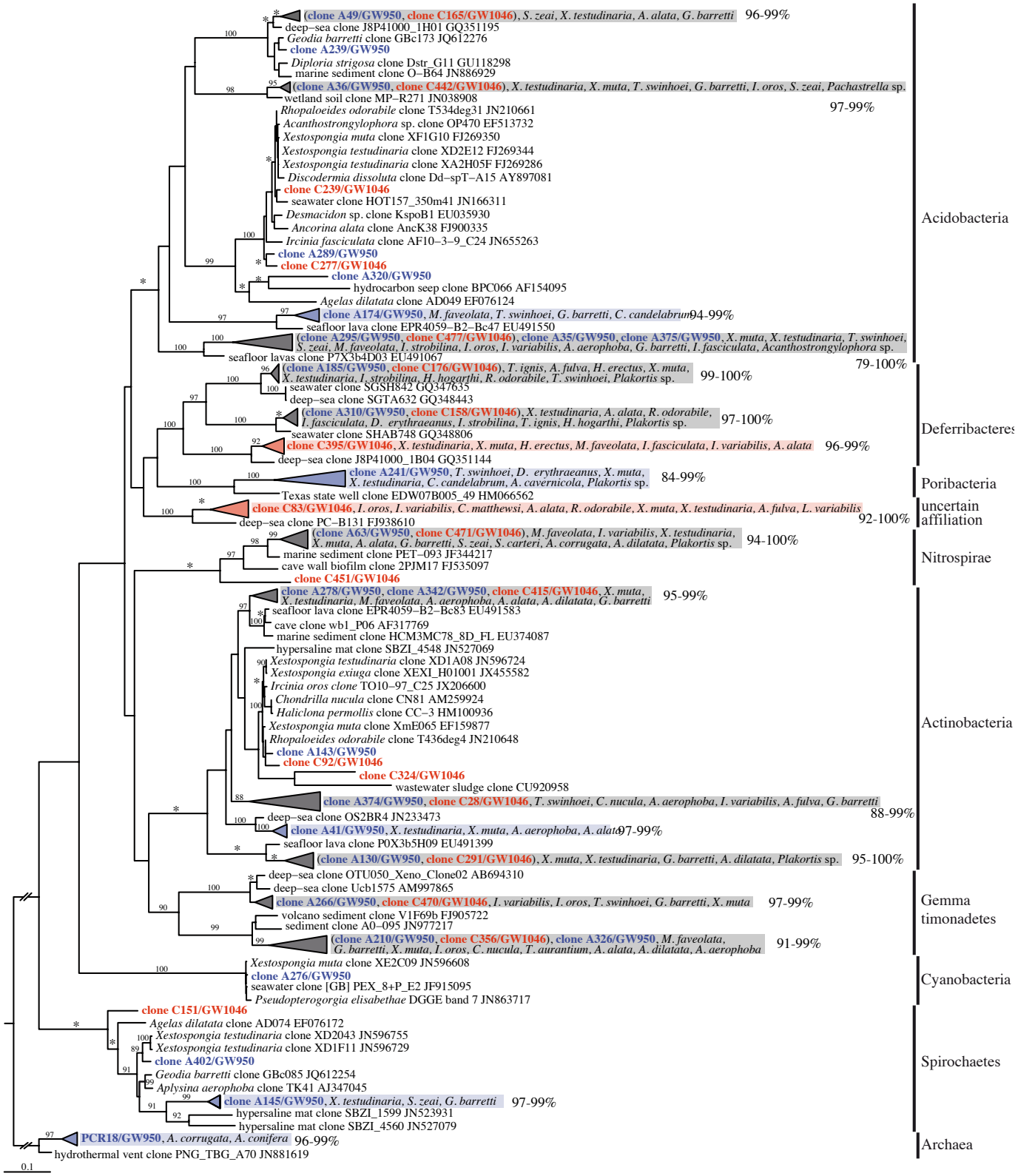
3.4. Discussion

To our knowledge this is the first study to address the diversity and composition of the microbial community associated with coralline sponges from the Red Sea, and it is the first investigation of the microbiota of *A. willeyana* over its wide Indo-Pacific range, from the Red Sea to the central Pacific. The 16S rRNA gene-based characterization of the microbial diversity of *A. willeyana* from the Red Sea revealed a highly complex and rich symbiotic community, including representatives of eight bacterial phyla and one uncertain bacterial group. A comparison with a previously assessed 16S clone library of *A. willeyana* from the GBR (Karlińska-Batres & Wörheide 2013b) showed significant differences in community composition as well as some similarities between the microbiota. The DGGE analysis of bacterial 16S rRNA genes obtained from *A. willeyana* specimens covering the vast area of their appearance confirmed a high microbial diversity in all of

the investigated samples and revealed a closer association between the microbial communities with respect to their geographical provenance.

The Red Sea, with its perennial high temperatures and high salinity of seawater bodies, constitutes a unique ecosystem on a global scale and a natural habitat for corals and sponges (Ilan et al. 2004). More than two decades of research on Red Sea sponges have brought significant findings regarding natural products and bioactive compounds as well as and their ecological importance to coral reefs (Ilan et al. 2004). However, from among about the 240 sponge species that were recorded in the Red Sea (Radwan et al. 2010), the microbial communities of only a few were investigated (Hentschel et al. 2002, Oren et al. 2005, Radwan et al. 2010, Lee et al. 2011). Our investigations on the microbial community of *A. willeyana* gave the first ever insight into the microbiota of coralline sponges from the Red Sea. This was also the first study on Red Sea sponges with a very high number of selected clones. Phyla commonly associated with marine sponges dominated in the microbial community of *A. willeyana* (Taylor et al. 2007, Webster & Taylor 2012); however, these phyla were significantly differentiated from the communities associated with other Red Sea sponges that had previously been studied using a similar 16S rRNA cloning approach (Hentschel et al. 2002, Oren et al. 2005, Radwan et al. 2010, Lee et al. 2011). The level of diversity could be compared with the microbiota of Red Sea *Hyrtios erectus* (42 selected clones), but this symbiotic community revealed members of *Bacteroidetes*, *Firmicutes*, TM7 and *Betaproteobacteria* (Radwan et al. 2010), and lacked bacteria of an uncertain affiliation that were found in the Red Sea *A. willeyana*. In the same study Radwan et al. (2010) also investigated the microbial community from *Amphimedon* sp. (39

Figure 3.4. The maximum-likelihood phylogeny of *A. willeyana*-derived 16S rRNA sequences affiliated to several phyla and to the domain *Archaea*, with the next most similar sequences obtained from other sponges or corals and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones obtained during this study from *A. willeyana* samples (*blue* from the GBR; *red* from the Red Sea); *clone names in brackets* indicate shared OTUs. *Shaded boxes* represent sponge-specific clusters: *grey* - clusters shared, *blue* with the clones from the GBR, *red* with the clones from the Red Sea; *numbers in parenthesis next to sponge names* indicate the number of sequences per sponge. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisks*. *Scale bar* signifies 10% sequence divergence →


(clone A49/GW950, clone C165/GW1046), S. zeai, X. testudinaria, A. alata, G. barretti 96-99%
deep–sea clone J8P41000_1H01 GQ351195
Geodia barretti clone GBc173 JQ612276
clone A239/GW950
Diploria strigosa clone Dstr_G11 GU118298
marine sediment clone O–B64 JN886929
(clone A36/GW950, clone C442/GW1046), X. testudinaria, X. muta, T. swinhoei, G. barretti, I. oros, S. zeai, Pachastrella sp.
wetland soil clone MP–R271 JN038908
97-99%
Rhopaloeides odorabile clone T534deg31 JN210661
Acanthostrongylophora sp. clone OP470 EF513732
Xestospongia muta clone XF1G10 FJ269350
Xestospongia testudinaria clone XD2E12 FJ269344
Xestospongia testudinaria clone XA2H05F FJ269286
Discodermia dissoluta clone Dd–spT–A15 AY897081
clone C239/GW1046
seawater clone HOT157_350m41 JN166311
Desmacidon sp. clone KspoB1 EU035930
Ancorina alata clone AncK38 FJ900335
Ircinia fasciculata clone AF10–3–9_C24 JN655263
clone A289/GW950
clone C277/GW1046
clone A320/GW950
hydrocarbon seep clone BPC066 AF154095
Agelas dilatata clone AD049 EF076124
clone A174/GW950, M. faveolata, T. swinhoei, G. barretti, C. candelabrum 94-99%
seafloor lava clone EPR4059–B2–Bc47 EU491550
(clone A295/GW950, clone C477/GW1046), clone A35/GW950, clone A375/GW950, X. muta, X. testudinaria, T. swinhoei, S. zeai, M. faveolata, I. strobilina, I. oros, I. variabilis, A. aerophoba, G. barretti, I. fasciculata, Acanthostrongylophora sp.
seafloor lavas clone P7X3b4D03 EU491067
79-100%
(clone A185/GW950, clone C176/GW1046), T. ignis, A. fulva, H. erectus, X. muta, X. testudinaria, I. strobilina, H. hogarthi, R. odorabile, T. swinhoei, Plakortis sp. 99-100%
seawater clone SGSH842 GQ347635
deep–sea clone SGTA632 GQ348443
(clone A310/GW950, clone C158/GW1046), X. testudinaria, A. alata, R. odorabile, I. fasciculata, D. erythraeanus, I. strobilina, T. ignis, H. hogarthi, Plakortis sp. 97-100%
seawater clone SHAB748 GQ348806
clone C395/GW1046, X. testudinaria, X. muta, H. erectus, M. faveolata, I. fasciculata, I. variabilis, A. alata 96-99%
deep–sea clone J8P41000_1B04 GQ351144
clone A241/GW950, T. swinhoei, D. erythraeanus, X. muta, X. testudinaria, C. candelabrum, A. cavernicola, Plakortis sp. 84-99%
Texas state well clone EDW07B005_49 HM066562
clone C83/GW1046, I. oros, I. variabilis, C. matthewsi, A. alata, R. odorabile, X. muta, X. testudinaria, A. fulva, L. variabilis
92-100%
deep–sea clone PC–B131 FJ938610
(clone A63/GW950, clone C471/GW1046), M. faveolata, I. variabilis, X. testudinaria, X. muta, A. alata, G. barretti, S. zeai, S. carteri, A. corrugata, A. dilatata, Plakortis sp. 94-100%
marine sediment clone PET–093 JF344217
cave wall biofilm clone 2PJM17 FJ535097
clone C451/GW1046
clone A278/GW950, clone A342/GW950, clone C415/GW1046, X. muta, X. testudinaria, M. faveolata, A. aerophoba, A. alata, A. dilatata, G. barretti 95-99%
seafloor lava clone EPR4059–B2–Bc83 EU491583
cave clone wb1_P06 AF317769
marine sediment clone HCM3MC78_8D_FL EU374087
hypersaline mat clone SBZI_4548 JN527069
Xestospongia testudinaria clone XD1A08 JN596724
Xestospongia exiuga clone XEXI_H01001 JX455582
Ircinia oros clone TO10–97_C25 JX206600
Chondrilla nucula clone CN81 AM259924
Haliclona permollis clone CC–3 HM100936
Xestospongia muta clone XmE065 EF159877
Rhopaloeides odorabile clone T436deg4 JN210648
clone A143/GW950
clone C92/GW1046
clone C324/GW1046
wastewater sludge clone CU920958
clone A374/GW950, clone C28/GW1046, T. swinhoei, C. nucula, A. aerophoba, I. variabilis, A. fulva, G. barretti
88-99%
deep–sea clone OS2BR4 JN233473
clone A41/GW950, X. testudinaria, X. muta, A. aerophoba, A. alata 97-99%
seafloor lava clone P0X3b5H09 EU491399
(clone A130/GW950, clone C291/GW1046), X. muta, X. testudinaria, G. barretti, A. dilatata, Plakortis sp. 95-100%
deep–sea clone OTU050_Xeno_Clone02 AB694310
deep–sea clone Ucb1575 AM997865
clone A266/GW950, clone C470/GW1046, I. variabilis, I. oros, T. swinhoei, G. barretti, X. muta 97-99%
volcano sediment clone V1F69b FJ905722
sediment clone A0–095 JN977217
(clone A210/GW950, clone C356/GW1046), clone A326/GW950, M. faveolata, G. barretti, X. muta, I. oros, C. nucula, T. aurantium, A. alata, A. dilatata, A. aerophoba 91-99%
Xestospongia muta clone XE2C09 JN596608
clone A276/GW950
seawater clone [GB] PEX_8+P_E2 JF915095
Pseudopterogorgia elisabethae DGGE band 7 JN863717
clone C151/GW1046
Agelas dilatata clone AD074 EF076172
Xestospongia testudinaria clone XD2043 JN596755
Xestospongia testudinaria clone XD1F11 JN596729
clone A402/GW950
Geodia barretti clone GBc085 JQ612254
Aplysina aerophoba clone TK41 AJ347045
clone A145/GW950, X. testudinaria, S. zeai, G. barretti 97-99%
hypersaline mat clone SBZI_1599 JN523931
hypersaline mat clone SBZI_4560 JN527079
PCR18/GW950, A. corrugata, A. conifera 96-99%
hydrothermal vent clone PNG_TBG_A70 JN881619
0.1
Acidobacteria
Deferribacteres
Poribacteria
uncertain affiliation
Nitrospirae
Actinobacteria
Gemma timonadetes
Cyanobacteria
Spirochaetes
Archaea

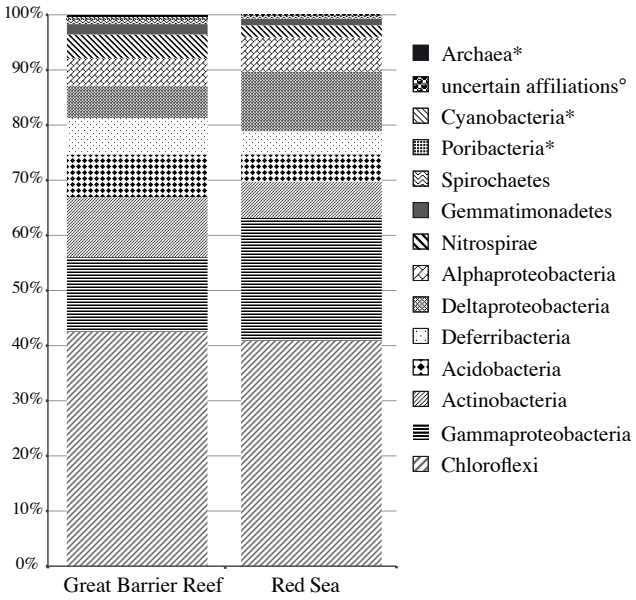


Figure 3.5. Distribution of the 16S rRNA gene clones among particular phylogenetic groups in the clone libraries obtained from the *A. willeyana* from Yonge Reef, GBR, Australia (left) and from the Red Sea (right). Phylogenetic groups found only in one of the clone libraries are indicated with asterisks (GBR) or with a degree symbol (Red Sea)

clones), but this microbiota revealed strikingly lower diversity and only slight overlap with *A. willeyana*. The microbial diversity associated with *A. willeyana* also significantly exceeded the diversity of the Red Sea sponge *Diacarnus erythraenus* (37 clones) dominated by *Cyanobacteria* (Bergman et al. 2011). However, if we also consider sequences obtained from larvae (38 clones) and from isolates from adult sponges (88) and larvae (40), the magnitude of diversity increases considerably (Bergman et al. 2011), which makes the results comparable to those of the community obtained from *A. willeyana*. The first pyrosequencing analysis of microbiota associated with three Red Sea sponges – *Hyrtios erectus*, *Stylissa carteri* and *Xestospongia testudinaria* (Lee et al. 2011) – significantly expanded the magnitude of microbial diversity in sponges from this biogeographic region; however, the differences in the level of phylogenetic resolution between the techniques that were used made a direct comparison of this study with our analyses difficult. One of the striking findings of the study by Lee et al. (2011) was a very high abundance of *Archaea* – up to 300 archaeal species estimated from a single sponge (up to 100 OTUs revealed). *A. willeyana* from the Red Sea lacked *Archaea* entirely. Interestingly, the clone library of *A. willeyana* from the

GBR revealed only a single archaeal OTU that was similar to a clone library constructed from another coralline sponge, *Vaceletia crypta*, which co-occurs and was sampled from the same site (Yonge Reef, GBR) (Karlińska-Batres & Wörheide 2013a). These findings raise the question as to whether coralline sponges indeed form very limited associations with *Archaea*.

The comparison of 16S rRNA clone libraries obtained from *A. willeyana* from the Red Sea and from the GBR (Karlińska-Batres & Wörheide 2013b) showed identical participation of clones from both communities in the SSC/SCC, thus confirming the uniqueness of symbiotic associations in sponges; they also revealed a more complex structure of the microbial community associated with the GBR specimen. The microbiota differed in terms of presence of some minor members of the communities (*Poribacteria, Cyanobacteria*, *Archaea* and the unclassified clade) and in the abundance of other groups (*Gammaproteobacteria*, *Deltaproteobacteria, Actinobacteria,* and *Acidobacteria*). According to Shade and Handelsman (2012), an abundant microorganism that is shared among all samples within a given habitat must play a significant function in the community. Therefore, recognizing core microbiomes (microbes that are common to two or more samples) in complex microbial habitats is the first step in understanding systems ecology (Shade & Handelsman 2012, Webster et al. 2013). Most studies compare the microbial communities of sponges from the same geographical region or sea (Erwin et al. 2012, Schmitt et al. 2012b, Webster et al. 2013). A study of 13 GBR sponge species revealed a high core microbiome within each species but a low microbiome shared between the species – a maximum of five sponge species shared OTUs and 91% of the OTUs were species-specific (Webster et al. 2013). In another study of three sympatric Mediterranean *Ircinia* sp., Erwin at al. (2012) identified host species-specific OTUs, OTUs shared between the two most phylogenetically related species and OTUs common to two species sharing the same cryptic habitat. These results suggested that host-specific factors have an impact on structuring microbial symbiont communities (Erwin et al. 2012). Montalvo and Hill (2011) compared, for the first time, the microbial symbionts of two closely related sponges from different oceans and revealed that the bacterial communities associated with *X. muta* and *X. testudinaria* were specific to each of the sponge species and to the genus *Xestospongia*. Our results are comparable with the study on *Xestospongia* sp. (Montalvo & Hill 2011), since the investigated *A. willeyana* samples

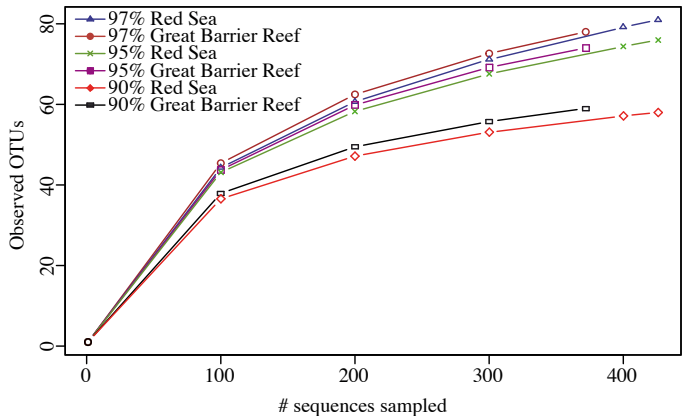


Figure 3.6. Rarefaction curves for the 16S rRNA gene sequences obtained from *A. willeyana* from the GBR and from the Red Sea. Operational Taxonomic Units (OTUs) were defined at the 97%, 95% and 90% similarity criteria

Table 3.3. Diversity analysis of the 16S rRNA gene clone libraries constructed at distance 0.03 for the *A. willeyana* samples. Lower and upper 95% confidence intervals are shown in parentheses where available. ACE: abundance-base coverage estimator

Sample source	No. of clones	No. of OTUs	Chao estimate	ACE	Shannon index	Simpson index
Great Barrier Reef	372	79	100 (87-136)	100 (89-125)	3.88 (3.77-3.98)	0.028 (0.023-0.033)
Red Sea	426	81	124 (98-191)	113 (96-148)	3.84 (3.73-3.94)	0.029 (0.025-0.033)

from the GBR and Red Sea shared 31 OTUs as defined on the 0.03 similarity criterion which grouped over half of both clone libraries. However, despite the fact that more clones were selected from the Red Sea specimen, we detected less diversity (predicted 65% of OTUs). Hence, Red Sea *A. willeyana* might harbor more abundant and heterogeneous microbiota; therefore, although our results indicate high species-specific associations in this coralline sponge, a further investigation of samples is necessary to confirm the host-specific nature of *A. willeyana* microbial communities.

The DGGE analysis of 42 microbial communities of *A. willeyana* over species widespread geographical distribution from the Red Sea to French Polynesia confirmed both high diversity and complex composition in all microbial communities. Moreover, the results exhibited a closer relationship between microbial

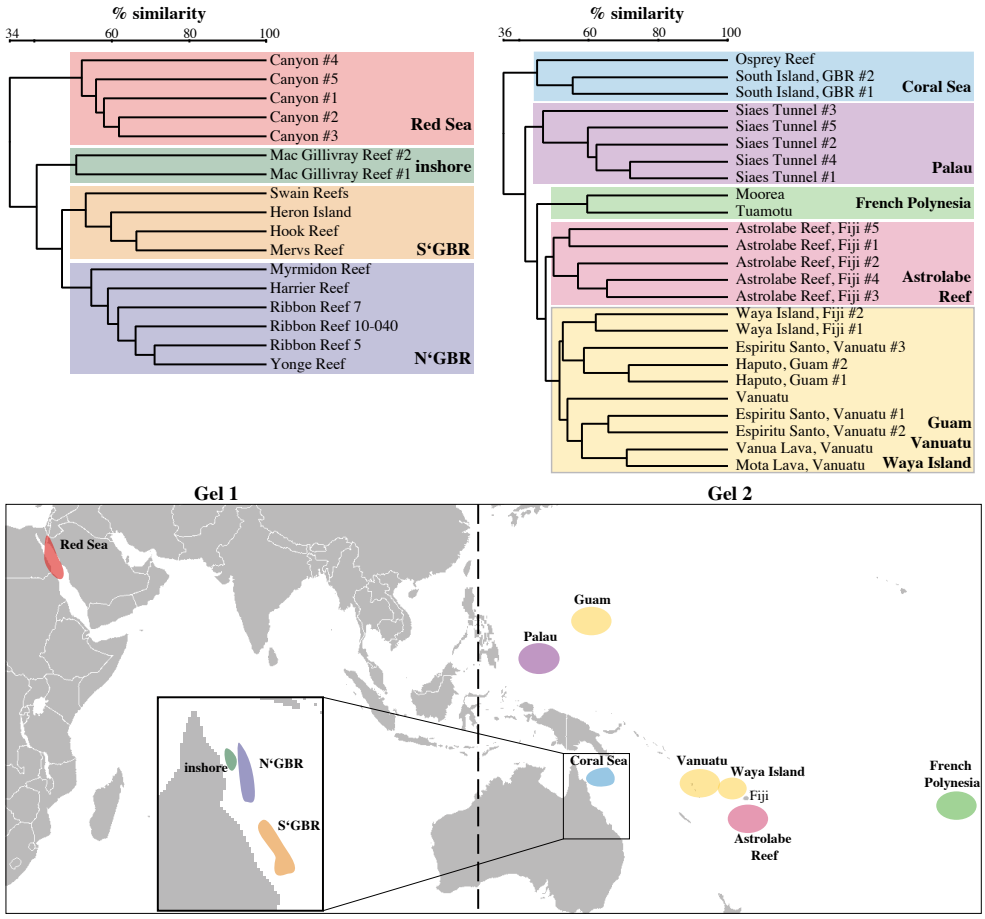


Figure 3.7. UPGMA dendrograms obtained with DGGE results of the PCR-amplified bacterial 16S rRNA genes of the microbial community associated with *A. willeyana* from different localities; colors of different clades (trees) accordingly to the colors of the geographical origin of the samples (map). Samples are named according to Table 3.1 (column: Site)

communities depending on geographical origin, which was similar to observations in samples from the GBR (Karlińska-Batres & Wörheide 2013b). Karlińska-Batres and Wörheide (2013b) suggested that the split between the southern and northern GBR microbial communities associated with *A. willeyana* might be an additional indicator of the existence of cryptic species (Wörheide 1998, Wörheide et al. 2002a, Wörheide 2006). In this study, not only on a small scale (GBR) but also through the wide range of *A. willeyana* in the Indo-Pacific, the microbial communities derived from specimens obtained from geographically closer populations clustered together (Fig. 3.7) partly confirmed these assumptions. The present data support the separation of a distinct Red Sea population of *A. willeyana*,

as has been primarily evidenced by analyses of ITS and COI (Wörheide et al. 2002a, Wörheide 2006). However, the separation of microbial communities from the Coral Sea and Palau, together with the clustering of microbial communities from Guam with microbiota of *A. willeyana* from Vanuatu and Waya Island (Fiji), as well as a clear split of the Astrolabe Reef population from the rest of Fiji, suggests closer affiliation of geographically more distant populations. This pattern could be explained, though, by ancient gene flow regimes determined by historical events that were controlled by different current systems during sea level low stands (Benzie 1999, Wörheide et al. 2002a) as was evidenced for some other coral reef organisms, such as the sponge *Leucetta 'chagosensis'*, starfishes *Acanthaster plancii* and *Linckia levigata* as well as the giant clam *Tridacna gigas* (Benzie 1994, 1999, Wörheide et al. 2002b).

Our study demonstrates the high diversity of microorganisms associated with *A. willeyana* from the Indo-Pacific. We cloned single samples from the GBR (Karlińska-Batres & Wörheide 2013b) and from the Red Sea, but by selecting an exceptionally high number of clones we provided a comprehensive picture of the core microbial community of the investigated *A. willeyana*, which was complemented through the application of DGGE for analysis of numerous samples. In our results the absence (Red Sea) or low occurrence (GBR) of *Archaea* and *Poribacteria* is surprising. Future studies with specific primers could bring more insight into the diversity of these phylogenetic groups. However, another coralline sponge, *Vaceletia crypta*, which co-occurs and was sampled from the same site (Yonge Reef, GBR), showed similar results (Karlińska-Batres & Wörheide 2013a). Furthermore, exploring the differences between the microbial communities of these two coralline sponges could bring more insight into the microbial associations that are specific for coralline sponges.

Acknowledgements

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Chapter 4

Microbial duel between coralline sponges – a comparison of the symbiotic communities of *Astrosclera willeyana* and *Vaceletia crypta*

This chapter is currently in preparation for standalone publication:

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Microbial duel between coralline sponges – a comparison of the symbiotic communities of *Astrosclera willeyana* and *Vaceletia crypta*

Abstract

Coralline sponges remain among the most understudied sponges in terms of their associations with microbial symbionts, although their Silurian fossils point to close interactions with microorganisms and might indicate an early stage of sponge-microbial symbiosis. Here we compare, for the first time, the microbial communities of two coralline sponges co-occurring at the Great Barrier Reef and demonstrate that, despite some differences, these sponges share phylogenetic highly similar symbiotic consortia. Both *Astrosclera willeyana* and *Vaceletia crypta*, considered to be living representatives of reef-building sclerosponges of the Mesozoic and Paleozoic, harbored very rich and diverse microbial communities with strikingly comparable composition of phyla, which are commonly affiliated with marine sponges. However, the coralline sponges differed in the abundance of members of particular phylogenetic groups. In this respect, *V. crypta* revealed a slightly more complex community structure showing a higher number of OTUs and the presence of members of *Betaproteobacteria*, *Deinococcus-Thermus*, and *Bacteroidetes*. Both coralline sponges exhibited very high numbers of OTUs with next similar sequences obtained from other sponges. *A. willeyana* and *V. crypta* shared over 30% of the 93 here identified sponge-specific clusters to which the majority of their microbial 16S rRNA sequences were affiliated. Furthermore, the coralline sponges shared a high number of bacterial species exceeding the level of OTUs characteristic for other sponges, and thus indicated specific patterns for the constitution of microbial communities in sclerosponges. Our results imply that at least a fraction of the symbionts of both *A. willeyana* and *V. crypta* must have been transmitted vertically.

4.1. Introduction

Associations of sponges (phylum Porifera) with extremely dense and diverse communities of microorganisms are believed to have their origin in the Precambrian (Wilkinson 1984), making them one of the most ancient microbe-metazoan symbiosis. So far, at least 32 bacterial phyla and candidate phyla as well as several archaeal lineages were reported from sponges (Hentschel et al. 2012, Webster & Taylor 2012, Webster et al. 2013), though members of the *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Proteobacteria*, (especially *Alpha*, *Delta*, *Gamma* classes) and the candidate phylum "*Poribacteria*" are considered as "core" taxa and the dominant sponge symbionts (Taylor et al. 2007b). Despite recently being reported from seawater (Webster et al. 2010) microbial symbionts are highly specific for sponges and their associations show low temporal and spatial variability (Hentschel et al. 2002, Taylor et al. 2007b). Analysis of numerous physical, chemical, and biological conditions, which may have an impact on the structure of symbiotic communities in marine sponges, showed that host-specific factors, such as mesohyl conditions, shape the structure of sponge-associated microbiota (Erwin et al. 2012). Moreover the microbial communities in sponges have been recently reported as specific to particular sponge species (Webster et al. 2010), and there is apparently a lack of correlation between host phylogeny and the arrangement of the symbionts (Schmitt et al. 2012a, Webster et al. 2013). However, after three decades of research the clear picture of microbial diversity in sponges remain afar and many issues unsolved; the ongoing studies bring new insights and lead to better understanding of those interactions (Webster & Blackall 2008, Webster & Taylor 2012).

Coralline sponges, or other called sclerosponges, belong to the most understudied sponges in term of associations with microbial symbionts. This unique group of Porifera constructs a solid secondary calcareous skeleton and enclose approximately 15 living taxa with *Astrosclera willeyana* and *Vaceletia crypta* among others (Reitner 1992, Chombard et al. 1997). Slerosponges contributed to reef-building in the Paleozoic and Mesozoic (Vacelet 1985) until they were displaced by corals approximately since late Jurassic (Reitner 1992). Both coralline sponges are regarded as 'living fossils': *A. willeyana,* the most common coralline sponge throughout the Indo-Pacific, is considered to represent the long-extinct "Stroma-

toporoidea," (Wood 1987, Chombard et al. 1997) and *V. crypta* is the only recent member of the so-called 'sphinctozoan-type' sponges (Reitner & Wörheide 2002). The discovery of Silurian fossil stromatoporoids neighbouring ubiquitous microbial laminae or less commonly encrusted by Cyanobacteria denote close associations (Soja et al. 2003) and might indicate an early stage of sponge-microbial symbiosis. It implies that investigations of coralline sponges might contribute to the elucidation of the evolution of sponge-microbe symbiosis.
Recently Karlinska-Batres and Wörheide described the microbial diversity of single sclerosponge species (2013a, 2013b), but microbiota of different coralline sponge species were never compared. Here we aim to close this gap and to examine how those previously assed microbial communities of the two co-occurring coralline sponges *A. willeyana* and *V. crypta* differ, and whether they show any specific patterns. The comprehensive analysis of the symbiotic communities will contribute to the limited knowledge of the insufficiently explored microbial diversity in coralline sponges and will elucidate if factors modeling modern microbial communities have also influenced the ancient associations of microorganisms in sclerosponges.

4.2. Material and methods

4.2.1. *Sample collection and construction of the 16S rRNA gene clone library*

Samples of *A. willeyana* (sample No. GW950) and *V. crypta* (sample No. GW947) were collected in 2006 during one SCUBA diving in a cave at a depth of 8 meters at Yonge Reef on the Great Barrier Reef (GBR) (14°34'20" S, 145°36'54" E). Collection, processing of both samples, and construction of the clone libraries were described previously in detail by Karlińska-Batres and Wörheide (2013a, 2013b).

4.2.2. *Phylogenetic analyses of microbial 16S rRNA clone libraries*

The sequences obtained from the *A. willeyana* and *V. crypta*, together with the most similar sequences found by BLAST (http://blast.ncbi.nlm.nih.gov/) were incorporated into the ARB database used to run phylogenetic analyses (Ludwig et al. 2004). The partial sequences were added to the ARB database using the ARB parsimony "quick add" tool. For this study the sequences were aligned using the ARB Integrated Aligner and the alignment was checked and corrected manually

for alignment errors. The neighbor-joining method (Jukes-Cantor correction) was used to calculate the initial phylogenetic using ARB. Subsequently, the alignment was exported from the ARB database and maximum likelihood phylogenies were constructed using RAxML v.7.2.5 (Stamatakis 2006) using 1000 bootstrap replicates and the GTR+GAMMA model of sequence evolution. The resulting trees were visualized using a program FigTree v.1.3.1.

4.2.3. *Sponge-specific and sponge-coral clusters*

In order to define the monophyletic, sponge-specific clusters the BLAST search results were checked for similar sequences obtained from different sponges, corals and non-sponges sources, which subsequently were incorporated in the ARB database and used to calculate phylogenies using neighbor-joining (ARB) and maximum likelihood methods (RAxML). Based on the criteria established by Hentschel (2002), as sponge-specific and/or sponge-coral cluster (SSC/SCC) were regarded group of sequences from sponges and corals that cluster together in one clade independent of the tree reconstruction method.

4.2.4. *Estimation of microbial diversity and statistical analysis of clone library*

The sequences obtained from the samples of *A. willeyana* and *V. crypta* were grouped as OTUs (operational taxonomic units) using Mothur (Schloss et al. 2009) and based on the distance matrix generated by ARB with a cut-off value of 0.03 (Schloss & Handelsman 2005). To determine the abundance and richness of the bacterial communities associated with each sponge, the Shannon and Simpson diversity indices (Spellerberg & Fedor 2003) were calculated to describe species diversity. The Chao1 and ACE (abundance-base coverage estimator) richness index (Colwell & Coddington 1994) were used to estimate total species richness. In order to determine the significance of differences between the clone libraries, the LIBSHUFF method was applied (Schloss et al. 2004). It compares more than two libraries at once with the same distance matrix to determine whether two libraries were drawn from the same population. The calculations were performed using Mothur. Also Mothur was used to generate rarefaction curves for observed OTUs and Venn diagram to compare the richness shared between both sponges. The rarefaction curves were plotted using the R software package (http://www.R-project.org).

4.2.5. Nucleotide sequence accession numbers

The 16S rDNA sequences obtained from *V. crypta* under the accession numbers HE817775 to HE817870 (Karlińska-Batres & Wörheide 2013a) and from *A. willeyana* were deposited in EMBL database under the accession numbers HE985081 to HE985159 (Karlińska-Batres & Wörheide 2013b).

4.3. Results

4.3.1. Clone libraries construction and OTU assignment

16S rRNA clone libraries were constructed as previously described (Karlińska-Batres & Wörheide 2013a). Mothur clustering analyses of the *V. crypta* sequences resulted in 96 OTUs from the remaining 250 bacterial sequences and a single archaeal sequence based on a similarity criterion of 97%. The remaining 174 clones were assigned to particular OTUs based on their restriction patterns (Karlińska-Batres & Wörheide 2013a). From the 16S rRNA clone library amplified from the *A. willeyana*, 380 clones were selected. From those clones, 298 were sequenced, and 9 sequences were discarded as chimeras. The remaining 289 clone sequences together with a single archaeal 16S rRNA sequence, which was retrieved, were clustered into 79 OTUs using a 97% similarity criterion. Further 82 clones were assigned to a particular OTU based on their restriction patterns (Karlińska-Batres & Wörheide 2013b). The clone libraries differ with respect to amount of singletons - 32% of the clone library of *A. willeyana* and 41% of the *V. crypta* (Fig. 4.1).

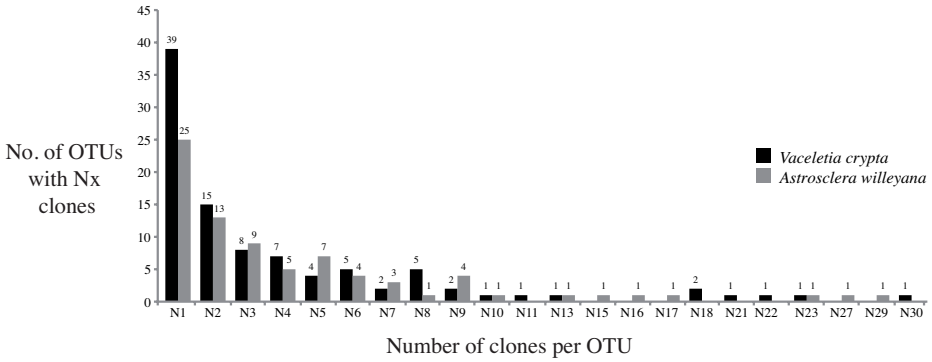
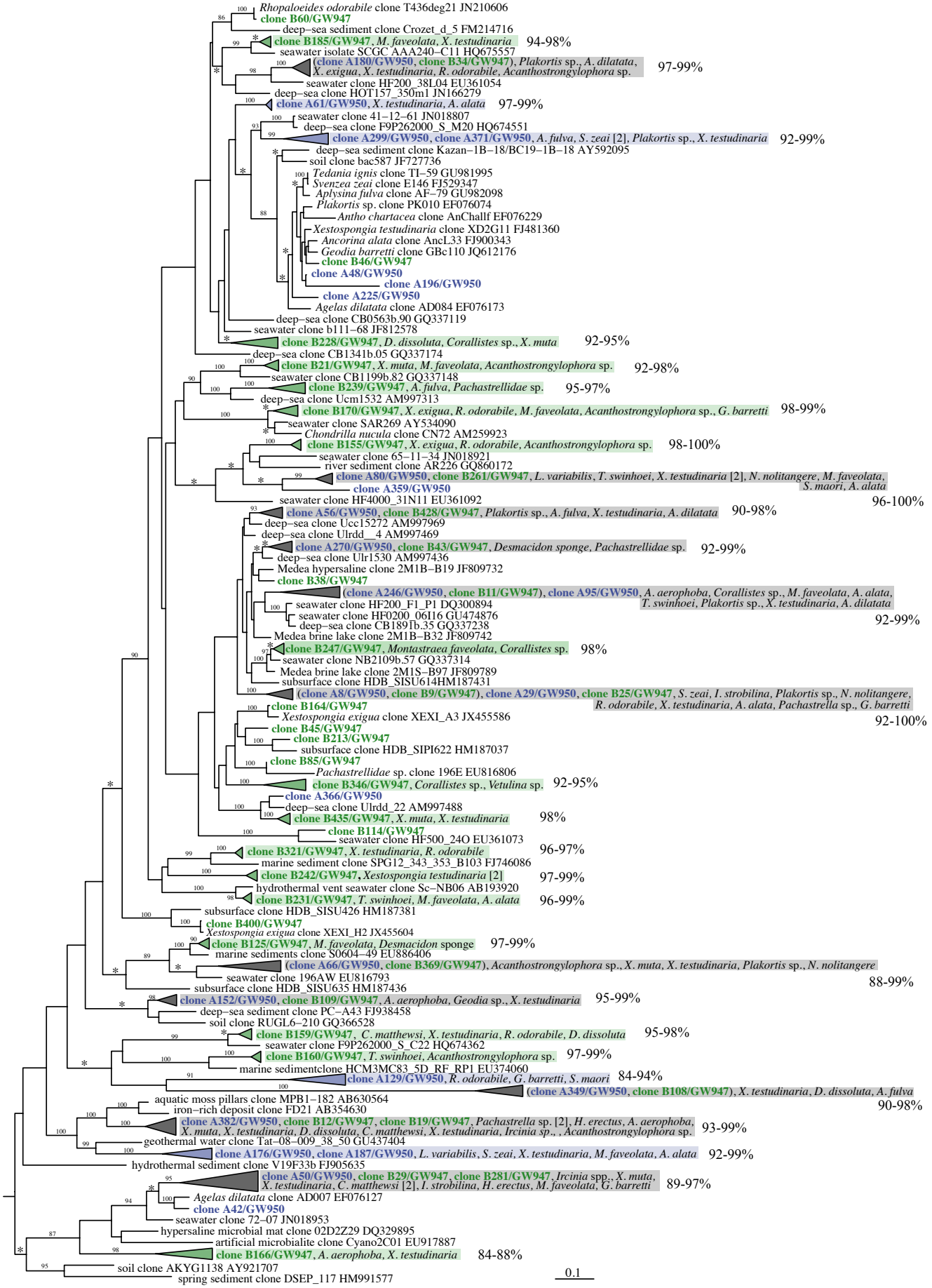


Figure 4.1. Distribution of 16S rRNA gene clones among the OTUs. * N1 represents the number of singletons, N2 the number of doubletons, etc.

4.3.2. Closest relatives

V. crypta revealed a slightly smaller fraction of OTUs with sponge-associated closest relatives, 71% (68 OTUs) in comparison to 82% (65 OTUs) from *A. willeyana*. However, the *V. crypta*-associated closest relatives were obtained from 23 different sponge species and those of *A. willeyana* from only 13 species. Of those sponge species, 12 (*Ancorina alata*, *Agelas dilatata*, *Aplysina fulva*, *Acanthostrongylophora* sp., *Axinella corrugata*, *Geodia* sp., *Plakortis* sp., *Rhopaloeides odorabile*, *Svenzea zeai*, *Theonella swinhoei*, *Xestospongia muta*, *Xestospongia testudinaria*) were found in BLAST results from both investigated coralline sponges. The constantly growing number of new sponge-derived sequences in public databases influenced the BLAST results from *V. crypta*, as a new search for the clone B400/GW947 revealed as the closest match sponge-derived sequences (previously the closest similar sequence was from the environment). *V. crypta* revealed closest relatives from further 10 sponges (*Discodermia dissoluta*, *Desmacidon* sponge, *Hyrtios erectus*, *Ircinia strobilina*, *Neofibularia nolitangere*, *Pachastrellidae* sp., *Tedania ignis*, *Tsitsikamma favus*, *Vetulina* sp., *Xestospongia exigua*); *A. willeyana* from one further sponge *Phyllospongia papyracea*. Both coralline sponges differed in the fraction of OTUs with closest relatives obtained from corals (18% *V. crypta*; *A. willeyana* 11%), however, for both the most numerous of those sequences were obtained from *Montastraea faveolata* (14 OTUs from *V. crypta*, and 8 OTUs from *A. willeyana*). Furthermore, *V. crypta* revealed single OTUs with closest sequences obtained from three corals (*Oculina patagonica, Diploria strigos* and *Erythropodium caribaeorum*) and *A. willeyana* revealed single OTU with closest sequence from *Pseudopterogorgia elisabethae*, an octocoral. Only *V. crypta* exposed one OTU with its closest sequence derived from a validly described organism (99% similarity), as well as one with distantly related (91%) 16S rRNA sequence from the chloroplast of a red alga (AY731517).

Figure 4.2. Maximum likelihood phylogeny of *V. crypta*- and *A. willeyana*-derived 16S rRNA sequences affiliated to the *Chloroflexi* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones analyzed during this study. *The parentheses* enclose shared OTUs defined at distance 0.03. *Shaded boxes* represent sponge-specific clusters: *grey* – shared between both coralline sponges, *blue* – with only *A. willeyana* clones, *green* – with only *V. crypta* clones. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence


Rhopaloeides odorabile clone T436deg21 JN210606
clone B60/GW947
deep–sea sediment clone Crozet_d_5 FM214716
clone B185/GW947, M. faveolata, X. testudinaria
94-98%
seawater isolate SCGC AAA240–C11 HQ675557
(clone A180/GW950, clone B34/GW947), Plakortis sp., A. dilatata, X. exigua, X. testudinaria, R. odorabile, Acanthostrongylophora sp.
97-99%
seawater clone HF200_38L04 EU361054
deep–sea clone HOT157_350m1 JN166279
clone A61/GW950, X. testudinaria, A. alata
97-99%
seawater clone 41–12–61 JN018807
deep–sea clone F9P262000_S_M20 HQ674551
clone A299/GW950, clone A371/GW950, A. fulva, S. zeai [2], Plakortis sp., X. testudinaria
92-99%
deep–sea sediment clone Kazan–1B–18/BC19–1B–18 AY592095
soil clone bac587 JF727736
Tedania ignis clone TI–59 GU981995
Svenzea zeai clone E146 FJ529347
Aplysina fulva clone AF–79 GU982098
Plakortis sp. clone PK010 EF076074
Antho chartacea clone AnChallf EF076229
Xestospongia testudinaria clone XD2G11 FJ481360
Ancorina alata clone AncL33 FJ900343
Geodia barretti clone GBc110 JQ612176
clone B46/GW947
clone A48/GW950
clone A196/GW950
clone A225/GW950
Agelas dilatata clone AD084 EF076173
deep–sea clone CB0563b.90 GQ337119
seawater clone b111–68 JF812578
clone B228/GW947, D. dissoluta, Corallistes sp., X. muta
92-95%
deep–sea clone CB1341b.05 GQ337174
clone B21/GW947, X. muta, M. faveolata, Acanthostrongylophora sp.
92-98%
seawater clone CB1199b.82 GQ337148
clone B239/GW947, A. fulva, Pachastrellidae sp.
95-97%
deep–sea clone Ucm1532 AM997313
clone B170/GW947, X. exigua, R. odorabile, M. faveolata, Acanthostrongylophora sp., G. barretti
98-99%
seawater clone SAR269 AY534090
Chondrilla nucula clone CN72 AM259923
clone B155/GW947, X. exigua, R. odorabile, Acanthostrongylophora sp.
98-100%
seawater clone 65–11–34 JN018921
river sediment clone AR226 GQ860172
clone A80/GW950, clone B261/GW947, L. variabilis, T. swinhoei, X. testudinaria [2], N. nolitangere, M. faveolata, S. maori, A. alata
clone A359/GW950
96-100%
seawater clone HF4000_31N11 EU361092
clone A56/GW950, clone B428/GW947, Plakortis sp., A. fulva, X. testudinaria, A. dilatata
90-98%
deep–sea clone Ucc15272 AM997969
deep–sea clone Ulrdd__4 AM997469
clone A270/GW950, clone B43/GW947, Desmacidon sponge, Pachastrellidae sp.
92-99%
deep–sea clone Ulr1530 AM997436
Medea hypersaline clone 2M1B–B19 JF809732
clone B38/GW947
(clone A246/GW950, clone B11/GW947), clone A95/GW950, A. aerophoba, Corallistes sp., M. faveolata, A. alata, T. swinhoei, Plakortis sp., X. testudinaria, A. dilatata
92-99%
seawater clone HF200_F1_P1 DQ300894
seawater clone HF0200_06I16 GU474876
deep–sea clone CB189Ib.35 GQ337238
Medea brine lake clone 2M1B–B32 JF809742
clone B247/GW947, Montastraea faveolata, Corallistes sp.
98%
seawater clone NB2109b.57 GQ337314
Medea brine lake clone 2M1S–B97 JF809789
subsurface clone HDB_SISU614HM187431
(clone A8/GW950, clone B9/GW947), clone A29/GW950, clone B25/GW947, S. zeai, I. strobilina, Plakortis sp., N. nolitangere, R. odorabile, X. testudinaria, A. alata, Pachastrella sp., G. barretti
92-100%
clone B164/GW947
Xestospongia exigua clone XEXI_A3 JX455586
clone B45/GW947
clone B213/GW947
subsurface clone HDB_SIPI622 HM187037
clone B85/GW947
Pachastrellidae sp. clone 196E EU816806
clone B346/GW947, Corallistes sp., Vetulina sp.
92-95%
clone A366/GW950
deep–sea clone Ulrdd_22 AM997488
clone B435/GW947, X. muta, X. testudinaria
98%
clone B114/GW947
seawater clone HF500_24O EU361073
clone B321/GW947, X. testudinaria, R. odorabile
96-97%
marine sediment clone SPG12_343_353_B103 FJ746086
clone B242/GW947, Xestospongia testudinaria [2]
97-99%
hydrothermal vent seawater clone Sc–NB06 AB193920
clone B231/GW947, T. swinhoei, M. faveolata, A. alata
96-99%
subsurface clone HDB_SISU426 HM187381
clone B400/GW947
Xestospongia exigua clone XEXI_H2 JX455604
clone B125/GW947, M. faveolata, Desmacidon sponge
97-99%
marine sediments clone S0604–49 EU886406
(clone A66/GW950, clone B369/GW947), Acanthostrongylophora sp., X. muta, X. testudinaria, Plakortis sp., N. nolitangere
seawater clone 196AW EU816793
88-99%
subsurface clone HDB_SISU635 HM187436
clone A152/GW950, clone B109/GW947, A. aerophoba, Geodia sp., X. testudinaria
95-99%
deep–sea sediment clone PC–A43 FJ938458
soil clone RUGL6–210 GQ366528
clone B159/GW947, C. matthewsi, X. testudinaria, R. odorabile, D. dissoluta
95-98%
seawater clone F9P262000_S_C22 HQ674362
clone B160/GW947, T. swinhoei, Acanthostrongylophora sp.
97-99%
marine sedimentclone HCM3MC83_5D_RF_RP1 EU374060
clone A129/GW950, R. odorabile, G. barretti, S. maori
84-94%
(clone A349/GW950, clone B108/GW947), X. testudinaria, D. dissoluta, A. fulva
90-98%
aquatic moss pillars clone MPB1–182 AB630564
iron–rich deposit clone FD21 AB354630
clone A382/GW950, clone B12/GW947, clone B19/GW947, Pachastrella sp. [2], H. erectus, A. aerophoba, X. muta, X. testudinaria, D. dissoluta, C. matthewsi, X. testudinaria, Ircinia sp., , Acanthostrongylophora sp.
93-99%
geothermal water clone Tat–08–009_38_50 GU437404
clone A176/GW950, clone A187/GW950, L. variabilis, S. zeai, X. testudinaria, M. faveolata, A. alata
92-99%
hydrothermal sediment clone V19F33b FJ905635
clone A50/GW950, clone B29/GW947, clone B281/GW947, Ircinia spp., X. muta, X. testudinaria, C. matthewsi [2], I. strobilina, H. erectus, M. faveolata, G. barretti
89-97%
Agelas dilatata clone AD007 EF076127
clone A42/GW950
seawater clone 72–07 JN018953
hypersaline microbial mat clone 02D2Z29 DQ329895
artificial microbialite clone Cyano2C01 EU917887
clone B166/GW947, A. aerophoba, X. testudinaria
84-88%
soil clone AKYG1138 AY921707
spring sediment clone DSEP_117 HM991577
0.1

Vaceletia had slightly higher ratio (9%) of OTUs with closest relatives derived from environment (*A. willeyana* nearly 6%).

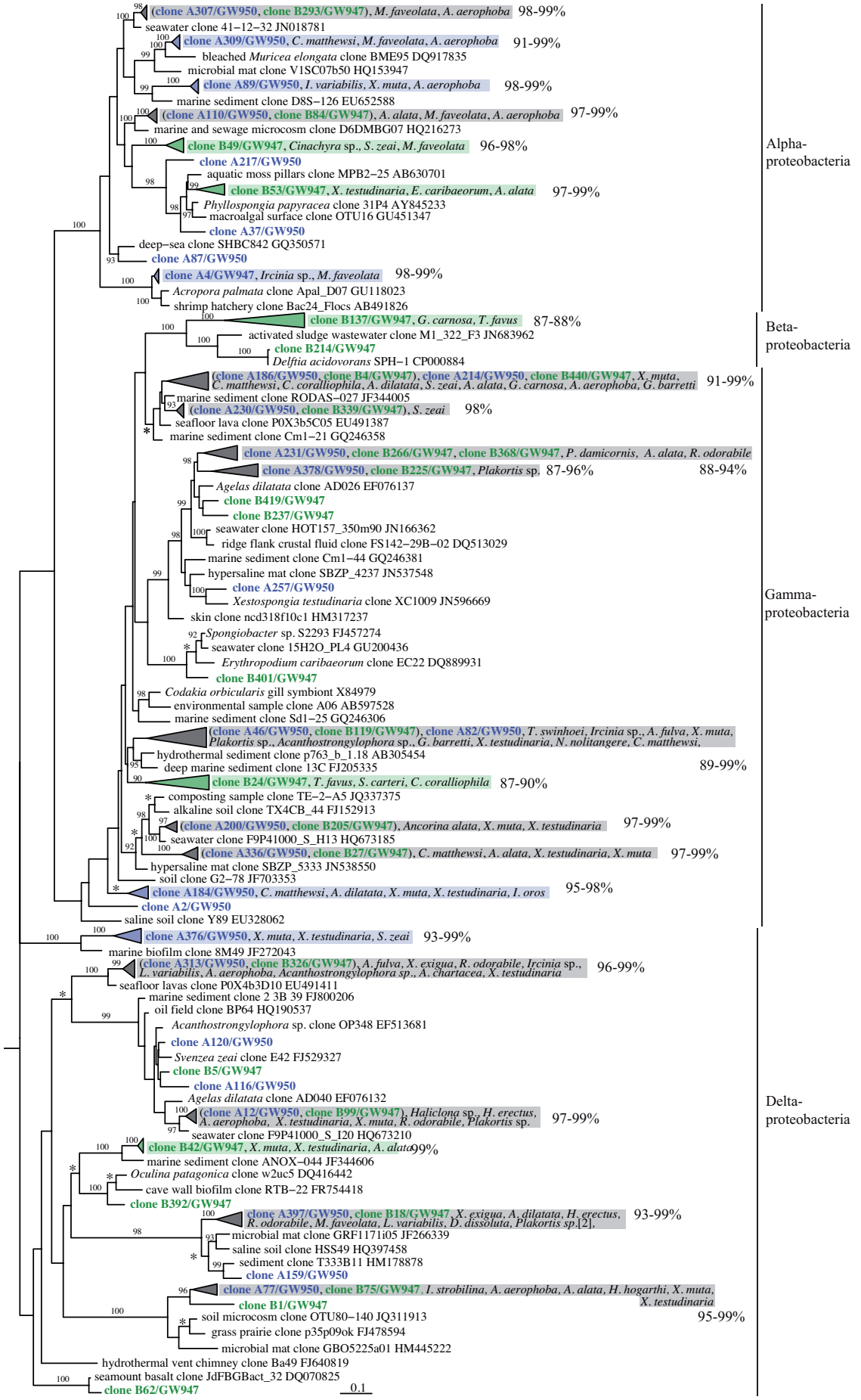
4.3.3. Phylogenetic analyses

The analyzed coralline sponges differ mostly by the presence of *Betaproteobacteria*, *Deinococcus-Thermus*, and *Bacteroidetes* in the clone library of *V. crypta*. Further sequences obtained from both sponges were assigned to *Chloroflexi, Gammaproteobacteria, Gemmatimonadetes*, *Actinobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Alphaproteobacteria*, *Poribacteria*, *Nitrospirae, Deferribacteres, Spirochaetes*, *Cyanobacteria*, and *Crenarchaeota*. The phylogenetic trees present the OTUs from both coralline sponges with nearest similar sequences assigned to the *Chloroflexi* (Fig. 4.2), *Proteobacteria* (Fig. 4.3) and to all other phyla (Fig. 4.4). The details of clones and OTUs assignments to particular phylogenetic groups are presented in Table 4.1.

4.3.4. Shared OTUs

21 OTUs defined at the 97% criterion, representing 35% of the *V. crypta* clones (n=141) and 28% of the *A. willeyana* clones (n=105), were found in both investigated samples. The largest number of shared OTUs was found in the phylum *Proteobacteria* (9 OTUs), which grouped 45 clones of *V. crypta* and 33 clones of *A. willeyana*. Five shared OTUs belonged to the *Chloroflexi* and grouped 43 clones of *V. crypta* and 39 clones of *A. willeyana*. *V. crypta* shared five OTUs with 13-22 clones in contrast to *A. willeyana*, which shared only 2 OTUs with 13 and 15 clones. In the phylogenetic trees (Fig. 4.2-4.4) shared OTUs in are indicated as clone names in brackets.

Figure 4.3. Maximum likelihood phylogeny of *V. crypta*- and *A. willeyana*-derived 16S rRNA sequences affiliated to the *Proteobacteria* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** *t* signifies clones analyzed during this study. *The parentheses* enclose shared OTUs defined at distance 0.03. *Shaded boxes* represent sponge-specific clusters: *grey* – shared between both coralline sponges, *blue* – with only *A. willeyana* clones, *green* – with only *V. crypta* clones. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence →


(clone A307/GW950, clone B293/GW947), M. faveolata, A. aerophoba 98-99%
seawater clone 41−12−32 JN018781
clone A309/GW950, C. matthewsi, M. faveolata, A. aerophoba 91-99%
bleached Muricea elongata clone BME95 DQ917835
microbial mat clone V1SC07b50 HQ153947
clone A89/GW950, I. variabilis, X. muta, A. aerophoba 98-99%
marine sediment clone D8S−126 EU652588
(clone A110/GW950, clone B84/GW947), A. alata, M. faveolata, A. aerophoba 97-99%
marine and sewage microcosm clone D6DMBG07 HQ216273
clone B49/GW947, Cinachyra sp., S. zeai, M. faveolata 96-98%
clone A217/GW950
aquatic moss pillars clone MPB2−25 AB630701
clone B53/GW947, X. testudinaria, E. caribaeorum, A. alata 97-99%
Phyllospongia papyracea clone 31P4 AY845233
macroalgal surface clone OTU16 GU451347
clone A37/GW950
deep−sea clone SHBC842 GQ350571
clone A87/GW950
clone A4/GW947, Ircinia sp., M. faveolata 98-99%
Acropora palmata clone Apal_D07 GU118023
shrimp hatchery clone Bac24_Flocs AB491826
clone B137/GW947, G. carnosa, T. favus 87-88%
activated sludge wastewater clone M1_322_F3 JN683962
clone B214/GW947
Delftia acidovorans SPH−1 CP000884
(clone A186/GW950, clone B4/GW947), clone A214/GW950, clone B440/GW947, X. muta, C. matthewsi, C. coralliophila, A. dilatata, S. zeai, A. alata, G. carnosa, A. aerophoba, G. barretti 91-99%
marine sediment clone RODAS−027 JF344005
(clone A230/GW950, clone B339/GW947), S. zeai 98%
seafloor lava clone P0X3b5C05 EU491387
marine sediment clone Cm1−21 GQ246358
clone A231/GW950, clone B266/GW947, clone B368/GW947, P. damicornis, A. alata, R. odorabile
clone A378/GW950, clone B225/GW947, Plakortis sp. 87-96% 88-94%
Agelas dilatata clone AD026 EF076137
clone B419/GW947
clone B237/GW947
seawater clone HOT157_350m90 JN166362
ridge flank crustal fluid clone FS142−29B−02 DQ513029
marine sediment clone Cm1−44 GQ246381
hypersaline mat clone SBZP_4237 JN537548
clone A257/GW950
Xestospongia testudinaria clone XC1009 JN596669
skin clone ncd318f10c1 HM317237
Spongiobacter sp. S2293 FJ457274
seawater clone 15H2O_PL4 GU200436
Erythropodium caribaeorum clone EC22 DQ889931
clone B401/GW947
Codakia orbicularis gill symbiont X84979
environmental sample clone A06 AB597528
marine sediment clone Sd1−25 GQ246306
(clone A46/GW950, clone B119/GW947), clone A82/GW950, T. swinhoei, Ircinia sp., A. fulva, X. muta, Plakortis sp., Acanthostrongylophora sp., G. barretti, X. testudinaria, N. nolitangere, C. matthewsi,
hydrothermal sediment clone p763_b_1.18 AB305454
deep marine sediment clone 13C FJ205335 89-99%
clone B24/GW947, T. favus, S. carteri, C. coralliophila 87-90%
composting sample clone TE−2−A5 JQ337375
alkaline soil clone TX4CB_44 FJ152913
(clone A200/GW950, clone B205/GW947), Ancorina alata, X. muta, X. testudinaria 97-99%
seawater clone F9P41000_S_H13 HQ673185
(clone A336/GW950, clone B27/GW947), C. matthewsi, A. alata, X. testudinaria, X. muta 97-99%
hypersaline mat clone SBZP_5333 JN538550
soil clone G2−78 JF703353
clone A184/GW950, C. matthewsi, A. dilatata, X. muta, X. testudinaria, I. oros 95-98%
clone A2/GW950
saline soil clone Y89 EU328062
clone A376/GW950, X. muta, X. testudinaria, S. zeai 93-99%
marine biofilm clone 8M49 JF272043
(clone A313/GW950, clone B326/GW947), A. fulva, X. exigua, R. odorabile, Ircinia sp., L. variabilis, A. aerophoba, Acanthostrongylophora sp., A. chartacea, X. testudinaria 96-99%
seafloor lavas clone P0X4b3D10 EU491411
marine sediment clone 2 3B 39 FJ800206
oil field clone BP64 HQ190537
Acanthostrongylophora sp. clone OP348 EF513681
clone A120/GW950
Svenzea zeai clone E42 FJ529327
clone B5/GW947
clone A116/GW950
Agelas dilatata clone AD040 EF076132
(clone A12/GW950, clone B99/GW947), Haliclona sp., H. erectus, A. aerophoba, X. testudinaria, X. muta, R. odorabile, Plakortis sp. 97-99%
seawater clone F9P41000_S_I20 HQ673210
clone B42/GW947, X. muta, X. testudinaria, A. alata 99%
marine sediment clone ANOX−044 JF344606
Oculina patagonica clone w2uc5 DQ416442
cave wall biofilm clone RTB−22 FR754418
clone B392/GW947
clone A397/GW950, clone B18/GW947, X. exigua, A. dilatata, H. erectus, R. odorabile, M. faveolata, L. variabilis, D. dissoluta, Plakortis sp.[2], 93-99%
microbial mat clone GRF1171i05 JF266339
saline soil clone HSS49 HQ397458
sediment clone T333B11 HM178878
clone A159/GW950
clone A77/GW950, clone B75/GW947, I. strobilina, A. aerophoba, A. alata, H. hogarthi, X. muta, X. testudinaria
clone B1/GW947
soil microcosm clone OTU80−140 JQ311913 95-99%
grass prairie clone p35p09ok FJ478594
microbial mat clone GBO5225a01 HM445222
hydrothermal vent chimney clone Ba49 FJ640819
seamount basalt clone JdFBGBact_32 DQ070825
clone B62/GW947
0.1
Alpha-proteobacteria
Beta-proteobacteria
Gamma-proteobacteria
Delta-proteobacteria

4.3.5. Sponge-specific and sponge-coral clusters

For both coralline sponges the majority of OTUs that were closely related to other sponge- or coral-derived sequences fell into 93 sponge-specific or sponge-coral clusters (SSC/SCC), however for *V. crypta* the percentage was slightly higher (87%, 74 OTUs) compared to *A. willeyana* (81%, 60 OTUs). The coralline sponges shared 32 SSC/SCC with 20 shared OTUs, and additionally 54 OTUs of *V. crypta* were assigned to 33 SSC/SCC, and 40 OTUs of *A. willeyana* to 18 SSC/SCC. The largest numbers of clusters belonged to the phyla *Chloroflexi* and *Proteobacteria* (31 and 23 clusters), but the most shared clusters were defined among the *Proteobacteria* (13 clusters with 20 OTUs of both coralline sponges, and thereof 9 shared OTUs). The SSC/SCC are indicated with shaded boxes in the phylogenetic trees (Figs. 4.2, 4.3 and 4.4), grey boxes indicate shared clusters, green boxes indicate clusters with clones only from *V. crypta*, and blue boxes indicate clusters with clones only from *A. willeyana*. The percentage values next to the grey-shaded boxes, ranging from 84 to 100%, indicate the degree of similarity between the sequences belonging to the clusters.

4.3.6. Microbial diversity and community structure

Analysis of the composition of the clone libraries revealed both microbial communities to be very complex and diverse (Fig. 4.5), however the microbiota of *V. crypta* was more diverse, because *A. willeyana* lacks of the *Betaproteobacteria, Deinococcus-Thermus*, and *Bacteroidetes*. Furthermore, the both communities vary significantly in the abundance of the *Gemmatimonadetes* (11.2% in *V. crypta* and 1.9% in *A. willeyana*), as well as slightly in *Alphaproteobacteria* and *Nitrospirae* (2.7% and 7.3% in *V. crypta* and 5.1% and 4.3% in *A. willeyana*, respectively). In

Figure 4.4. Maximum likelihood phylogeny of *V. crypta*- and *A. willeyana*-derived 16S rRNA sequences affiliated to several phyla and to the domain *Archaea* with next similar sequences obtained from other sponges or corals, and from the environment. Reference sequences are listed with their GenBank numbers. ***Bold text*** signifies clones analyzed during this study. *The parentheses* enclose shared OTUs defined at distance 0.03. *Shaded boxes* represent sponge-specific clusters: *grey* – shared between both coralline sponges, *blue* – with only *A. willeyana* clones, *green* – with only *V. crypta* clones. Bootstrap analysis was based on 1000 replicates – the support values 70-85% are indicated by *asterisk*. *Scale bar* signifies 10% sequence divergence →

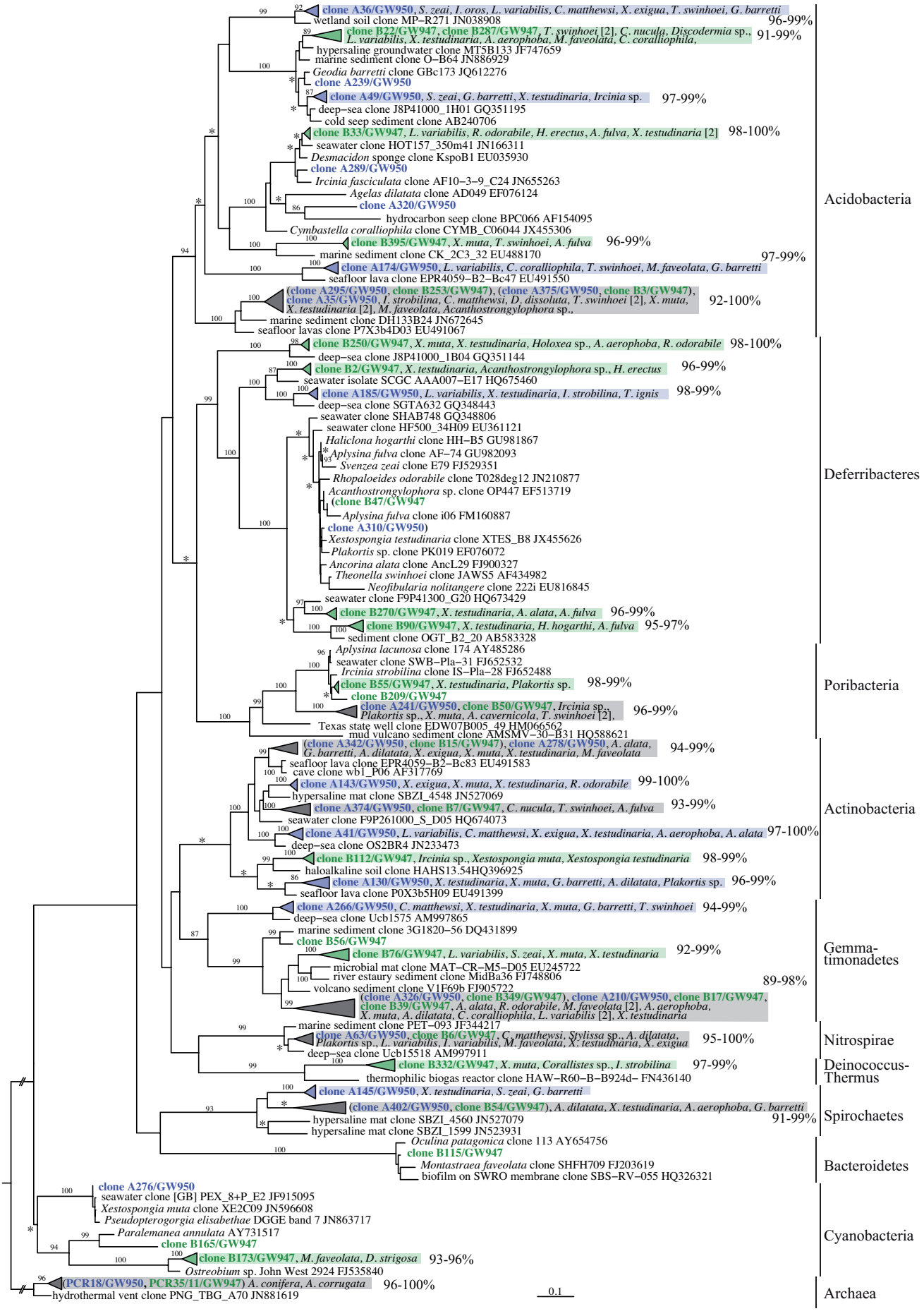

clone A36/GW950, S. zeai, I. oros, L. variabilis, C. matthewsi, X. exigua, T. swinhoei, G. barretti
wetland soil clone MP−R271 JN038908
96-99%
clone B22/GW947, clone B287/GW947, T. swinhoei [2], C. nucula, Discodermia sp., L. variabilis, X. testudinaria, A. aerophoba, M. faveolata, C. coralliophila,
91-99%
hypersaline groundwater clone MT5B133 JF747659
marine sediment clone O−B64 JN886929
Geodia barretti clone GBc173 JQ612276
clone A239/GW950
clone A49/GW950, S. zeai, G. barretti, X. testudinaria, Ircinia sp.
97-99%
deep−sea clone J8P41000_1H01 GQ351195
cold seep sediment clone AB240706
clone B33/GW947, L. variabilis, R. odorabile, H. erectus, A. fulva, X. testudinaria [2]
98-100%
seawater clone HOT157_350m41 JN166311
Desmacidon sponge clone KspoB1 EU035930
clone A289/GW950
Ircinia fasciculata clone AF10−3−9_C24 JN655263
Agelas dilatata clone AD049 EF076124
clone A320/GW950
hydrocarbon seep clone BPC066 AF154095
Cymbastella coralliophila clone CYMB_C06044 JX455306
clone B395/GW947, X. muta, T. swinhoei, A. fulva
96-99%
marine sediment clone CK_2C3_32 EU488170
97-99%
clone A174/GW950, L. variabilis, C. coralliophila, T. swinhoei, M. faveolata, G. barretti
seafloor lava clone EPR4059−B2−Bc47 EU491550
(clone A295/GW950, clone B253/GW947), (clone A375/GW950, clone B3/GW947), clone A35/GW950, I. strobilina, C. matthewsi, D. dissoluta, T. swinhoei [2], X. muta, X. testudinaria [2], M. faveolata, Acanthostrongylophora sp.,
92-100%
marine sediment clone DH133B24 JN672645
seafloor lavas clone P7X3b4D03 EU491067
Acidobacteria
clone B250/GW947, X. muta, X. testudinaria, Holoxea sp., A. aerophoba, R. odorabile
98-100%
deep−sea clone J8P41000_1B04 GQ351144
clone B2/GW947, X. testudinaria, Acanthostrongylophora sp., H. erectus
96-99%
seawater isolate SCGC AAA007−E17 HQ675460
clone A185/GW950, L. variabilis, X. testudinaria, I. strobilina, T. ignis
98-99%
deep−sea clone SGTA632 GQ348443
seawater clone SHAB748 GQ348806
seawater clone HF500_34H09 EU361121
Haliclona hogarthi clone HH−B5 GU981867
Aplysina fulva clone AF−74 GU982093
Svenzea zeai clone E79 FJ529351
Rhopaloeides odorabile clone T028deg12 JN210877
Acanthostrongylophora sp. clone OP447 EF513719
(clone B47/GW947
Aplysina fulva clone i06 FM160887
clone A310/GW950)
Xestospongia testudinaria clone XTES_B8 JX455626
Plakortis sp. clone PK019 EF076072
Ancorina alata clone AncL29 FJ900327
Theonella swinhoei clone JAWS5 AF434982
Neofibularia nolitangere clone 222i EU816845
seawater clone F9P41300_G20 HQ673429
clone B270/GW947, X. testudinaria, A. alata, A. fulva
96-99%
clone B90/GW947, X. testudinaria, H. hogarthi, A. fulva
95-97%
sediment clone OGT_B2_20 AB583328
Deferribacteres
Aplysina lacunosa clone 174 AY485286
seawater clone SWB−Pla−31 FJ652532
Ircinia strobilina clone IS−Pla−28 FJ652488
clone B55/GW947, X. testudinaria, Plakortis sp.
98-99%
clone B209/GW947
clone A241/GW950, clone B50/GW947, Ircinia sp., Plakortis sp., X. muta, A. cavernicola, T. swinhoei [2],
96-99%
Texas state well clone EDW07B005_49 HM066562
mud vulcano sediment clone AMSMV−30−B31 HQ588621
Poribacteria
(clone A342/GW950, clone B15/GW947), clone A278/GW950, A. alata, G. barretti, A. dilatata, X. exigua, X. muta, X. testudinaria, M. faveolata
94-99%
seafloor lava clone EPR4059−B2−Bc83 EU491583
cave clone wb1_P06 AF317769
clone A143/GW950, X. exigua, X. muta, X. testudinaria, R. odorabile
99-100%
hypersaline mat clone SBZI_4548 JN527069
clone A374/GW950, clone B7/GW947, C. nucula, T. swinhoei, A. fulva
93-99%
seawater clone F9P261000_S_D05 HQ674073
clone A41/GW950, L. variabilis, C. matthewsi, X. exigua, X. testudinaria, A. aerophoba, A. alata
97-100%
deep−sea clone OS2BR4 JN233473
clone B112/GW947, Ircinia sp., Xestospongia muta, Xestospongia testudinaria
98-99%
haloalkaline soil clone HAHS13.54HQ396925
clone A130/GW950, X. testudinaria, X. muta, G. barretti, A. dilatata, Plakortis sp.
96-99%
seafloor lava clone P0X3b5H09 EU491399
Actinobacteria
clone A266/GW950, C. matthewsi, X. testudinaria, X. muta, G. barretti, T. swinhoei
94-99%
deep−sea clone Ucb1575 AM997865
marine sediment clone 3G1820−56 DQ431899
clone B56/GW947
clone B76/GW947, L. variabilis, S. zeai, X. muta, X. testudinaria
92-99%
microbial mat clone MAT−CR−M5−D05 EU245722
river estuary sediment clone MidBa36 FJ748806
89-98%
volcano sediment clone V1F69b FJ905722
(clone A326/GW950, clone B349/GW947), clone A210/GW950, clone B17/GW947, clone B39/GW947, A. alata, R. odorabile, M. faveolata [2], A. aerophoba, X. muta, A. dilatata, C. coralliophila, L. variabilis [2], X. testudinaria
Gemma-timonadetes
marine sediment clone PET−093 JF344217
clone A63/GW950, clone B6/GW947, C. matthewsi, Stylissa sp., A. dilatata, Plakortis sp., L. variabilis, L. variabilis, M. faveolata, X. testudinaria, X. exigua
95-100%
deep−sea clone Ucb15518 AM997911
Nitrospirae
clone B332/GW947, X. muta, Corallistes sp., I. strobilina
97-99%
thermophilic biogas reactor clone HAW−R60−B−B924d− FN436140
Deinococcus-Thermus
clone A145/GW950, X. testudinaria, S. zeai, G. barretti
(clone A402/GW950, clone B54/GW947), A. dilatata, X. testudinaria, A. aerophoba, G. barretti
91-99%
hypersaline mat clone SBZI_4560 JN527079
hypersaline mat clone SBZI_1599 JN523931
Spirochaetes
Oculina patagonica clone 113 AY654756
clone B115/GW947
Montastraea faveolata clone SHFH709 FJ203619
biofilm on SWRO membrane clone SBS−RV−055 HQ326321
Bacteroidetes
clone A276/GW950
seawater clone [GB] PEX_8+P_E2 JF915095
Xestospongia muta clone XE2C09 JN596608
Pseudopterogorgia elisabethae DGGE band 7 JN863717
Paralemanea annulata AY731517
clone B165/GW947
clone B173/GW947, M. faveolata, D. strigosa
93-96%
Ostreobium sp. John West 2924 FJ535840
Cyanobacteria
(PCR18/GW950, PCR35/11/GW947) A. conifera, A. corrugata
96-100%
hydrothermal vent clone PNG_TBG_A70 JN881619
Archaea
0.1

Table 4.1. Distribution of the 16S rRNA clones and OTUs defined at distance 0.03 among particular phylogenetic groups in the clone libraries obtained from the coralline sponges

	Vaceletia crypta			*Astrosclera willeyana*		
Phylogenetic group	**Proportion of clones in the library**	**No. of clones**	**No. of OTUs**	**Proportion of clones in the library**	**No. of clones**	**No. of OTUs**
Chloroflexi	**35.2%**	144	39	**42.9%**	156	25
Gammaproteobacteria	**11.2%**	46	13	**13.7%**	51	12
Gemmatimonadetes	**11.2%**	46	5	**1.9%**	7	3
Actinobacteria	**8.3%**	34	3	**11.0%**	41	6
Nitrospirae	**7.3%**	30	1	**4.3%**	16	1
Deferribacteres	**7.3%**	30	5	**6.7%**	25	2
Deltaproteobacteria	**7.1%**	29	9	**5.9%**	22	8
Acidobacteria	**5.9%**	24	6	**7.8%**	29	9
Alphaproteobacteria	**2.7%**	11	4	**5.1%**	19	8
Poribacteria	**1.0%**	4	3	**0.3%**	1	1
Betaproteobacteria	**0.7%**	3	2	**-**	-	-
Cyanobacteria	**0.7%**	3	2	**0.3%**	1	1
Spirochaetes	**0.5%**	2	1	**0.8%**	3	2
Deinococcus-Thermus	**0.2%**	1	1	**-**	-	-
Bacteroidetes	**0.2%**	1	1	**-**	-	-
Archaea	**0.2%**	1	1	**0.3%**	1	1
		409	96		372	79

both communities the most abundant taxa were the *Chloroflexi* (35.2% *V. crypta* and 42.9% *A. willeyana*), followed by *Gammaproteobacteria* (11.2% in *V. crypta* and 13.7% in *A. willeyana*).

Libshuff statistical analysis of the libraries confirmed a highly significant difference between the microbial communities of *V. crypta* and *A. willeyana* ($P<0.0001$). A Venn diagram of OTU distributions at distance 0.03 revealed that from 154 defined different OTUs, and thereof 21 OTUs were shared between the communities of *V. crypta* and *A. willeyana*. According to the Chao1 index and abundance-base coverage estimator (ACE; Tab. 4.2), we sequenced nearly 70% of the predicted number of microbial species in the community associated with *V. crypta* and 80% of the *A. willeyana* one. Also, the rarefaction analysis confirmed more successful sampling for *A. willeyana* (Fig. 4.6). Although the rarefaction curves for

both samples calculated for the 0.03, 0.05, and 0.1 cut-off criteria didn't reach clear saturation, they were very flat indicating that the sampled diversity provide a comprehensive picture of the core microbial communities of both coralline sponges (Schmitt et al. 2011). The slightly higher Shannon-Wiener index for the *V. crypta* confirmed a greater complexity of its microbiota. However the *A. willeyana*-community revealed a slightly higher value of the Simpson index, which gives a strong weighting to the dominants.

4.4. Discussion

This study represents the first comparison of microbial communities of taxonomically diverse coralline sponges from the GBR and despite some variations indicates a very high degree of similarity of these microbiota. Both, *V. crypta* and *A. willeyana* harbored very rich and diverse consortia with strikingly comparable phyla composition, however, they differed in the abundance of the members of the particular phylogenetic groups and *V. crypta* revealed slightly more complex community structure. Coralline sponges shared also a high number of bacterial species, exceeding the level of OTUs shared with other sponges from the same location. Both sponges exhibited very high numbers of OTUs with next similar sequences obtained from other sponges, though the number was slightly higher for the *A. willeyana*. Furthermore the sequences fell into numerous SSC/SCC and thereof over 30% were shared between *A. willeyana* and *V. crypta*. A large fraction of the SSC/SCC with sequences of both coralline sponges also contained sequences obtained from other sponges from the GBR.

Core microbiomes – abundant microbes, shared between all samples taken from some complex microbial habitat – must fulfill functions important for the maintenance of the microbial community (Shade & Handelsman 2012, Webster et al. 2013). The microbial communities of *A. willeyana* and *V. crypta* contained phyla commonly affiliated with marine sponges (Taylor et al. 2007b, Webster & Taylor 2012), with the most abundant *Chloroflexi* followed by *Gammaproteobacteria*. The third most abundant phylum in the microbial community of *V. crypta* - the *Gemmatimonadetes*, constituted only minor component of the microbiota of *A. willeyana*, where the *Actinobacteria* were the third most abundant phylum. Kamke and colleagues (2010) reported the *Gemmatimonadetes* as active members of sponge

microbiota, nevertheless the features of their functions in the community remain still unspecified. Furthermore, the microbial communities of both coralline sponges differed with respect to the abundance of *Alphaproteobacteria* and also *Nitrospirae* involved in the two steps of the nitrification, and thereby indicating pathways for nitrogen metabolism in the host tissues (Bayer et al. 2007). Altogether *V. crypta* revealed a higher microbial diversity due to the higher number of OTUs and to the presence of members of the *Betaproteobacteria*, *Deinococcus-Thermus*, and *Bacteroidetes*. Libshuff statistical analysis of the libraries confirmed significant differences in the structure of the symbiotic communities hosted by both coralline sponges.

Recently Erwin et al. (2012) classified numerous factors shaping the symbiotic communities and suggested that host-specific factors determined microbial consortia of *Ircinia* sp. from the Mediterranean Sea. Our results confirm this hypothesis since *A. willeyana* and *V. crypta* share restricted cryptic habitats (Wörheide 1998). At the sampling site at Yonge Reef coralline sponges appear in caves mostly on the outer seaward slope in a water depth starting between 8-15 m and *A. willeyana* and *V. crypta* are restricted to the darker zones (0-2 lux) of the caves (Wörheide 1998). Generally *A. willeyana* occurs scarcely in the very darkest areas of the caves (Wörheide 1998), but actually, *V. crypta* revealed a higher ratio of the light-dependent *Cyanobacteria*; nevertheless they constituted only a minor part of the microbial communities in both investigated coralline sponges (Tab. 4.1). Moreover, the herein investigated sponges were collected in one cave during the same dive, thus definitely were exposed to the similar environmental conditions.

A. willeyana and *V. crypta* harbor symbiotic communities comprised primarily of sequences closely related to microbial sequences from other sponges and the majority of those sequences formed numerous SSC/SCC (93) with nearest relatives, indicating that the microbiota of coralline sponges comprise a particular assembly of generalist sponge symbionts. The proportion of sequences within the SSC/SCC in both coralline sponges belong to the highest ever reported, even if slightly decreased for *V. crypta*, however increased for *A. willeyana* compared to a previous single analysis (Karlińska-Batres & Wörheide 2013a, Karlińska-Batres & Wörheide 2013b). The symbiotic communities in sponges have been reported not only as sponge-specific (Hentschel et al. 2002, Taylor et al. 2007b), but recently moreover as specific to particular sponge species (Webster et al. 2010). Montalvo

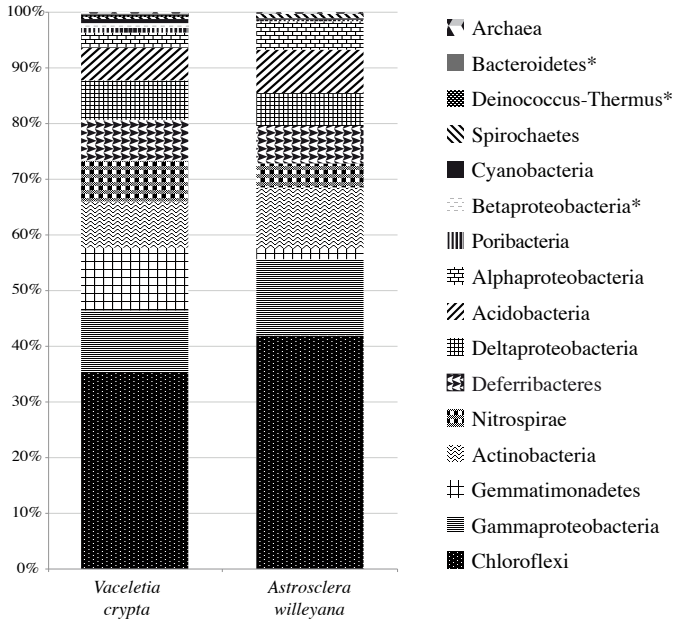


Figure 4.5. Distribution of the 16S rRNA gene clones among particular phylogenetic groups in the clone libraries obtained from two coralline sponges – *V. crypta* (left) and *A. willeyana* (right) from Yonge Reef, GBR, Australia. Phylogenetic groups found only in the clone library of *V. crypta* are indicated with *asterisks*

and Hill (2011) analyzed the microbial diversity of the two closely related, but geographically distant giant barrel sponges *Xestospongia muta* (Atlantic) and *Xestospongia testudinaria* (Pacific) and referred the microbial communities as specific to each of the sponge species and to the genus *Xestospongia*. Recently Schmitt and colleagues (2012a) demonstrated the absence of correlation between host phylogeny and arrangement of the symbiotic communities in three species each within the genera *Aplysina*, *Hyrtios*, and *Ircinia* from the Mediterranean Sea. Likewise three sympatric Mediterranean *Ircinia* sp., which exposed host species-specific communities of symbionts, with fractional ratio of OTUs (12-14%) shared between all three species (Erwin et al. 2012). Interestingly, *I. variabilis* and *I. fasciculata*, whose sequences can not be distinguished with the mitochondrial COI marker, shared slightly higher ratio (21% and 24% of the detected OTUs, respectively) (Erwin et al. 2012). Also a previous study of symbionts in putative cryptic species of the coralline sponge *A. willeyana* confirmed the species-specific character of the sponge microbiota showing differences in microbial communities from different geographical localities (Red Sea vs. GBR) (Karlińska-Batres and Wör-

Table 4.2. Diversity analysis of the 16S rRNA gene clone libraries constructed at distance 0.03 for the coralline sponges samples. Lower and upper 95% confidence intervals are shown in parentheses where available. ACE: abundance-base coverage estimator

Sample source	No. of clones	No. of OTUs	Chao estimate	ACE	Shannon index	Simpson index
Vaceletia crypta	409	96	142 (117-200)	140 (119-181)	4.02 (3.92-4.12)	0.025 (0.021-0.029)
Astrosclera willeyana	372	79	100 (87-136)	100 (89-125)	3.88 (3.77-3.98)	0.028 (0.023-0.033)

heide, in review). A recent study of 13 diverse GBR sponge species revealed microbial communities largely conserved within different individuals of each species, but particular low microbiome shared between species – no OTU was common for all species (Webster et al. 2013). Moreover, the most ubiquitous OTUs were shared by maximal five sponge species and 91% of OTUs were species-specific (Webster et al. 2013). In comparison, our result showed that taxonomically distant *A. willeyana* and *V. crypta*, collected also from the GBR, shared remarkable high number of bacterial species (21 OTUs representing app. 30% of each clone library) significantly outnumbering microbiome shared between other GBR sponge species (Webster et al. 2013). Consequently, these results together with very high ratio of *A. willeyana* and *V. crypta* 16S r RNA gene sequences within the SSC/SCC are an indication of a particularly tight bonding of coralline sponges with their symbionts. Furthermore, this indicates that microbial communities in coralline sponges both are shaped by factors that are host-dependent, but also represent specific patterns, which deviate from the patterns shown by other sponges. These specific patterns likely correlate with the evolutionary age of the sclerosponge host species (Karlińska-Batres & Wörheide 2013a).

Recent studies indicated that combination of vertical and horizontal transmission forms microbial communities in marine sponges (Hentschel et al. 2012, Schmitt et al. 2012a). Nevertheless several issues concerning the evolution of the strategies shaping the sponge-microbe associations remain unresolved. Did these mechanisms develop simultaneously? Or maybe one has more primary origin, and the other has evolved over time, leading to strategy where both, vertical and hori-

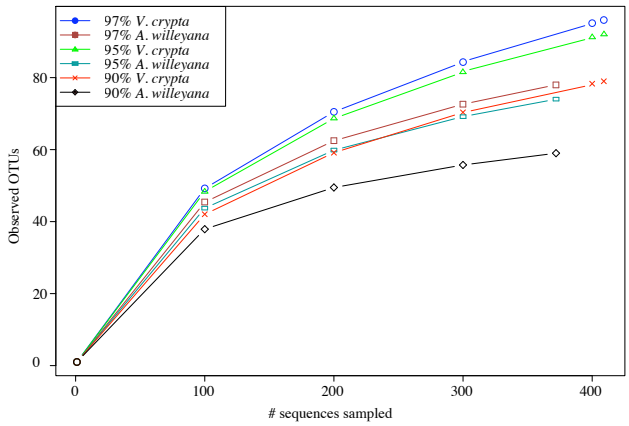


Figure 4.6. Rarefaction curves for the 16S rRNA gene sequences obtained from *A. willeyana* and *V. crypta* from Yonge Reef, GBR. Operational Taxonomic Units (OTUs) were defined at the 97%, 95% and 90% similarity criteria

zontal transmission, complement themselves? *V. crypta* and *A. willeyana* reproduce sexually with development of parenchymella larva, which enclose numerous bacteria as evidenced for *A. willeyana* (Wörheide 1998, Vacelet 2002). Our results imply that at least a fraction of symbionts of both coralline sponges must have been vertically transmitted. This suggestion base on distant similarity of their microbial 16S rRNA gene sequences to the next related sequences in the SSC/SCC and on criterion for the proposal of a novel species (< 97% 16S rRNA gene sequence similarity), and a new genus (<93%) (Rohwer et al. 2002). Those criteria revealed approximately 30% of novel symbiotic species in both coralline sponges, and thereof 15% and 11% (*V. crypta* and *A. willeyana*, respectively) novel at genus level. Simultaneously, very high ratio of the microbial 16S rRNA gene sequences from both coralline sponges in SSC/SCC, closely related to other microbial sequences, together with very high similarity of microbiota between scleorsponges, might indicate maintenance their microbial communities mainly through environmental transmission (Hentschel et al. 2002, Taylor et al. 2007b). Moreover, if we consider that coralline sponges of the genus *Vaceletia* belong to the keratose sponges (Wörheide 2008), forming an early-branching lineage in the Demospongiae (Philippe et al. 2009), suggesting environmental acquisition as more primary mechanism.

However we compared only single specimens of *A. willeyana* and *V. crypta*, their clone libraries enclosed remarkably high number of clones enabling comprehensive analysis. Nevertheless, the example of the clone B400/GW947 - primarily

distantly related to an environmental sequence (Karlińska-Batres & Wörheide 2013a), after re-analysis with BLAST revealed 98% similarity to recently published *Xestospongia exigua* clone XEXI_H2 (Fig. 4.2), shows that we still lack a full picture of microbial diversity in sponges and new studies would extend our knowledge and lead to more precise and certain conclusions.

This first ever comparison of microbial communities in *A. willeyana* and *V. crypta* demonstrates, despite some differences, very high similarity in phylogenetic composition of both symbiotic consortia. Coralline sponges share high number of bacterial species, far exceeding the amount of shared OTUs characteristic for other sponges and thus indicate by sclerosponges specific patterns for the constitution of microbial communities. Our results confirm indirectly vertical transmission of microbial symbionts in coralline sponges; however simultaneously indicate horizontal transmission as more original mechanism. Further studies involving reproductive stages and wider range of specimens of *A. willeyana* and *V. crypta*, as well as other coralline sponges, would bring more information on shared "core microbiome" and elucidate if those microorganisms might be functionally important for the ecology and evolution of sclerosponges.

Summary of results

The 16S rRNA gene-based analysis of microorganisms associated with the coralline sponge *Vaceletia crypta* from the Great Barrier Reef revealed a highly diverse symbiotic community with a complex composition demonstrating a relatively homogeneous phylogenetic distribution (**Chapter 1**). The majority of the microbial sequences were closely related to other sponge-derived sequences and also fell into sponge- or sponge-coral specific clusters, denoting that the "living fossil" coralline sponge *V. crypta* shares features of its microbial community with other sponges. The denaturing gradient gel electrophoresis cluster analysis confirmed a high microbial diversity associated with *V. crypta* and indicated distinct microbial communities in the different growth forms (solitary and colonial).

Exploration of microbial diversity in *Astrosclera willeyana* from the Great Barrier Reef exposed the presence of a complex symbiotic community with high diversity (**Chapter 2**) and also confirmed the uniqueness of the microbial consortia in sponges, as the majority of the *A. willeyana*-associated sequences grouped together with other sponge-derived sequences and formed numerous sponge specific clusters. The DGGE results showed clear divisions according to the geographical origin of the samples, indicating closer relationships between the microbial communities with respect to their geographic origin (northern vs. southern GBR) and suggesting that differences in symbiotic community composition might be an additional indicator of cryptic species.

Additional DGGE analyses of numerous *A. willeyana* specimens from virtually the entire area wherein *A. willeyana* occurs – from the Red Sea to the central Pacific – confirmed high microbial diversity and a complex composition in all samples that were investigated (**Chapter 3**). Closer associations between the microbiota with respect to their geographic origin were also confirmed for the whole distribution range, thus supporting separation of *A. willeyana* populations. Moreover, this study provided initial insight into the hitherto undetermined diversity and composition of microbial communities associated with sclerosponges from the Red Sea. Subsequent comparison with previously assessed 16S clone library

of the *A. willeyana* from the GBR showed that, in spite of many similarities, microbiota associated with the Red Sea specimen have a less complex structure. Nevertheless, both sponges shared 40% of defined OTUs, which represents about 60% of both clone libraries.

A comparison of microbial communities associated with *A. willeyana* and *V. crypta* from the Great Barrier Reef demonstrated, despite some differences, a highly similar phylogenetic composition of both symbiotic consortia (**Chapter 4**). Both coralline sponges harbored rich and diverse microbial communities with strikingly comparable composition of phyla; they differed, however, in the abundance of the members of the particular phylogenetic groups. *V. crypta* revealed a slightly more complex community structure. *A. willeyana* and *V. crypta* shared a large number of bacterial species, far exceeding the amount of shared OTUs characteristic for other sponges. Our results indirectly confirmed vertical transmission of microbial symbionts in coralline sponges; however, our results simultaneously indicated horizontal transmission as the more primary mechanism.

The first ever characterization of microbial symbionts associated with coralline sponges clearly showed a very complex structure and a high diversity of their communities. Sclerosponges harbor microbial consortia composed mainly of members of phyla typically associated with other sponges. Microbial 16S rRNA gene sequences obtained from coralline sponges show a high similarity with other sponge-derived sequences and fall into abundant sponge specific clusters. In contrast to other sponges, distantly related sclerosponges share a much higher degree of microbial species and thus indicate specific patterns for the constitution of microbial communities. Further studies involving wider ranges of specimens of *A. willeyana* and *V. crypta*, as well as other coralline sponges and reproductive stages, would bring more information on shared "core microbiome" and clarify whether or not those microorganisms are functionally important for the ecology and evolution of sclerosponges.

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Supplementary material

Table S1.1. List of the OTUs obtained from *Vaceletia crypta* with phylogenetic affiliations and closest relative sequences from BLAST search

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B125/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118592	Uncultured bacterium clone Mfav_D06	*Chloroflexi*	100%
B287/GW947	1	*Discodermia dissoluta* (sponge)	Caribbean Sea: coast of Curacao	AY897104	Uncultured Acidobacteria bacterium clone Dd-spU-49	*Acidobacteria*	99%
B253/GW947	1	*Ircinia strobilina* (sponge)	USA: Florida, Key Largo, Conch Reef, 18 m	EF629591	Uncultured Acidobacteria bacterium clone W04IS5A01	*Acidobacteria*	99%
B33/GW947	8	*Rhopaloeides odorabile* (sponge)	Australia: Pelorus island, 15 m	EU183850	Uncultured bacterium clone 27aD12	*Acidobacteria*	99%
B3/GW947	8	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado, 20 m	FJ269280	Uncultured Acidobacteria bacterium clone XA2F04F	*Acidobacteria*	99%
B112/GW947	3	*Xestospongia muta* (sponge)	Indonesia: the coast of Manado	FJ229954	Uncultured actinobacterium clone XB3G12F	*Actinobacteria*	99%
B15/GW947	22	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	FJ229964	Uncultured actinobacterium clone XF1D12	*Actinobacteria*	99%
B84/GW947	6	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118617	Uncultured bacterium clone Mfav_B20	*Alphaproteobacteria*	99%
B53/GW947	1	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596714	Uncultured alpha proteobacterium clone XD1D10	*Alphaproteobacteria*	99%
B214/GW947	2	Isolate		CP000884	*Delftia acidovorans* SPH-1	*Betaproteobacteria*	99%
B34/GW947	5	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	EF513742	Uncultured bacterium clone OP491	*Chloroflexi*	99%
B155/GW947	1	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	FJ543137	Uncultured bacterium clone OP446	*Chloroflexi*	99%
B19/GW947	7	*Discodermia dissoluta* (sponge)	Caribbean Sea: the coast of Curacao	AY897114	Uncultured bacterium clone Dd-spT-A21	*Chloroflexi*	99%
B170/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118572	Uncultured bacterium clone Mfav_A13	*Chloroflexi*	99%
B369/GW947	2	*Neofibularia nolitangere* (sponge)	Caribbean Sea	EU816835	Uncultured bacterium clone 222CK	*Chloroflexi*	99%
B25/GW947	10	*Rhopaloeides odorabile* (sponge)	Australia: GBR, Rib Reef	JN210620	Uncultured Chloroflexi bacterium clone T028deg56	*Chloroflexi*	99%
B60/GW947	1	*Rhopaloeides odorabile* (sponge)	Australia: GBR, Rib Reef	JN210606	Uncultured Chloroflexi bacterium clone T436deg21	*Chloroflexi*	99%

Table S1.1 continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B261/GW947	4	*Theonella swinhoei* (sponge)	Palau: Western Caroline Islands, 20–30 m	AF186417	Uncultured sponge symbiont PAWS52f	*Chloroflexi*	99%
B160/GW947	1	*Theonella swinhoei* (sponge)	Japan: Hachijo-jima Island, 15 m	AF434973	Uncultured sponge symbiont JAWS17	*Chloroflexi*	99%
B231/GW947	1	*Theonella swinhoei* (sponge)	Japan: Hachijo-jima Island, 15 m	AF434974	Uncultured sponge symbiont JAWS18	*Chloroflexi*	99%
B12/GW947	7	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	FJ481328	Uncultured Chloroflexus sp. clone XB3D09F	*Chloroflexi*	99%
B332/GW947	1	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	HQ270396	Uncultured Truepera sp. clone XE1H04	*Deinococcus-Thermus*	99%
B42/GW947	4	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	HQ270283	Uncultured bacterium clone XB1B12F	*Deltaproteobacteria*	99%
B18/GW947	6	*Rhopaloeides odorabile* (sponge)	Australia: GBR, Rib Reef	JN210659	Uncultured delta proteobacterium clone T436deg77	*Deltaproteobacteria*	99%
B119/GW947	4	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	EF513715	Uncultured bacterium clone OP437	*Gammaproteobacteria*	99%
B4/GW947	18	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076171	Uncultured gamma proteobacterium clone AD082	*Gammaproteobacteria*	99%
B27/GW947	4	*Agelas dilatata* (sponge)	USA: Florida, Key Largo	HQ270284	Uncultured gamma proteobacterium clone XB1C07F	*Gammaproteobacteria*	99%
B205/GW947	2	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	JN596624	Uncultured gamma proteobacterium clone XE3B06	*Gammaproteobacteria*	99%
B76/GW947	11	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	HQ270202	Uncultured bacterium clone XA1E03F	*Gemmatimonadetes*	99%
B6/GW947	30	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	HQ270417	Uncultured Nitrospira sp. clone XF1F12	*Nitrospirae*	99%
B55/GW947	1	*Plakortis sp.* (sponge)	Bahamas	EU071662	Uncultured Poribacteria bacterium clone P20	*Poribacteria*	99%
B50/GW947	2	*Plakortis sp.* (sponge)	Bahamas: Little San Salvador Island	EF076079	Uncultured Poribacteria bacterium clone PK_Pori20	*Poribacteria*	99%
B54/GW947	2	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596755	Uncultured Spirochaetes bacterium clone XD2043	*Spirochaetes*	99%
B250/GW947	2	*Rhopaloeides odorabile* (sponge)	Australia: GBR, Rib Reef	JN210869	Uncultured Deferribacteres bacterium clone T536deg33	*uncertain affiliation*	99%

Table S1.1 continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B47/GW947	18	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	EF513719	Uncultured bacterium clone OP447	*uncertain affiliation*	99%
B2/GW947	5	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	EF513682	Uncultured bacterium clone OP354	*uncertain affiliation*	99%
B22/GW947	3	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529304	Uncultured Acidobacteria bacterium clone A105	*Acidobacteria*	98%
B7/GW947	9	*Theonella swinhoei* (sponge)	Palau: Western Caroline Islands, 20–30 m	AF186411	Uncultured sponge symbiont PAUC32f	*Actinobacteria*	98%
B49/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118606	Uncultured bacterium clone Mfav_A19	*Alphaproteobacteria*	98%
B293/GW947	3	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118690	Uncultured bacterium clone Mfav_G17	*Alphaproteobacteria*	98%
B115/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea	FJ203619	Uncultured bacterium clone SHFH709	*Bacteroidetes*	98%
B46/GW947	6	*Ancorina alata* (sponge)	New Zealand: north-eastern, Jones Bay	FJ900343	Uncultured bacterium clone AncL33	*Chloroflexi*	98%
B159/GW947	3	*Discodermia dissoluta* (sponge)	Caribbean Sea: the coast of Curacao	AY897115	Uncultured bacterium clone Dd-spT-C35	*Chloroflexi*	98%
B11/GW947	15	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118655	Uncultured bacterium clone Mfav_L15	*Chloroflexi*	98%
B21/GW947	7	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118650	Uncultured bacterium clone Mfav_F22	*Chloroflexi*	98%
B185/GW947	2	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118534	Uncultured bacterium clone Mfav_C17	*Chloroflexi*	98%
B247/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118590	Uncultured bacterium clone Mfav_G13	*Chloroflexi*	98%
B428/GW947	1	*Tedania ignis* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU981989	Uncultured bacterium clone TI-53	*Chloroflexi*	98%
B9/GW947	11	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596671	Uncultured Chloroflexi bacterium clone XC1025	*Chloroflexi*	98%
B242/GW947	3	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596746	Uncultured Chloroflexi bacterium clone XD2008	*Chloroflexi*	98%
B435/GW947	1	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	FJ481247	Uncultured Chloroflexus sp. clone XA2H04F	*Chloroflexi*	98%

Table S1.1 continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B75/GW947	7	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	HQ270347	Uncultured bacterium clone XC1F12	*Deltaproteobacteria*	98%
B326/GW947	1	*Acanthostrongylophora sp.* (sponge)	Indonesia: the coast of Manado	EF513695	Uncultured bacterium clone OP401	*Deltaproteobacteria*	98%
B99/GW947	6	*Rhopaloeides odorabile* (sponge)	Australia: GBR, Rib Reef	JN210625	Uncultured Desulfurellaceae bacterium clone T534deg62	*Deltaproteobacteria*	98%
B270/GW947	2	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900328	Uncultured bacterium clone AncL47	*uncertain affiliation*	98%
B395/GW947	3	*Theonella swinhoei* (sponge)	Palau: Western Caroline Islands, 20–30 m	AF186458	Uncultured sponge symbiont PAWS72	*Acidobacteria*	97%
B239/GW947	1	*Aplysina fulva* (sponge)	Brasil: Río de Janeiro, Búzios	FM160787	Uncultured bacterium partial clone d131	*Chloroflexi*	97%
B109/GW947	1	*Geodia sp.* (sponge)	Croatia: Limski Channel	HM485641	Uncultured bacterium clone GspT-5	*Chloroflexi*	97%
B281/GW947	5	*Hyrtios erectus* (sponge)	Red Sea: Egypt, Sinai, Ras Mohamed, 10 m	GQ163736	Uncultured sponge bacterium clone JZ59-15	*Chloroflexi*	97%
B321/GW947	4	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado, 20 m	FJ481358	Uncultured Chloroflexus sp. clone XD2D08	*Chloroflexi*	97%
B1/GW947	1	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	HQ270363	Uncultured bacterium clone XD2D12	*Delta/Nitrospina*	97%
B62/GW947	1	basalt glass from seamount	Pacific: Juan de Fuca Ridge, Cobb Seamount	DQ070825	Uncultured delta proteobacterium clone JdFBGBact_32	*Deltaproteobacteria*	97%
B368/GW947	1	*Rhopaloeides odorabile* (sponge)	Australia: Pelorus island, 15 m	EU183759	Uncultured bacterium clone 27B11	*Gammaproteobacteria*	97%
B339/GW947	1	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island, 12 m	FJ529263	Uncultured gamma proteobacterium clone A16	*Gammaproteobacteria*	97%
B17/GW947	9	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118544	Uncultured bacterium clone Mfav_K21	*Gemmatimonadetes*	97%
B90/GW947	3	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596662	Uncultured Deferribacteres bacterium clone XC1007	*uncertain affiliation*	97%
B38/GW947	8	madea	Mediterranean Sea	JF809732	Uncultured bacterium clone 2M1B-B19	*Chloroflexi*	96%
B173/GW947	1	*Diploria strigosa* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU119661	Uncultured organism clone Dstr_C23	*Cyanobacteria*	96%

Table S1.1 continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B5/GW947	1	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529327	Uncultured delta proteobacterium clone E42	*Deltaproteobacteria*	96%
B56/GW947	2	marine sediment	Gulf of Mexico: 4-10 cm	DQ431899	Uncultured Gemmatimonadetes bacterium clone 3G1820-56	*Gemmatimonadetes*	96%
B349/GW947	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118605	Uncultured bacterium clone Mfav_L08	*Gemmatimonadetes*	96%
B209/GW947	1	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596758	Uncultured Clostridium sp. clone XD2045	*Poribacteria*	96%
PCR35/GW947	1	*Axinella corrugata* (sponge)	USA: Florida, Florida Keys	DQ299275	Uncultured crenarchaeote clone A137.2	*Archaea*	95%
B228/GW947	1	*Discodermia dissoluta* (sponge)	Caribbean Sea: the coast of Curacao	AY897077	Uncultured Chloroflexi bacterium clone Dd-spT-A3	*Chloroflexi*	95%
B85/GW947	1	*Pachastrellidae sp.* (sponge)	Caribbean Sea	EU816806	Uncultured Chloroflexi bacterium clone 196E	*Chloroflexi*	95%
B346/GW947	2	*Verulina sp.* (sponge)	Caribbean Sea	EU816857	Uncultured bacterium clone 229DK	*Chloroflexi*	95%
B440/GW947	1	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159837	Uncultured bacterium clone XmA074	*Gammaproteobacteria*	95%
B108/GW947	2	*Aplysina fulva* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU982057	Uncultured bacterium clone AF-37	*Chloroflexi*	94%
B29/GW947	10	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118666	Uncultured bacterium clone Mfav_P24	*Chloroflexi*	94%
B213/GW947	2	subsurface	USA: Washington, Hanford Site 300 Area	HM187037	Uncultured bacterium clone HDB_SIPI622	*Chloroflexi*	94%
B419/GW947	1	*Agelas dilatata* (sponge)	Bahamas: San Salvador Island	EF076137	Uncultured gamma proteobacterium clone AD026	*Gammaproteobacteria*	94%
B43/GW947	4	*Desmacidon* sponge	Norway	EU035947	Uncultured bacterium clone KspoC9	*Chloroflexi*	93%
B392/GW947	2	*Oculina patagonica* (coral)	Mediterranean Sea: Israel, Sedot Yam	DQ416442	Uncultured bacterium clone w2uc5	*Deltaproteobacteria*	93%
B266/GW 947	1	marine water	Pacific Ocean: Station ALOHA	JN166362	Uncultured marine microorganism clone HOT157_350m90	*Gammaproteobacteria*	93%
B39/GW947	23	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118605	Uncultured bacterium clone Mfav_L08	*Gemmatimonadetes*	93%

Table S1.1 continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
B45/GW947	1	subsurface	USA: Washington, Hanford Site 300 Area	HM187037	Uncultured bacterium clone HDB_SIP1622	*Chloroflexi*	92%
B237/GW947	1	*Agelas dilatata* (sponge)	Bahamas: San Salvador Island	EF076137	Uncultured gamma proteobacterium clone AD026	*Gammaproteobacteria*	92%
B165/GW947	2	red algae		AY731517	Paralemanea annulata; chloroplast	*Cyanobacteria*	91%
B164/GW947	4	deep-sea sediment	depth: 2725m	FJ205348	Uncultured Chloroflexi bacterium clone I2C	*Chloroflexi*	90%
B114/GW947	5	marine water	Hawaii: Ocean Station ALOHA, 500 m	EU361073	Uncultured Chloroflexi bacterium clone HF500_24O	*Chloroflexi*	90%
B400/GW947	1	subsurface	USA: Washington, Hanford Site 300 Area	HM187381	Uncultured bacterium clone HDB_SISU426	*Chloroflexi*	90%
B401/GW947	1	*Erythropodium caribaeorum* (octocoral)	USA: Florida, coast of Fort Lauderdale, 9 m	DQ889931	Uncultured Spongiobacter sp. clone EC22	*Gammaproteobacteria*	90%
B137/GW947	1	*Tsitsikamma favus* (sponge)	South Africa: Algoa Bay	HQ241805	Uncultured marine bacterium clone Sp02sw-26	*Betaproteobacteria*	88%
B166/GW947	1	*Xestospongia testudinaria* (sponge)	Indonesia: the coast of Manado	JN596702	Uncultured Chloroflexi bacterium clone XC2G01	*Chloroflexi*	88%
B225/GW947	3	marine water	Northeast subarctic Pacific Ocean, 2000m depth	HQ673993	Uncultured bacterium clone F9P122000_S_O13	*Gammaproteobacteria*	88%
B24/GW947	8	*Tsitsikamma favus* (sponge)	South Africa: Algoa Bay	HQ241812	Uncultured marine bacterium clone Sp02sw-31	*Gammaproteobacteria*	88%

Figure S1.1 Image of the DGGE gel; sample names according to the Table 1.1 (column: Site)

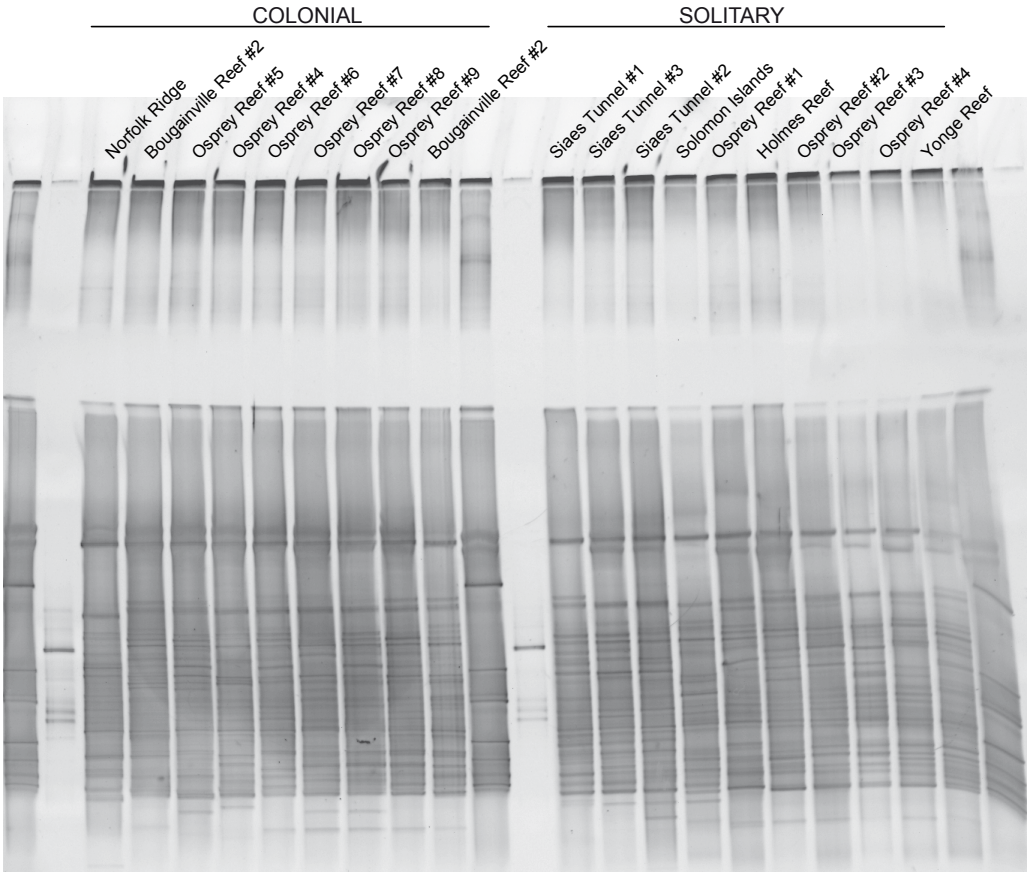


Table S2.1. List of the OTUs obtained from *Astrosclera willeyana* from GBR with phylogenetic affiliations and closest relative sequences from BLAST search.

		Closest phylogenetic affiliation					
OTU	**No. of clones**	**Host**	**Source**	**EMBL accession no.**	**Strain**	**Taxonomic group**	**Similarity GenBank**
A89/GW950	4	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	JN596639	Uncultured alpha proteobacterium clone XE3H04	*Alphaproteobacteria*	99%
A342/GW950	1	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	FJ229964	Uncultured actinobacterium clone XF1D12	*Actinobacteria*	99%
A187/GW950	9	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159846	Uncultured bacterium clone XmA159	*Chloroflexi*	99%
A375/GW950	7	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269280	Uncultured Acidobacteria bacterium clone XA2F04F	*Acidobacteria*	99%
A8/GW950	13	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481354	Uncultured Chloroflexus sp. clone XD2B09	*Chloroflexi*	99%
A36/GW950	5	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269287	Uncultured Acidobacteria bacterium clone XA2H07F	*Acidobacteria*	99%
A41/GW950	5	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ229956	Uncultured actinobacterium clone XC1C08	*Actinobacteria*	99%
A299/GW950	5	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596713	Uncultured Chloroflexi bacterium clone XD1E10	*Chloroflexi*	99%
A12/GW950	4	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270236	Uncultured delta proteobacterium clone XA2D07F	*Deltaproteobacteria*	99%
A145/GW950	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596734	Uncultured Spirochaetes bacterium clone XD1D08	*Spirochaetes*	99%
A82/GW950	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270351	Uncultured gamma proteobacterium clone XD2A08	*Gammaproteobacteria*	99%
A295/GW951	7	*Acanthostrongylophora sp.* (sponge)	Indonesia: Manado	EF513642	Uncultured bacterium clone OP101	*Acidobacteria*	99%
A61/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596723	Uncultured Chloroflexi bacterium clone XD1B05	*Chloroflexi*	99%
A4/GW950	7	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118636	Uncultured bacterium clone Mfav_M11	*Alphaproteobacteria*	99%
A110/GW950	2	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118617	Uncultured bacterium clone Mfav_B20	*Alphaproteobacteria*	99%
A326/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118605	Uncultured bacterium clone Mfav_L08	*Gemmatimonadetes*	99%
A309/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118526	Uncultured bacterium clone Mfav_F06	*Alphaproteobacteria*	99%

Table S2.1. continued

		Closest phylogenetic affiliation					
OTU	**No. of clones**	**Host**	**Source**	**EMBL accession no.**	**Strain**	**Taxonomic group**	**Similarity GenBank**
A307/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118690	Uncultured bacterium clone Mfav_G17	*Alphaproteobacteria*	99%
A63/GW950	16	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076129	Uncultured Nitrospira sp. clone AD017	*Nitrospirae*	99%
A185/GW950	10	*Plakortis sp.* (sponge)	Bahamas: Little San Salvador Island	EF076105	Uncultured bacterium clone PK072	*Deferribacteres*	99%
A246/GW950	9	*Plakortis sp.* (sponge)	Bahamas: Little San Salvador Island	EF076111	Uncultured Chloroflexi bacterium clone PK064	*Chloroflexi*	99%
A186/GW950	8	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076171	Uncultured gamma proteobacterium clone AD082	*Gammaproteobacteria*	99%
A130/GW950	3	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076188	Uncultured actinobacterium clone AD028	*Actinobacteria*	99%
A397/GW950	1	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076122	Uncultured delta proteobacterium clone AD037	*Deltaproteobacteria*	99%
A276/GW950	1	*Pseudopterogorgia elisabethae* (coral)	Bahamas	JN863717	Uncultured bacterium isolate DGGE gel band 7	*Cyanobacteria*	99%
A143/GW950	29	*Rhopaloeides odorabile* (sponge)	Australia: Great Barrier Reef, Rib Reef	JN210648	Uncultured Acidimicrobidae bacterium clone T436deg4	*Actinobacteria*	99%
A310/GW950	15	*Rhopaloeides odorabile* (sponge)	Australia: Great Barrier Reef, Rib Reef	JN210877	Uncultured Deferribacteres bacterium clone T028deg12	*Deferribacteres*	99%
A180/GW950	6	*Rhopaloeides odorabile* (sponge)	Australia: Great Barrier Reef, Davies Reef	AF333544	Uncultured bacterium clone R43	*Chloroflexi*	99%
A49/GW950	2	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529305	Uncultured Acidobacteria bacterium clone A106	*Acidobacteria*	99%
A266/GW950	2	*Theonella swinhoei* (sponge)	Palau: Western Caroline Islands	AF186415	Uncultured sponge symbiont PAUC43f	*Gemmatimonadetes*	99%
A382/GW950	2	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	FJ481375	Uncultured Chloroflexus sp. clone XF1B12	*Chloroflexi*	98%
A214/GW950	6	*Geodia barretti* (sponge)	Norway: west coast	JQ612250	Uncultured bacterium clone GBc034	*Gammaproteobacteria*	98%
A239/GW950	1	*Geodia barretti* (sponge)	Norway: west coast	JQ612276	Uncultured bacterium clone GBc173	*Acidobacteria*	98%
A80/GW950	5	*Ancorina alata* (sponge)	New Zealand: Jones Bay	FJ900580	Uncultured bacterium clone AncE9	*Chloroflexi*	98%

Table S2.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
A200/GW950	3	*Ancorina alata* (sponge)	New Zealand: Jones Bay	FJ900318	Uncultured bacterium clone AncL1	*Gammaproteobacteria*	98%
A196/GW950	3	*Ancorina alata* (sponge)	New Zealand: Jones Bay	FJ900513	Uncultured bacterium clone AncD14	*Chloroflexi*	98%
A48/GW950	27	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481360	Uncultured Chloroflexus sp. clone XD2G11	*Chloroflexi*	98%
A77/GW950	5	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270341	Uncultured bacterium clone XC1C09	*Deltaproteobacteria*	98%
A336/GW950	3	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270337	Uncultured gamma proteobacterium clone XC1B08	*Gammaproteobacteria*	98%
A313/GW950	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270280	Uncultured delta proteobacterium clone XA3H07F	*Deltaproteobacteria*	98%
A402/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596755	Uncultured Spirochaetes bacterium clone XD2043	*Spirochaetes*	98%
A35/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118645	Uncultured bacterium clone Mfav_K01	*Acidobacteria*	98%
A174/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118537	Uncultured bacterium clone Mfav_O17	*Acidobacteria*	98%
A184/GW950	6	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076133	Uncultured gamma proteobacterium clone AD023	*Gammaproteobacteria*	98%
A278/GW950	2	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076144	Uncultured actinobacterium clone AD035	*Actinobacteria*	98%
A241/GW950	1	*Plakortis sp.* (sponge)	Bahamas: Little San Salvador Island	EF076079	Uncultured Poribacteria bacterium clone PK_Pori20	*Poribacteria*	98%
A29/GW950	3	*Geodia barretti* (sponge)	Norway: west coast	JQ612203	Uncultured bacterium clone GBc159	*Chloroflexi*	97%
A66/GW950	9	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596717	Uncultured Chloroflexi bacterium clone XD1C12	*Chloroflexi*	97%
A289/GW950	3	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269286	Uncultured Acidobacteria bacterium clone XA2H05F	*Acidobacteria*	97%
A152/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481356	Uncultured Chloroflexus sp. clone XD2C08	*Chloroflexi*	97%
A95/GW950	23	*Plakortis sp.* (sponge)	Bahamas: Little San Salvador Island	EF076111	Uncultured Chloroflexi bacterium clone PK064	*Chloroflexi*	97%

Table S2.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
A56/GW950	5	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076128	Uncultured Chloroflexi bacterium clone AD031	*Chloroflexi*	97%
A230/GW950	1	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529263	Uncultured gamma proteobacterium clone A16	*Gammaproteobacteria*	97%
A231/GW950	2	*Ancorina alata* (sponge)	New Zealand: Jones Bay	FJ900321	Uncultured bacterium clone AncL24	*Gammaproteobacteria*	96%
A225/GW950	6	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596744	Uncultured Chloroflexi bacterium clone XD2016	*Chloroflexi*	96%
A120/GW950	1	*Acanthostrongylophora sp.* (sponge)	Indonesia: Manado	EF513681	Uncultured bacterium clone OP348	*Deltaproteobacteria*	96%
A176/GW950	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118628	Uncultured bacterium clone Mfav_O03	*Chloroflexi*	96%
A42/GW950	17	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076127	Uncultured Chloroflexi bacterium clone AD007	*Chloroflexi*	96%
A210/GW950	4	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076125	Uncultured Gemmatimonadetes bacterium clone AD004	*Gemmatimonadetes*	96%
A366/GW950	1	deep-sea	Atlantic Ocean: Angola Basin	AM997488	Uncultured deep-sea clone Ulrdd_22	*Chloroflexi*	96%
A376/GW950	5	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529303	Uncultured delta proteobacterium clone A102	*Deltaproteobacteria*	96%
PCR18 /GW950	1	*Axinella corrugata* (sponge)	USA: Florida Keys	DQ299275	Uncultured Cenarchaeaceae crenarchaeote clone A137.2	*Archaea*	96%
A50/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481255	Uncultured Chloroflexus sp. clone XA3C04F	*Chloroflexi*	95%
A270/GW950	1	deep-sea	Atlantic Ocean: Angola Basin	AM997436	Uncultured deep-sea clone Ulr1530	*Chloroflexi*	95%
A159/GW950	3	sediment	Australia: Great Barrier Reef, Davies Reef	HM178878	Uncultured bacterium clone T333B11	*Deltaproteobacteria*	95%
A371/GW950	2	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529310	Uncultured Chloroflexi bacterium clone A124	*Chloroflexi*	95%
A257/GW950	4	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596669	Uncultured gamma proteobacterium clone XC1009	*Gammaproteobacteria*	94%
A349/GW950	2	*Aplysina fulva* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU982057	Uncultured bacterium clone AF-37	*Chloroflexi*	94%

Table S2.1. continued

		Closest phylogenetic affiliation					
OTU	**No. of clones**	**Host**	**Source**	**EMBL accession no.**	**Strain**	**Taxonomic group**	**Similarity GenBank**
A374/GW950	1	*Aplysina fulva* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU982106	Uncultured bacterium clone AF-90	*Actinobacteria*	94%
A129/GW950	3	*Rhopaloeides odorabile* (sponge)	Australia: Great Barrier Reef, Rib Reef	JN210584	Uncultured Chloroflexi bacterium clone T534deg63	*Chloroflexi*	94%
A87/GW950	2	deep-sea	Saanich Inlet, 120 m depth	GQ350571	Uncultured alpha proteobacterium clone SHBC842	*Alphaproteobacteria*	93%
A37/GW950	1	*Phyllospongia papyracea* (sponge)	Palau: KB Channel, seamount	AY845233	Uncultured bacterium clone 31P4	*Alphaproteobacteria*	93%
A359/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596752	Uncultured Chloroflexi bacterium clone XD2046	*Chloroflexi*	92%
A217/GW950	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596714	Uncultured alpha proteobacterium clone XD1D10	*Alphaproteobacteria*	92%
A116/GW950	1	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529327	Uncultured delta proteobacterium clone E42	*Deltaproteobacteria*	92%
A46/GW950	9	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	HQ270412	Uncultured gamma proteobacterium clone XF1E08	*Gammaproteobacteria*	90%
A378/GW950	4	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076137	Uncultured gamma proteobacterium clone AD026	*Gammaproteobacteria*	90%
A2/GW950	3	saline soil	China	EU328062	Uncultured gamma proteobacterium clone Y89	*Gammaproteobacteria*	88%
A320/GW950	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269249	Uncultured bacterium clone XA1C08F	*Acidobacteria*	87%

Table S3.1. List of the OTUs obtained from *Astrosclera willeyana* from Red Sea with phylogenetic affiliations and closest relative sequences from BLAST search.

		Closest phylogenetic affiliation					
OTU	**No. of clones**	**Host**	**Source**	**EMBL accession no.**	**Strain**	**Taxonomic group**	**Similarity GenBank**
C457/GW1046	1	*Porites astreoides* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU119137	uncultured bacterium clone Past_P10	*Gammaproteobacteria*	100%
C471/GW1046	8	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076129	uncultured *Nitrospira* sp. clone AD017	*Nitrospira*	99%
C211/GW1046	2	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	HQ270424	uncultured proteobacterium clone XF1H12	*Gammaproteobacteria*	99%
C1/GW1046	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270219	uncultured proteobacterium clone XA1H12F	*Deltaproteobacteria*	99%
C155/GW1046	15	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076122	uncultured proteobacterium clone AD037	*Deltaproteobacteria*	99%
C87/GW1046	21	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	FJ481372	uncultured *Chloroflexus* sp. clone XE1H05	*Chloroflexi*	99%
C198/GW1046	8	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481354	uncultured *Chloroflexus* sp. clone XD2B09	*Chloroflexi*	99%
C369/GW1046	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ481239	uncultured *Chloroflexus* sp. clone XA2E03F	*Chloroflexi*	99%
C395/GW1046	1	*Xestospongia testudinaria* (sponge)	Australia: Orpheus Island, GBR	JX455624	uncultured bacterium clone XTES_G10068	*Deferribacteres*	99%
C158/GW1046	15	*Xestospongia testudinaria* (sponge)	Australia: Orpheus Island, GBR	JX455631	uncultured bacterium clone XTES_E11	*Deferribacteres*	99%
C92/GW1046	1	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159877	uncultured bacterium clone XmE065	*Actinobacteria*	99%
C348/GW1046	9	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159846	uncultured bacterium clone XmA159	*Chloroflexi*	99%
C442/GW1046	3	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269287	uncultured bacterium clone XA2H07F	*Acidobacteria*	99%
C124/GW1046	4	*Ircinia oros* (sponge)	Spain: Catalunya	JX206590	uncultured bacterium clone TO10-97_C14	*Gammaproteobacteria*	99%
C176/GW1046	2	*Rhopaloeides odorabile* (sponge)	Australia: Rib Reef, GBR	JN210881	uncultured bacterium clone T028deg15	*Deferribacteres*	99%
C37/GW1046	6	*Rhopaloeides odorabile* (sponge)	Australia: Davies Reef, GBR	AF333544	uncultured bacterium clone R43	*Chloroflexi*	99%
C58/GW1046	1	*Rhopaloeides odorabile* (sponge)	Australia: Davies Reef, GBR	AF333544	uncultured bacterium clone R43	*Chloroflexi*	99%

Table S3.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
C419/GW1046	5	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118690	uncultured bacterium clone Mfav_G17	*Alphaproteobacteria*	99%
C454/GW1046	8	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118617	uncultured bacterium clone Mfav_B20	*Alphaproteobacteria*	99%
C165/GW1046	1	*Svenzea zeai* (sponge)	Bahamas: San Salvador Island	FJ529305	uncultured bacterium clone A106	*Acidobacteria*	99%
C83/GW1046	1	*Rhopaloeides odorabile* (sponge)	Australia: Pelorus island, 15 m	EU183804	uncultured bacterium clone 27H6	uncertain affiliation	99%
C239/GW1046	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596703	uncultured *Acidobacterium* sp. clone XD2B08	*Acidobacteria*	99%
C105/GW1046	1	coastal sediment water		AB030085	*Pseudomonas sp. MBIC2027*	*Gammaproteobacteria*	99%
C9/GW1046	1	copepod	Indian Ocean	FJ457171	*Pseudoalteromonas piscicida* strain S1948	*Gammaproteobacteria*	99%
C456/GW1046	5	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076171	uncultured proteobacterium clone AD082	*Gammaproteobacteria*	98%
C463/GW1046	5	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	HQ270280	uncultured proteobacterium clone XA3H07F	*Deltaproteobacteria*	98%
C480/GW1046	11	*Plakortis* sp. (sponge)	Bahamas: Little San Salvador Island	EF076111	uncultured bacterium clone PK064	*Chloroflexi*	98%
C120/GW1046	1	*Pachastrella* sp. (sponge)	USA: Gulf of Mexico	AB453749	uncultured bacterium GM-WBS-cloneBB1	*Chloroflexi*	98%
C435/GW1046	13	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159856	uncultured bacterium clone XmA254	*Deltaproteobacteria*	98%
C411/GW1046	6	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596744	uncultured bacterium clone XD2016	*Chloroflexi*	98%
C275/GW1046	2	*Ircinia oros* (sponge)	Spain: Catalunya	JX206616	uncultured bacterium clone TO10-919_C9	*Gammaproteobacteria*	98%
C452/GW1046	1	*Rhopaloeides odorabile* (sponge)	Australia: Davies Reef, GBR	AF333545	uncultured bacterium clone R6	*Chloroflexi*	98%
C477/GW1046	12	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118645	uncultured bacterium clone Mfav_K01	*Acidobacteria*	98%
C334/GW1046	1	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118644	uncultured bacterium clone Mfav_I13	*Gammaproteobacteria*	98%

Table S3.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
C294/GW1046	3	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118526	uncultured bacterium clone Mfav_F06	*Alphaproteobacteria*	98%
C374/GW1046	6	*Haliclona hogarthi* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU981894	uncultured bacterium clone HH-D11	*Deltaproteobacteria*	98%
C363/GW1046	4	*Geodia barretti* (sponge)	Norway	JQ612250	uncultured bacterium clone GBc034	*Gammaproteobacteria*	98%
C140/GW1046	27	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900580	uncultured bacterium clone AncE9	*Chloroflexi*	98%
C19/GW1046	3	*Aplysina fulva* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU982102	uncultured bacterium clone AF-85	*Chloroflexi*	98%
C414/GW1046	1	*Pachastrella* sp. (sponge)	USA: Gulf of Mexico	AB453749	uncultured bacterium GM-WBS-cloneBB1	*Chloroflexi*	98%
C291/GW1046	2	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076188	uncultured actinobacterium clone AD028	*Actinobacteria*	98%
C474/GW1046	7	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596721	uncultured bacterium clone XD1A06	*Chloroflexi*	97%
C154/GW1046	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596656	uncultured bacterium clone XC1043	*Chloroflexi*	97%
C206/GW1046	1	*Pachastrella* sp. (sponge)	USA: Gulf of Mexico	AB453749	uncultured bacterium GM-WBS-cloneBB1	*Chloroflexi*	97%
C470/GW1046	1	*Geodia barretti* (sponge)	Norway	JQ612300	uncultured bacterium clone GBc171	*Gemmatimonadetes*	97%
C190/GW1046	11	*Geodia barretti* (sponge)	Norway	JQ612203	uncultured bacterium clone GBc159	*Chloroflexi*	97%
C407/GW1046	2	marine sediment	Spain: Cies Islands-Galicia	JF344637	uncultured proteobacterium clone ANOX-075	*Alphaproteobacteria*	97%
C277/GW1046	3	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	FJ269344	uncultured bacterium clone XD2E12	*Acidobacteria*	97%
C356/GW1046	4	*Agelas dilatata* (sponge)	Bahamas: Little San Salvador Island	EF076125	uncultured bacterium clone AD004	*Gemmatimonadetes*	96%
C475/GW1046	4	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	JN596772	uncultured bacterium clone XF2G06	*Chloroflexi*	96%
C224/GW1046	3	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596723	uncultured bacterium clone XD1B05	*Chloroflexi*	96%

Table S3.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
C260/GW1046	1	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596721	uncultured bacterium clone XD1A06	*Chloroflexi*	96%
C373/GW1046	3	*Axinella corrugata* (sponge)		EF092260	uncultured *Bdellovibrio* sp. clone MBrad_A7	*Deltaproteobacteria*	96%
C466/GW1046	1	*Axinella corrugata* (sponge)		EF092260	uncultured *Bdellovibrio* sp. clone MBrad_A7	*Deltaproteobacteria*	96%
C430/GW1046	35	*Ircinia variabilis* (sponge)	Spain: Catalunya	JX206713	uncultured bacterium clone TV10-912_C1	*Gammaproteobacteria*	96%
C415/GW1046	7	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118532	uncultured bacterium clone Mfav_N05	*Actinobacteria*	96%
C343/GW1046	9	*Montastraea faveolata* (coral)	Caribbean Sea: Panama, Bocas del Toro	GU118534	uncultured bacterium clone Mfav_C17	*Chloroflexi*	96%
C296/GW1046	4	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900321	uncultured bacterium clone AncL24	*Gammaproteobacteria*	96%
C469/GW1046	10	deep-sea surface sediment	South-Atlantic Ocean: Angola Basin	AM997436	uncultured deep-sea bacterium clone ulr1530	*Chloroflexi*	95%
C428/GW1046	1	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900321	uncultured bacterium clone AncL24	*Gammaproteobacteria*	95%
C324/GW1046	17	*Rhopaloeides odorabile* (sponge)	Australia: Rib Reef, GBR	JN210648	uncultured bacterium clone T436deg4	*Actinobacteria*	95%
C340/GW1046	21	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596767	uncultured bacterium clone XD1D01	*Chloroflexi*	94%
C386/GW1046	1	*Xestospongia muta* (sponge)	USA: Florida, Key Largo	EF159856	uncultured bacterium clone XmA254	*Deltaproteobacteria*	94%
C467/GW1046	1	*Ircinia variabilis* (sponge)	Spain: Catalunya	JX206713	uncultured bacterium clone TV10-912_C1	*Gammaproteobacteria*	94%
C339/GW1046	4	*Geodia barretti* (sponge)	Norway	JQ612189	uncultured bacterium clone GBc029	*Alphaproteobacteria*	94%
C300/GW1046	1	soil	China: Yellow River Delta	JQ800936	uncultured bacterium clone BJGMM-3s-176	*Alphaproteobacteria*	94%
C352/GW1046	1	*Sigmadocia fibulata* (sponge)	Arabian Sea: Lakshadweep Islands	HQ908691	*Pseudovibrio denitrificans* strain *F71059*	*Alphaproteobacteria*	94%
C151/GW1046	2	*Xestospongia testudinaria* (sponge)	Indonesia: Manado	JN596729	uncultured bacterium clone XD1F11	*Spirochaetes*	93%

Table S3.1. continued

OTU	No. of clones	Closest phylogenetic affiliation					
		Host	Source	EMBL accession no.	Strain	Taxonomic group	Similarity GenBank
C36/GW1046	2	deep-sea	South-Atlantic Ocean: Guinea Basi	AM997761	uncultured deep-sea bacterium clone ucb15714	*Gammaproteobacteria*	93%
C401/GW1046	5	*Rhopaloeides odorabile* (sponge)	Australia: Rib Reef, GBR	JN210584	uncultured bacterium clone T534deg63	*Chloroflexi*	93%
C433/GW1046	3	*Ircinia strobilina* (sponge)	Bahamas: Sweetings Cay, Mangrove	GU982157	uncultured bacterium clone IS-55	*Chloroflexi*	93%
C182/GW1046	1	salt water	British Columbia: Saanich Inlet, 120 m depth	GQ350571	uncultured proteobacterium clone SHBC842	*Alphaproteobacteria*	93%
C18/GW1046	1	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900580	uncultured bacterium clone AncE9	*Chloroflexi*	92%
C298/GW1046	1	*Haliclona simulans* (sponge)	Ireland: Galway, Kilkieran Bay	EU350869	uncultured proteobacterium clone HAL-T-12	*Deltaproteobacteria*	91%
C431/GW1046	23	subseafloor sediment	Taiwan	JQ989838	uncultured bacterium clone ORIII-1368-22-G_S008-010_221B13	*Gammaproteobacteria*	90%
C319/GW1046	2	*Geodia barretti* (sponge)	Norway	JQ612187	uncultured bacterium clone GBc162	*Alphaproteobacteria*	90%
C358/GW1046	5	*Ancorina alata* (sponge)	New Zealand: northeastern, Jones Bay	FJ900321	uncultured bacterium clone AncL24	*Gammaproteobacteria*	90%
C254/GW1046	1	deep-sea hydrothermal vent	South-Atlantic Ocean: East Scotia Ridge	JN562618	uncultured prokaryote clone JR224_E9_II-C6	*Chloroflexi*	89%
C342/GW1046	3	*Desmacidon* sp. (sponge)	Norway	EU035927	uncultured bacterium clone KspoA10	*Gammaproteobacteria*	89%
C28/GW1046	1	*Geodia barretti* (sponge)	Norway	JQ612198	uncultured bacterium clone GBc125	*Actinobacteria*	89%
C451/GW1046	1	cave wall biofilm	Slovenia	FJ535097	uncultured bacterium clone 2PJM17	*Nitrospira*	87%

Figure S3.1. Image of the DGGE gel - Gel 1; sample names according to the Table 3.1 (column: Site)

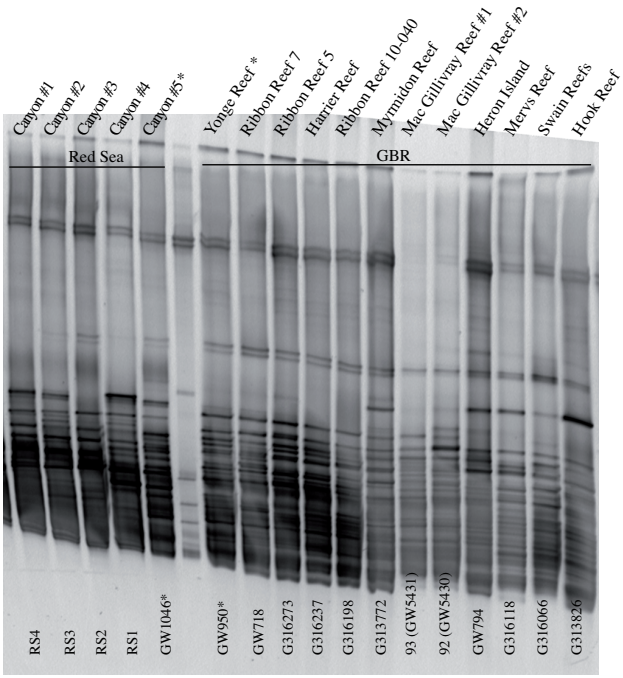
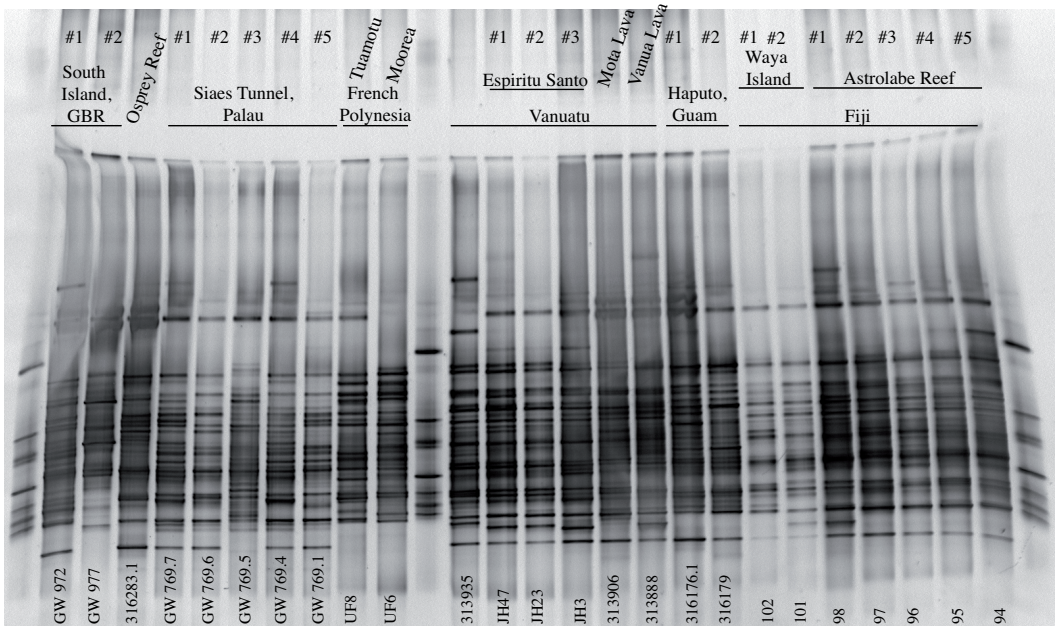


Figure S3.2. Image of the DGGE gel - Gel 2; sample names according to the Table 3.1 (column: Site)



Author contributions

Chapter 1. Microbial diversity in the coralline sponge *Vaceletia crypta*

Klementyna Karlińska-Batres designed this study, generated the sequences, performed the analyses, interpreted the results and wrote the manuscript. Gert Wörheide participated in design of the study and contributed to the improvement of the manuscript with critical comments.

Chapter 2. Phylogenetic diversity and community structure of the symbionts associated with the coralline sponge *Astrosclera willeyana* of the Great Barrier Reef

Klementyna Karlińska-Batres designed this study, generated the sequences, performed the analyses, interpreted the results and wrote the manuscript. Gert Wörheide participated in design of the study and contributed to the improvement of the manuscript with critical comments.

Chapter 3. Spatial variability of microbial communities of the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific

Klementyna Karlińska-Batres designed this study, generated the sequences, performed the analyses, interpreted the results and wrote the manuscript. Gert Wörheide participated in design of the study and contributed to the improvement of the manuscript with critical comments.

Chapter 4. Microbial duel between coralline sponges – a comparison of the symbiotic communities of *Astrosclera willeyana* and *Vaceletia crypta*

Klementyna Karlińska-Batres designed this study, generated the sequences, performed the analyses, interpreted the results and wrote the manuscript. Gert Wörheide participated in design of the study and contributed to the improvement of the manuscript with critical comments.