EXPLORING MICROBIAL DIVERSITY OF MARINE SPONGES BY CULTURE-DEPENDENT AND MOLECULAR APPROACH Mohd Azrul Naim Mohamad 2015

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Exploring microbial diversity of marine sponges by culture-dependent and molecular approaches

Mohd Azrul Naim

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Summary

Discovery of sponge-grade metazoans dated 650 million years ago proved that sponges have been around since the Precambrian era. Their resilience to ever-changing environmental conditions and their global distribution is one of the features attributed to the symbionts in sponges, which include *Archaea*, *Bacteria* and *Eukarya.* It is yet unknown how sponges attract and select their bacterial associates but mechanisms to maintain or newly acquire their symbionts have been demonstrated, such as vertical and horizontal transmission.

Discovery of species-specific bacterial communities in the marine sponges *H. panicea*, *H. oculata* and *H. xena* which are dominated by an alpha, beta- and gammaproteobacterium, respectively, confirmed host-specificity of bacterial associates in marine sponges from the North Sea, although their function remains unknown. Detection of *Chlamydiae* in high relative abundance raised the question as to what is their function in the sponge holobiont as they were only distantly related to other known *Chlamydiae*.

Little is known about the fungal community in marine sponges. This prompted the study of sponge-associated fungi based on molecular analysis. This was previously a difficult enterprise due the large amount of ‘ contaminating’ sponge DNA, which is susceptible to amplification with fungi-specific PCR primers as well. The advent of next generation sequencing technology now for the first time allowed to overcome this hurdle by the sheer numbers of sequences that can be generated. This lead to discovery of novel yeast lineages from the phyla *Ascomycota* and *Basidiomycota* in North Sea and Mediterranean marine sponges, indicating a much higher diversity of fungi yet to be explored. For instance, yeasts from the order *Malasseziales*, which are common pathogens of marine animals, were found as the dominant yeasts in many of the sponges tested that were without apparent disease.

A complementary cultivation-dependent approach provided access to fungal isolates. Fungi belonging to the genus *Penicillium* were found to be the dominant fungi recovered by isolation from the Mediterranean sponges *Aplysina aerophoba*, *Petrosia ficiformis* and *Corticium candelabrum*. In addition, fungi belonging to the order *Alternaria* and yeasts affiliated to the genus *Rhodotorula* were isolated multiple times. No overlap was found with

the fungal species observed through the molecular study, which indicates that the great plate anomaly also exists for fungi. Many of the fungal *Pencilillium* and *Alternaria* strains isolated were shown to have the genetic capacity for producing polyketide synthases (PKS) or PKS-non ribosomal peptide synthase (PKS-NRPS) hybrids. These enzyme complexes are generally responsible for the production of secondary metabolites with a high biological activity.

Isolation of bacteria from *H. panicea* in a cultivation experiment with a large diversification of media and growth conditions and subsequent comparison of the retrieved microorganisms to bacteria found in the sponge tissue by a molecular approach revealed the presence of bacterial genera that dominate the cultivation library, but comprise of represent minor components of the sponge microbiome. This includes genera such *as Bacillus*, *Paracoccus* and *Shewanella*. Another genus that was commonly isolated from many marine sponges, but only is found at low relative abundance in the sponge microbiome is *Pseudovibrio*. Phenotypic characterization based on antibiotic resistance and genotypic differentiation based on bacterial BOX elements and presence of halogenase-encoding genes could discriminate closely related strains that could not be distinguished based on their 16 rRNA gene sequence.

In conclusion, this thesis helps to bridge the gap between cultivation-dependent and cultivation-independent studies of sponge-associated bacteria and fungi by clearly defining the frontiers of the gap. The knowledge derived from this thesis could serve as a scientific foundation and inspiration for future microbial diversity studies and provides perspective for analysing and exploiting sponge symbionts.

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Chapter 1:

Introduction

General Introduction

The oceans currently cover nearly 71% of the Earth's surface and are on average 4 km deep. Our fascination with the ocean could originate from the idea that life began in the ocean approximately 3.1 to 3.4 billion years ago, based on evidence from stromatolites (Lowe, 1980, Wacey*, et al.*, 2011). In contrast, land dwellers appeared relatively recent in geological time, approximately 400 million years ago (Ahlberg & Clack, 2006, Standen*, et al.*, 2014). It was hypothesized that early life forms may have originated from deep hydrothermal vents, first discovered in the late 1970's, where living communities exist thousands of meters beneath the surface of the sea (Ballard & Grassle, 1979). Among the oldest multicellular animals that have been around are Metazoa, which arose through clonal cell division to attain more complex multicellular lifestyles (Grosberg & Strathmann, 2007). The most primitive of the metazoans are marine sponges, arising at least as early as the Precambrian era (Li*, et al.*, 1998). Molecular clocks suggested that the divergence of sponges from the ancestors of other metazoans may have occurred even earlier, around 1.3 billion years ago (Blair Hedges*, et al.*, 2004). Sponges are simple multicellular, benthic invertebrates and invaded a variety of habitats, from freshwater such as streams, springs, lakes and ponds (Manconi & Pronzato, 2008) to marine environments ranging from coastal waters to deep sea (Beazley*, et al.*, 2013), and they accounted for much of the biomass on marine reefs during subsequent periods of the Paleozoic era (Wood, 1995, Hooper & Van Soest, 2002). Today, sponges remain important members of both shallow- and deep-water marine communities, occupying up to 90% of available surfaces in some areas (Becking*, et al.*, 2013, Loh & Pawlik, 2014). Their continuity of surviving with vast numbers in seas (and in freshwater habitats) is linked with apparent adaptability of their body plan to dramatic changes in environmental characteristics and their symbionts (Hentschel*, et al.*, 2012). To this end, sponges represent an enormous and dynamic reservoir of genetic variability that is the basis for evolution by natural selection. As such, they are one of nature's hot spots for microbial diversity. However, unlike many other established and studied host-microbe associations, in which only a very small number of participants are involved (*e.g.* squid-*Vibrio fischeri* (Nyholm & McFall-Ngai, 2004), amoeba-*Chlamydiae* (Horn & Wagner, 2004), attine ant-microbe (Little & Currie, 2007) and *Bugula-"Endobugula*" symbioses (Haygood & Davidson, 1998,

Lim & Haygood, 2004), it is clear that sponge-associated microbial communities can be highly diverse, with a range of different microorganisms consistently associated with the same host species (Sipkema*, et al.*, 2009). In this chapter, firstly the sponge host is introduced followed by description of diversity of microbial communities associated with sponges and cultivation of sponge-associated bacteria. Salient features outlining various terminologies in sponge microbiology, sponge symbiosis and emerging research directions are also discussed.

An overview of sponge biology and ecology

Sponges have been around since at least 650 million years ago. This was supported by discovery of possible sponge-grade metazoan fossils from Australia (Maloof*, et al.*, 2010). Sponges are organized in the phylum *Porifera* consisting of four taxonomic classes: the *Calcarea* (calcareous sponges), the *Hexactinellida* (glass sponges), *Homoscleromorpha* and the *Demospongiae*, of which the latter comprises the vast majority (appr. 83%) of sponges living today (Borchiellini*, et al.*, 2001, Hooper & Van Soest, 2002, Gazave*, et al.*, 2012, Van Soest*, et al.*, 2012). The four distinct classes are further divided into 25 orders, 128 families and 680 genera (Hooper & Van Soest, 2002, Van Soest*, et al.*, 2012). To date, approximately 11,000 sponge species have been formally described with various morphological and molecular methods, and approximately 8,500 species are considered valid. Due to new insights obtained from molecular systematic methods and new considerations of the morphological characteristics of sponges, twice as many species are thought to exist (Van Soest*, et al.*, 2012). On top of that, there are several hundred (219 accepted) freshwater sponge species (Manconi & Pronzato, 2008) and fossil sponges which comprise a similar additional diversity (Rigby & RL, 2003).

Sponges are benthic, sessile invertebrates that are morphologically diverse and come in a large variety of colours, shapes and structural complexities (Figure 1.1). Underwater inspection of any marine sponge benthic cover will reveal a colourful array of encrusting, branching, cup-shaped, and massive (amorphous) types (Taylor*, et al.*, 2007, Hentschel*, et al.*, 2012). They range in size from a few millimeters to more than a meter in diameter (Hill*, et al.*, 2009). Morphology of a typical sponge can also reflect its ecological

function. For instance many cyanobacterium-containing sponge species have flattened shapes for allowing optimal light reception for their photosynthetic symbionts (Wilkinson, 1983, Sara*, et al.*, 1998, Alex*, et al.*, 2012). Siliceous or calcareous spicules confer structural integrity upon most sponges, and these skeletal components are the basis for sponge biology and taxonomy (Simpson, 1984) as sponge taxa are defined by a wide range of spicule types. Collagenous tissues such as spongin also play a role in providing structural support and together with spicules allow the development of very large sponge individuals, such as those found among many tropical species(Hooper & Van Soest, 2002, Van Soest*, et al.*, 2012).
Unlike any other taxon, sponge architecture and sponge morphology significantly affect many aspects of sponge biology, including interactions with microorganisms. The basic sponge body plan comprises several different cell layers (Figure 1.1). The outer surface of a sponge is called pinacoderm, formed by epithelial cells known as pinacocytes. These cells extend along the interior canals, which permeate the sponge up to pores called ostia on the sponge surface. Inside the sponge, specialized flagellated cells called choanocytes form a series of chambers where feeding takes place. Collectively, the flagellated choanocytes are called choanoderm and these chambers pump water in through the ostia along the elaborate aquiferous systems within the sponge (Ruppert, 2004). Choanocytes also filter out food particles (including bacteria and microalgae) from the water, and these are transferred to the mesohyl, the gelatinous matrix within a sponge, which resembles an extensive layer of connective tissue occupying the space between the external pinacoderm and the internal choanoderm (Figure 1.1). An exception to this are the so-called carnivorous sponges in which the aquiferous system is non-existent but instead, due to their sticky outer surface they are able to capture small prey animals in the deep seas (Vacelet & Boury-Esnault, 1995). In the mesohyl, another group of sponge cells called archaeocytes digests food particles after phagocytosis. These archaeocytes are capable of differentiating into any of the other sponge cell types. In addition, there are dense communities of microorganisms present in the mesohyl of many sponges (Vacelet & Donadey, 1977, Wilkinson, 1978, Wilkinson, 1978, Wilkinson, 1978, Friedrich*, et al.*, 1999, Hoffmann*, et al.*, 2006, Croué*, et al.*, 2013). The presence of these symbionts alongside bacterium-digesting archaeocytes indicates either recognition of different microbial types by the sponge cells or shielding of symbiont cells to prevent consumption

(Wilkinson*, et al.*, 1981). After the water is filtered in the choanocyte chambers, it is eventually expelled from the sponge via the exhalant opening called osculum. Sponges are estimated to pump approximately 24,000 L of seawater per kg of sponge every day with their choanocytes (Vogel, 1977).

One of the most important aspects of sponge biology is sexual and asexual reproduction. Asexual reproduction by sponges could happen in different ways such as regeneration of viable adults from fragments, production of gemmules, budding and possibly formation of asexual larvae (Brusca & Brusca, 1990). Gemmules are produced by freshwater sponges such as *Spongilla* as well as some marine sponges such as *Ficulina*, *Suberites*, and *Tethya* for survival during unfavourable conditions and could germinate to produce new sponges (Manconi & Pronzato, 2007). For sexual reproduction, sponges either brood larvae within their body (vivipary) or release eggs, which are generally fertilized externally to develop outside the sponge (ovipary). One of the focus areas of sponge microbiology revolves around maintenance of the sponge symbionts based on asexual and sexual reproduction. The sponge host can select and maintain its microorganisms in either of the following two ways: (i) inheritance of microbial symbionts from the parent sponge via reproductive stages (*i.e.,* vertical transmission), or (ii) recruitment of microbes from the surrounding water by filter feeding (*i.e.*, horizontal or environmental transmission). The advantage of vertical transmission is a “symbiont assurance” meaning that the offspring receives symbionts identical to those of the parent (Vrijenhoek, 2010); in that all offspring will immediately host the symbionts required for growth. Evidence of vertical transmission based on asexual reproduction has been demonstrated based on budding (Hentschel*, et al.*, 2002), and a protruding bud from the surface of the marine sponge *Tethya orphei* was found to contain a symbiotic cyanobacterium (Gaino*, et al.*, 2006). Moreover, some species of freshwater sponges such as *Spongilla lacustris* and *Tubella pennsylvanica* are known to produce gemmules which possess symbiotic zoochlorellae (Simpson & Gilbert, 1973). As for sexual reproduction, bacteria have been detected in embryos or larvae from all four classes of *Porifera*, including sponge species with highly varied reproductive strategies (Kaye, 1991, Gaino & SarÀ, 1994, Uriz*, et al.*, 2001, Ereskovsky*, et al.*, 2005, Usher*, et al.*, 2005, Webster*, et al.*, 2010). In addition, it was shown that a combination of vertical and horizontal transmission is required to maintain the association between the sponge

Ectyoplasia ferox and its diverse sponge-specific microbes (Schmitt*, et al.*, 2008) This is similar to another study on Great Barrier Reef sponges where, using tag pyrosequencing of PCR-amplified 16S ribosomal RNA gene fragments, it was shown that sequences which are present in low relative abundance in the seawater are highly enriched in sponge hosts (and often larvae) (Webster*, et al.*, 2010). It was suggested that the Great Barrier Reef sponges may harvest these organisms from the seawater in addition to their vertical transmission via the larvae of symbionts (Webster*, et al.*, 2010). This is an area that might need more attention is sponge microbiology, although recruitment and maintenance of symbionts via horizontal transmission have been demonstrated convincingly for several marine symbioses such as in bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* (Nyholm*, et al.*, 2000, Nyholm & McFall-Ngai, 2004), as well as for hydrothermal vent tubeworm-chemoautotrophic bacterium symbioses (Nussbaumer*, et al.*, 2006). It is not yet known how sponges attract and select their associated bacteria.

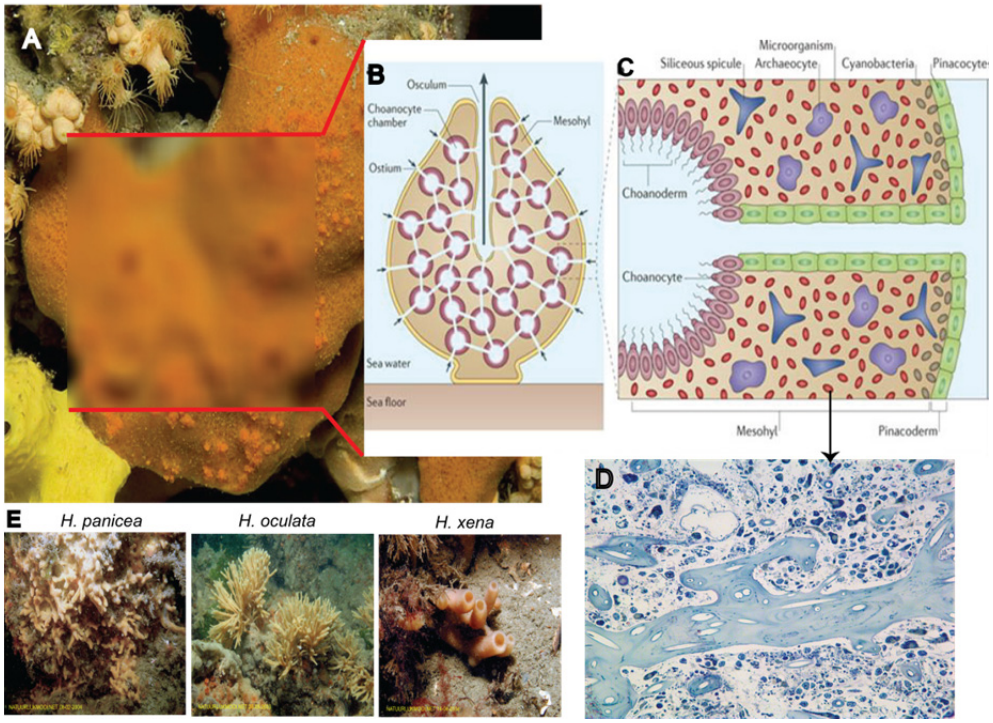

Figure 1.1. A - a typical underwater view of a sponge. **B** - schematic overview of a common demosponge. **C** - an enlargement of the internal structure of a typical demosponge. Please refer to the text for the description of the sponge cells and their function. **D** - Scanning Electron Microscopy (SEM) of *Axinella vaceleti* showing dense microorganisms inhabiting the sponge mesohyl. **E** - Underwater photograph of North Sea sponge species used in this thesis, from left to right, *Halichondria panicea*, *Haliclona oculata* and *Haliclona xena.* Images courtesy of J. Vacelet, Institut Méditerranéen de Biodiversité et d'Ecologie Marine et Continentale (IMBE), France (**D**); A. Frijsinger and M. Vestjens, http://www.natuurlijkmooi.net/ (**E**). Part **B** and **C** is adapted from (Hentschel*, et al.*, 2012).

Diversity of sponge-associated microorganisms

Sponges have been shown to be inhabited by a wide diversity of other invertebrates and a nurturing ground for many organisms such as shrimp, cnidarians, and other eukaryotes. As such, sponges are referred as 'veritable living hotels' and constitute, next to tropical coral reefs, one of the richest and most interesting biotopes (Klitgaard, 1995). Apart from the so-called macrofauna, sponges also possess their own microbial symbionts comprising archaea, bacteria, fungi and viruses (Webster & Taylor, 2012). In sponge microbiology, a recurring phenomenon is the high similarity among sponge-associated bacterial communities in which sponge-derived 16S rRNA gene sequences were shown to cluster together regardless of their host sponge and/or sampling location (Hentschel*, et al.*, 2002). Therefore, the term 'sponge-specific' was introduced for these clusters to describe a phylogenetically complex community that is repeatedly detected in sponges around the world but that is different from microbial seawater communities (Hentschel*, et al.*, 2002, Taylor*, et al.*, 2007). Recent evidence from pyrosequencing studies suggests that sponge-specific bacteria are also found in seawater, but only at very low abundance (Taylor*, et al.*, 2013). To date, at least 30 bacterial phyla (of which 18 are formally described and 12 candidate phyla known only from their 16S rRNA gene sequences) have been recorded to be associated with sponges (Hentschel*, et al.*, 2012, Webster*, et al.*, 2013). In addition, 2 archaeal phyla have also been described from sponge which are *Euryarchaeota* and *Thaumarchaeota* (Preston*, et al.*, 1996, Pape*, et al.*, 2006). This knowledge is derived from cultivation and/or conventional molecular approaches such as 16S rRNA gene library construction (Simister*, et al.*, 2012). Deep pyrosequencing of 16S rRNA genes have detected additional phyla, but since they are present at only very low relative abundance these taxa are considered to belong to the rare biosphere (Lee*, et al.*, 2011, Schmitt*, et al.*, 2012). Regardless of the detection technique used, the dominant sponge-associated bacterial phyla include *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Nitrospirae* and the candidate phylum *Poribacteria*. It is noteworthy to mention that this candidate phylum *Poribacteria* and the candidate phylum *Tectomicrobia* exist exclusively in marine sponges (Fieseler*, et al.*, 2004, Siegl*, et al.*, 2011, Simister*, et al.*, 2012, Wilson*, et al.*, 2014). Other phyla commonly encountered are *Bacteroidetes* and *Firmicutes*. However, sponge-derived bacteria belonging

to these phyla rarely belong to sponge-specific clusters (Schmitt*, et al.*, 2012). The importance of sponge-specific bacteria is that they are most likely to represent true symbionts of sponges, rather than food bacteria that were present at the time of sampling. It is noteworthy to highlight that the concept of sponge-specific clusters is unlike many other intimate and evolutionarily established host–symbiont interactions that are characterized by co-evolution and congruent phylogenies. Instead of 'one host, one symbiont' associations, sponges represent 'one host, many symbionts' associations, possibly due to the abundant exchange with seawater that occurs during filter feeding, and also the lack of microbial compartmentalization to specific cells or organs. Apart from Bacteria, Archaea (mostly of the phylum *Thaumarchaeota*) are often associated with sponges with some cases where they are present at high relative abundance, up to 60% of the sponge biomass (Preston*, et al.*, 1996, Pape*, et al.*, 2006, Radax*, et al.*, 2012, Jackson*, et al.*, 2013). In addition, eukaryotic microorganisms, which include fungi, diatoms and dinoflagellates are also present (Höller*, et al.*, 2000, Webster*, et al.*, 2004, Sipkema*, et al.*, 2009). The diversity of eukaryotes in sponges remains one of the final frontiers in sponge microbiology together with viruses of which virtually nothing is known.

In terms of bacterial diversity, it has been established that there are two groups of sponges with respect to their associated microbes. Firstly, there are sponges with high diversity of microbes in their mesohyl called High Microbial Abundance (HMA) sponges (Hentschel*, et al.*, 2003). Bacterial densities may reach 10^8 to 10^{10} bacterial cells per g (wet weight) in HMA sponges, which is generally two to four orders of magnitude higher than seawater concentrations (Hentschel*, et al.*, 2006, Schmitt*, et al.*, 2012). The second type includes sponges that harbour small populations of bacteria. These hosts are known as Low Microbial Abundance (LMA) sponges because they have bacterial densities that are up to fivefold lower than in HMA sponges with corresponding reductions in bacterial species richness (Gerçe*, et al.*, 2011, Giles*, et al.*, 2013). Sponge-specific microbes are mainly found in HMA sponges whereas LMA sponges usually harbour only one or two sponge-specific microbes (Hentschel*, et al.*, 2006, Kamke*, et al.*, 2010, Giles*, et al.*, 2013). HMA sponges and LMA sponges also have different chemical profiles, in which polyketide synthase (PKS) genes which are responsible for a large number of bioactive compounds have been detected only from HMA sponges (Hochmuth*, et al.*, 2010).

Since sponges are sessile organisms, they are reliant on production of chemicals as a form of defence against natural enemies and competitors (Pawlik, 2011). This form of defence is also true for other marine invertebrates such as corals, bryozoans and ascidians. Incentives to study sponges in particular came from the fact that they (or their symbionts) produce a wide variety of natural products (Mehbub*, et al.*, 2014). More novel bioactive metabolites are gained from sponges each year than from any other marine taxon with diverse pharmacological properties (Munro*, et al.*, 1999, Blunt*, et al.*, 2011). In addition, various ecological roles have also been proposed for these compounds such as defence against predators (Pawlik*, et al.*, 1995, Chanas*, et al.*, 1997, Becerro*, et al.*, 2003), competitors (Thacker*, et al.*, 1998, Turon*, et al.*, 2005, Enticknap*, et al.*, 2006), fouling organisms (Willemsen, 1994, Ganapiriya*, et al.*, 2012) and microbes (Becerro*, et al.*, 1994, Newbold*, et al.*, 1999, Thakur*, et al.*, 2003).

Sponge-associated fungi: An enigma of biodiversity

As succinctly put in the previous section, the association of microorganisms with sponges has been extensively reported, and microorganisms from all three domains of life namely Archaea, Bacteria and Eukaryotes have been identified (Vacelet & Donadey, 1977, Preston*, et al.*, 1996, Gao*, et al.*, 2008, Hoffmann*, et al.*, 2009, Wang*, et al.*, 2014). In a recent review Webster and co-workers (2012) outlined possible new research directions on sponge-associated microorganisms. This included research on sponge-associated archaea, viruses and fungi. In case of fungi for instance, little is known about their ecological function since most research until now focused on their bioactive molecules. So far, there is no morphological evidence that confirms the existence of fungal mycelia in sponges although cultivation-based approaches have demonstrated that fungi can easily be recovered from sponges (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006, Proksch*, et al.*, 2008, Wang*, et al.*, 2008, Ding*, et al.*, 2010, Liu*, et al.*, 2010, Paz*, et al.*, 2010, Passarini*, et al.*, 2012, Yu*, et al.*, 2012). Examination of the marine sponge *Axinella polypoides* using Transmission Electron Microscopy (TEM) showed filamentous structures commonly associated but not restricted to fungi (Figure 1.2). It has been previously suggested that this filamentous structure belongs to *Streptomyces axinellae,* an actinomycte that has been isolated from the marine sponge *Axinella polypoides* (Pimentel-Elardo*, et al.*, 2009), but there is no clear evidence

that *Streptomyces axinellae* is either localized in the sponge mesohyl or is the parasitic organism of axinellids and other demosponges. Furthermore, the β-glucan receptor, found in the marine sponge *Suberites domuncula*, suggests that sponge can actually recognize fungi which could be important in selection of symbionts (Perović-Ottstadt*, et al.*, 2004). The presence of endosymbiotic yeasts that are maternally transmitted from the marine sponge *Chondrilla* sp. has also been documented (Maldonado*, et al.*, 2005), highlighting the importance of fungi in sponges.

Isolation and cultivation of sponge-associated microorganisms

Many bacteria, archaea and fungi have been isolated from sponges but they do not represent the total phylogenetic diversity (Webster*, et al.*, 2004, Liu*, et al.*, 2010, Schippers*, et al.*, 2012, Esteves*, et al.*, 2013). For bacteria, cultivability values from only 0.1% to 11% have been reported despite the best effort from various research groups (Santavy*, et al.*, 1990, Friedrich*, et al.*, 2001, Webster & Hill, 2001, Olson & McCarthy, 2005). This is probably due to the fact that conventional strategies for cultivation including the use of nutrient-rich medium favours the growth of fast-growing microbes at the expense of slow-growing ones (Vartoukian*, et al.*, 2010). Diversification of media such as using a range of rich and poor formulations, and the use of innovative techniques such as floating filter culture could increase recovery by cultivation up to 14.5% of the bacterial species present in a sponge (Sipkema*, et al.*, 2011).

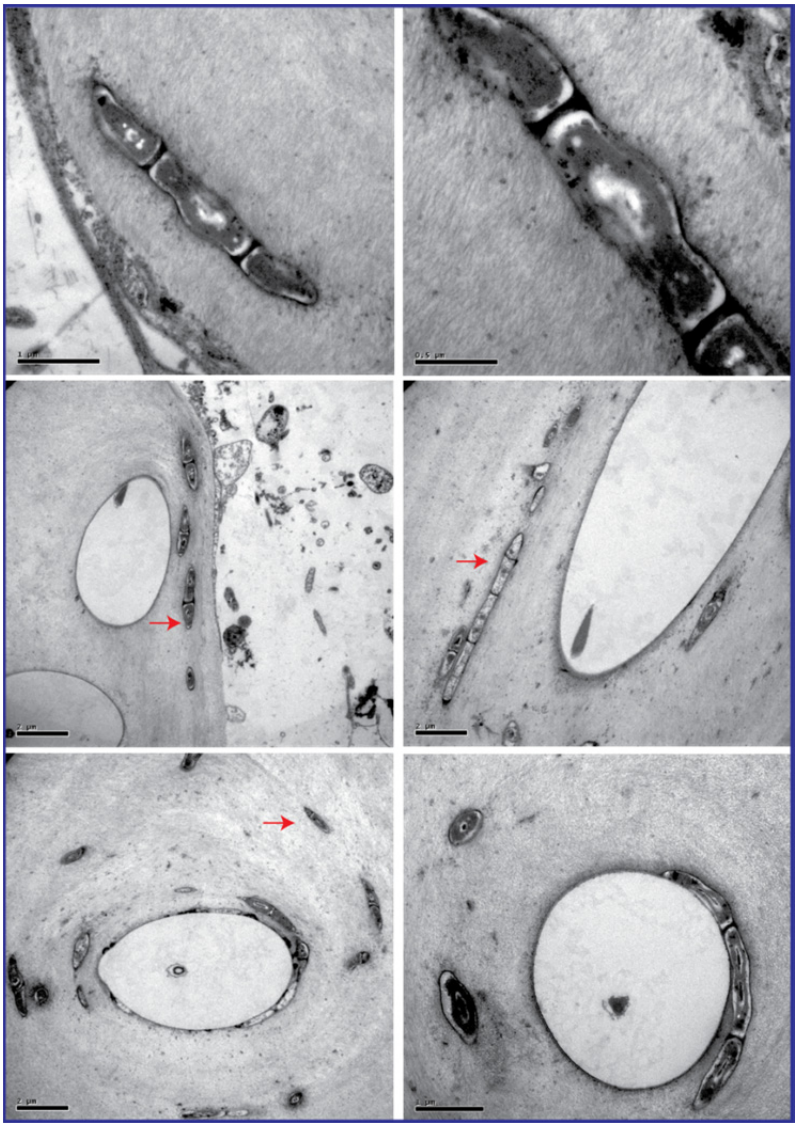

Figure 1.2. Transmission Electron Microscopy of marine sponge *Axinella polypoides*. The sponge spicules were reduced to their axial filament by desilicification before embedding with epoxy-resin material for electron microscopy. Images courtesy of N. Boury-Esnault, Institut Méditerranéen de Biodiversité et d'Ecologie Marine et Continentale (IMBE), France.

For growing sponge-specific microorganisms, one has to supply nutrients and provide an environment as close as possible to the natural environment in sponges. This is particularly important for strains residing in the mesohyl of marine sponges, where the microenvironment may differ from the macroenvironment (seawater) that is used in most cultivation experiments. For example, anoxic conditions frequently occur when the sponge temporarily ceases to pump water inside the sponge mesohyl (Hoffmann*, et al.*, 2005, Hoffmann*, et al.*, 2008). In addition, because of the presence of siderophores, iron may be available at higher concentrations in the mesohyl than in the surrounding seawater (Guan*, et al.*, 2000). Furthermore, it was shown that sponges host cyanobacteria which produce numerous bioactive compounds and require light for growth (Faulkner*, et al.*, 2000). Lectins, which are one of the core elements of the mesohyl could be important for the cultivation of obligate sponge-specific symbionts (Muller*, et al.*, 1981). Considering that bacteria from sponges (and other environments) have adapted to oligotrophic conditions (Connon & Giovannoni, 2002) and that the doubling time of many microorganisms in nature is in the order of 100 days (Whitman*, et al.*, 1998), which is much longer than most reported cultivation experiments, patience may prove to be of utmost importance (Sipkema*, et al.*, 2011).

Based on the work by Schippers and co-workers (2012), it has been demonstrated that bacteria repeatedly isolated from sponges are present in low abundance in the tissue, but generally they are not the dominant bacteria. Extensive phylogenetic analysis based on 16S rRNA gene sequences placed such groups of bacteria frequently isolated from sponges into what they term sponge cultivation clusters (SCC) (Schippers*, et al.*, 2012).

A motivation to grow sponge-associated microorganisms is derived from the fact that many of these microorganisms can produce chemically diverse secondary metabolites potentially relevant for biotechnological and medical purposes. For instance, antitumor compounds such as pederin, mycalamide A, and onnamide A were discovered to be produced by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei* (Piel*, et al.*, 2005). Moreover, the majority of natural products studied in the marine environment came from fungi associated with sponges (Bugni & Ireland, 2004). These sponge-associated fungi produce novel metabolites with potent antibacterial and anticancer activities (Jensen & Fenical, 2002), which have not been previously reported from terrestrial strains of the same

species (Hiort, *et al.*, 2004). The first novel metabolite discovered from sponge-associated fungi was Trichoharzin, a polyketide-derived fungicide isolated from *Trichoderma harzianum* associated with the sponge *Mycale cecilia* (Kobayashi, *et al.*, 1993). *Trichoderma harzianum* is a widespread soil *Ascomycotina* known for its production of compounds with antifungal activity against other microscopic fungi, but only the marine-derived strain produced Trichoharzin when tested simultaneously (Verbist, *et al.*, 2000). In addition, gymnastatins A, B and C were the first novel cytotoxic metabolites from sponge-associated fungi (Amagata, *et al.*, 1998). These studies highlighted the potential for discovery of novel natural products from sponge-associated bacteria and fungi.

From genes to genomes: Molecular toolbox to study sponge-associated microorganisms and function

Culture-independent analyses, mostly based on 16S rRNA genes, such as denaturing gradient gel electrophoresis (DGGE), clone libraries and terminal restriction fragment length polymorphism (T-RFLP) have provided resourceful insight into the sponge-associated microorganisms (Taylor, *et al.*, 2004, Kennedy, *et al.*, 2008, Anderson, *et al.*, 2010, Sipkema & Blanch, 2010, Yang & Li, 2012). Accompanied by imaging techniques such as fluorescence *in situ* hybridization (FISH) and microscopy techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), it was consistently shown that symbionts of sponges mostly reside in the mesohyl of the sponge body (Vacelet & Donadey, 1977, Wilkinson, 1978, Wilkinson, 1978, Wilkinson, 1978, Friedrich, *et al.*, 1999, Hoffmann, *et al.*, 2006, Croué, *et al.*, 2013). More recently, high-throughput culture-independent approaches such as metagenomic analysis, deep sequencing (pyrosequencing, Illumina MiSeq sequencing) and single cell genomic analysis have provided a deeper understanding of sponge-associated microorganisms. The first metagenomic analysis of sponge microbiota focused on mechanisms of bacterium–sponge interactions and highlighted ANK-containing proteins as putative symbiosis factors (Thomas, *et al.*, 2010). Similarly, the first study to apply single-cell analysis for investigating the genome of uncultivated bacterial symbionts of sponges was done for a bacterium belonging to the candidate phylum *Poribacteria* (Siegl, *et al.*, 2011). Siegl and co-workers (2011) identified several putative symbiosis factors such as adhesins and eukaryotic

domain-containing proteins (*i.e*. ankyrin repeats), which may be involved in mediating microbe–sponge interactions. Furthermore, metagenomic analysis of dominant sponge symbionts has provided insights into function and metabolic potential of sponge-associated bacteria and archaea (Hallam*, et al.*, 2006, Liu*, et al.*, 2011). In addition, genome reconstruction from metagenome sequencing provides invaluable information on the genetic potential of uncultivated bacteria. For example, the genome of the uncultured *Entotheonella* symbiont from the marine sponge *Theonella swinhoei* was found to harbour genes encoding for the potent anticancer agent polytheonamide (Freeman*, et al.*, 2012, Wilson*, et al.*, 2014). With the use of single cell genomics, it was revealed that *Entotheonella* has a large number of novel biosynthetic gene clusters, expanded genome size in comparison to *Streptomyces,* multiple modular assembly lines and high metabolic variability among closely related organisms (Wilson*, et al.*, 2014). Similarly, genome sequencing from *Pseudovibrio* isolates from the sponge *Mycale laxissima* and a diseased coral, revealed novel biosynthetic gene clusters and metabolic abilities involved in host-symbiont interactions (Bondarev*, et al.*, 2013).

Research aims and thesis outline

The picture of microbial diversity in sponges is far from complete. As such, using a combination of culture-dependent and culture-independent approaches, this thesis aims to pursue the discovery of the full diversity of bacteria and fungi associated with sponges and predict their ecological role in the sponge host. In addition, assessment of bacterial and fungal assemblages using a cultivation-based approach will provide novel insights on how to culture these organisms and allow direct access to their genomes for screening of their genetic potential to produce bioactive compounds.

Chapter 2 introduces the biodiversity of sponge-associated bacteria and archaea through deep-sequencing the microbial communities of three Dutch sponge species. In this chapter it is demonstrated how sponges living together in the same environment have their own specific bacterial community. Furthermore, for the first time, the presence of environmental *Chlamydiae* and their ecological significance in sponges is discussed.

The ecological importance of fungi inside sponges has been a long-standing debate due to the limit of detection and the low number of studies

on this topic. **Chapter 3** seeks to gain insight into community structure of fungi in sponges by using an 18S rRNA gene amplicon pyrosequencing approach. Novel assemblages of fungi were detected and provide new perspectives on a phylum that has been proven difficult to be cultured, such as *Basidiomycota.*

Prime interest to grow and isolate fungi from sponges arose from their capacity to produce diverse compounds which are interesting for pharmaceutical applications. In **Chapter 4** sponge-derived fungi are screened using a PCR-based approach by targeting the polyketide synthase (PKS) and PKS-NRPS hybrid genes to evaluate their genetic potential of producing such bioactive compounds. It is shown that sponge-derived fungi, in particular *Penicillium* spp. possess genetically diverse PKS genes and that a substantial genetic diversity among *Penicillium* strains is found.

Chapter 5 describes a comprehensive assessment of cultivable bacterial assemblages from the marine sponge *Halichondria panicea* using different media and cultivation conditions to increase the efficiency. This effort is combined with molecular analysis of the sponge microbiome using the pyrosequencing of 16S rRNA gene amplicons to assess the uncultured majority. The comparison between culture-dependent and culture independent approaches shows that the cultivation efficiency is about 3.8.%.

Bacteria from the genus *Pseudovibrio* are frequently isolated from sponges and are known to be metabolically versatile. In **Chapter 6** genotypic and phenotypic characteristics of *Pseudovibrio* spp. are analysed to understand the strain diversity and aid in selection for genome sequencing, in which the strains will be compared at genomic level. Strains of *Pseudovibrio* spp. showed different antibiotic resistance profiles and genomic fingerprints when analysed with plate assay and for BOX elements, respectively.

Chapter 7 concludes this thesis with a summary and discussion of the results that are presented in a way that it can contribute to the state-of-the-art knowledge. It also discusses and proposes new research directions in order to understand the ecology of microbial (and eukaryotic) diversity in sponges.

Chapter 2:

Host-specific microbial communities in three sympatric North Sea sponges

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Abstract:

The establishment of next generation technology sequencing has deepened our knowledge of marine sponge-associated microbiota with the identification of at least 32 phyla of bacteria and archaea from a large number of sponge species. In this study we assessed the diversity of the microbial communities hosted by three sympatric sponges living in a semi-enclosed North-Sea environment using pyrosequencing of bacterial and archaeal 16S ribosomal RNA gene fragments. The three sponges harbour species-specific communities each dominated by a different class of *Proteobacteria*. An α-proteobacterial *Rhodobacter*-like phylotype was confirmed as the predominant symbiont of *Halichondria panicea.* The microbial communities of *Haliclona xena* and *Haliclona oculata* are described for the first time in this study and are dominated by *Gammaproteobacteria* and *Betaproteobacteria*, respectively. Several common phylotypes belonging to *Chlamydiae*, TM6, *Actinobacteria* and *Betaproteobacteria* were detected in all sponge samples. A number of phylotypes of the phylum *Chlamydiae* were present at an unprecedentedly high relative abundance of up to 14.4% ± 1.4% of the total reads, which suggests an important ecological role in North Sea sponges. These *Chlamydiae*-affiliated OTUs may represent novel lineages at least at the genus level as they are only 86-92% similar to known sequences.

Keywords: sponge microbiota, pyrosequencing, host-specific, microbial diversity, *Chlamydiae, Proteobacteria*

Introduction

Sponges (phylum Porifera) are ancient sessile invertebrates that have been around for approximately 710 million years (Maloof*, et al.*, 2010). The sponge holobiont is a complex ecosystem comprising microbial representatives from three different domains namely *Eukarya, Bacteria* and *Archaea*, as well as viruses. To date, there are 32 different phyla and candidate phyla of *Archaea* and *Bacteria* that have been described in sponges (Hentschel*, et al.*, 2012, Schmitt*, et al.*, 2012, Simister*, et al.*, 2012). Based on a microscopic study of microbial communities in sponges (Vacelet & Donadey, 1977), sponges can be divided into two classes with respect to their microbial carriage, namely those with high microbial abundance (HMA sponges) and low microbial abundance (LMA sponges) (Hentschel*, et al.*, 2006). A number of studies investigating sponge–microbe associations have revealed that sponges share phylogenetically related and complex microbial communities that differ from benthic and planktonic microbial communities (Vacelet & Donadey, 1977, Taylor*, et al.*, 2007, Webster*, et al.*, 2010, Simister*, et al.*, 2012). Deep-sequencing studies have now revealed that bacteria that were previously thought to be only present in sponges are also found in seawater, albeit at a very low abundance (Taylor*, et al.*, 2013). In addition to a sponge-enriched bacterial community (bacteria that are present at high abundance in sponges only), it has been demonstrated that sponges also harbour a sponge species-enriched bacterial community (bacteria that are present at high abundance in one sponge species only) that is obtained via vertical transmission in many cases (Schmitt*, et al.*, 2008, Webster*, et al.*, 2010). The knowledge on sponge-bacterial symbioses gained over the past decade has helped us to understand their ecological and evolutionary implications and sparked new research in this field (Webster & Taylor, 2012). Nevertheless, most sponge–associated bacteria studies to date focussed on tropical and sub-tropical sponge species, and thus, sponge-associated bacterial diversity from cold water regions (Hoffmann*, et al.*, 2005, Wichels*, et al.*, 2006, Cardoso*, et al.*, 2013, Schöttner*, et al.*, 2013) such as the North Sea is understudied. To this end, the purpose of this study was to assess the diversity of the microbial communities in three sympatric sponges living in the semi-enclosed environment of the Eastern Scheldt (The Netherlands) estuary, which is separated from the North Sea by a storm surge barrier. To

address questions related to host-specificity and to identify intra- and inter-species commonalities and variation, we sampled triplicate individuals of three different sponge species, namely *Halichondria panicea, Haliclona xena* and *Haliclona oculata*, the latter two of which have not been previously studied with respect to their microbiota.

Materials and Methods

Sampling

Sponge samples were collected by SCUBA diving in December 2008 in the Oosterschelde, at the dive site Lokkersnol (N51°38'58.07", E3°53'5.11"). The Oosterschelde is an estuary located in Zeeland, between Schouwen-Duiveland and Tholen in the north and Noord- and Zuid-Beveland in the south. The Oosterschelde (The Netherlands) estuary has a storm surge barrier built at its seaward entrance during 1979-1986. Since then, it has also been disconnected from river inputs by construction of two large dams at the rear end of the system, and the freshwater inputs from the Scheldt, Rhine and Meuse rivers are diverted into the Westerschelde (Nienhuis & Smaal, 1994). Consequently the Oosterschelde was changed from a turbid estuary into a tidal bay.

Triplicate samples from *Halichondria panicea* (order: *Halichondrida*), *Haliclona xena* (order: *Haplosclerida*) and *Haliclona oculata* (order: *Haplosclerida*) were collected and stored in separate containers for each specimen in seawater at ambient temperature. Initial identification of sponges was done by Prof. Rene Wijffels and Dr. Klaske Schippers (Bioprocess Engineering Group, Wageningen University), and further confirmation of sponge identity was done by 18S rRNA gene sequencing. Habitat seawater (at the surface at the same location) was also collected in sterile container to compare the sponge-associated bacteria to bacterioplankton. Upon arrival at the laboratory (two hours after sampling) the sponges were washed three times with sterile artificial seawater (SASW) and were kept at -80 °C until further processing.

Table 2.1 Primers used in this study.

Primer	Sequence (5′-3′)	Target	Annealing temperature	Amplicon length (bp)	Use	Reference
MPRK341f	cct ayg ggr bgc asc ag	Bacteria and archaea 16S rRNA gene	56	466	pyrosequencing	Yu et al., (2005)
MPRK806r	gga cta cnn ggg tat cta at	Bacteria and archaea 16S rRNA gene	56		pyrosequencing	Yu et al., (2005)
1369F	cgg tga ata cgt tcy cgg	Bacteria 16S rRNA gene	52	123	qPCR	Suzuki et al., (2000)
1492R	ggw tac ctt gtt acg act	Bacteria 16S rRNA gene	52		qPCR	Suzuki et al., (2000)
A349F	gyg cas cag kcg mga aw	Archaea 16S rRNA gene	59	457	qPCR	Takai & Horikoshii (2000)
A806R	gga cta cvs ggg tat cta at	Archaea 16S rRNA gene	59		qPCR	Takai & Horikoshii (2000)
EUKF	aac ctg gtt gat cct gcc agt	Eukaryotes 18S rRNA gene	55	1450-1500	clone libraries	Medlin et al., (1988)
EUKR	tga tcc ttc tgc agg ttc acc tac	Eukaryotes 18S rRNA gene	55		clone libraries	Medlin et al., (1988)

DNA extraction, PCR amplification of 16S rRNA genes and bar-coded pyrosequencing

Total genomic DNA was extracted from sponge tissues (150-200 mg) using the DNAeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol for animal tissue. The sponge tissue was cut in a way so that tissue from both mesohyl and ectoderm was included. For the isolation of DNA from bacterioplankton, ten litres of seawater was pre-filtered using a 5 µm polycarbonate filter (GE Osmonics, Minnetonka, MN, USA), and subsequently filtered with a 0.22 µm polycarbonate filter (GE Osmonics) before DNA extraction. Total genomic DNA was extracted from the 0.22 µm filter using the FastDNA® Spin Kit for Soil (Q-Biogene, Carlsbad, CA). The concentration of extracted DNA from sponge tissue and seawater was determined with a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), whereas its integrity was visually examined by gel electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide. The extracted DNA (dissolved in TE buffer) was stored at -20°C until further analysis.

Bar-coded amplicon pyrosequencing was conducted for three individuals of each *Halichondria panicea* (P1-P3), *Haliclona xena* (X1-X3), and *Haliclona oculata* (O1-O3), and one seawater (W) sample. In this study, we used a two-step PCR protocol as previously described (Sutton*, et al.*, 2013). The universal prokaryote primers PRK341F and PRK806R were used to amplify an approximately 466 bp fragment of the 16S rRNA gene comprising the V3 and V4 regions (Table 2.1, Table S2.1 for barcodes used) (Yu*, et al.*, 2005). PCR amplification was performed in a volume of 40 µl containing 1× Phusion HF buffer, 2.5 mM magnesium chloride, 0.2 mM dNTP mixture, 0.8 U Phusion Hot Start II High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 0.5 µM of each primer and 20 ng template DNA. PCR was performed using the following conditions: an initial denaturation at 98°C for 30s, followed by 30 cycles of denaturation at 98°C for 5s, annealing at 56°C for 20s, elongation at 72°C for 20s, and a final elongation at 72°C for 5 min. After PCR amplification, the samples were held at 60°C for 3 min and then placed on ice before they were checked on a 1.25% (w/v) agarose gel. PCR-products were purified using the Millipore DNA Gel Extraction Kit (Millipore, Billerica, MA, USA). Subsequently, a second round of PCR was performed as described above, except that a pyrosequencing adapter and

ten different barcodes of 10 nucleotides length were introduced with the forward primer (Table 2.1, Table S2.1). Furthermore, the number of cycles of denaturation, annealing and elongation was reduced to fifteen. The PCR products were analyzed on 1% (w/v) agarose gel, and DNA was extracted from the agarose gel as described above, quantified using the Quant-iTdsDNA high-sensitivity assay kit and the Qubit fluorometer (Invitrogen, Grand Island, NY), and mixed in approximately equal concentrations ($4x10^6$ copies μl^{-1}) to ensure equal representation of each sample. These samples were pooled with other samples (total of 72 samples) and were sequenced on one of the two regions of a 70 x 75 GS PicoTiterPlate (PTP) by using the Titanium kit and GS FLX system according to the manufacturer's instructions (Roche, Branford, CT) at the Technical University of Copenhagen. Pyrosequencing data were deposited at the European Bioinformatics Institute under ERS 179001 – ERS179010 (Table S2.1).

Sequence processing, taxonomic assignment, and analysis of diversity and phylogeny

Pyrosequencing data were analyzed as previously reported (Sutton*, et al.*, 2013), using the QIIME 1.3.0 pipeline (http://qiime.sourceforge.net/) (Caporaso*, et al.*, 2010a). Firstly, retained pyrosequencing reads had to comply with the following default quality parameters: a perfect match to the sequence tag and the 16S rRNA gene primer, be at least 200 bp in length, have no ambiguous bases and have no homopolymer stretches longer than 6 nucleotides. Once trimmed and assigned to samples, sequences were processed using the QIIME´s UCLUST method in order to cluster the sequences in operational taxonomic units (OTUs) at the 97% identity level. The most abundant sequence of each OTU was selected as a representative sequence and subsequently aligned using PyNAST (DeSantis*, et al.*, 2006b) against the Greengenes core set (DeSantis*, et al.*, 2006a) version 4Feb2011. Possible chimeric sequences were identified using QIIME's ChimeraSlayer and subtracted from the previously generated OTU list, generating a non-chimeric non-redundant OTU list. The taxonomic affiliation was assigned to each OTU using the Ribosomal Data Project (RDP) Classifier at a confidence threshold of 80% (Wang*, et al.*, 2007).

Rarefaction curves and Good's coverage index (Good, 1953) were calculated by using the QIIME script alpha_rarefaction.py. In order to

compute Beta diversity of the samples, the number of reads of all samples was first normalized to the sample with the lowest number of reads (sample X1, 4922 reads) by using the QIIME script single_rarefaction_py. The normalized OTU table was then imported in the software package PRIMER 6 v6.1.13 (PRIMER-E Ltd) for cluster similarity, NMDS and ANOSIM analysis. Similarity was calculated using the Bray-Curtis index, and cluster analysis was conducted with complete linkage. OTU abundance heat maps were built in Excel 2007 (Microsoft Office). A list of OTUs found in all nine sponge samples) was obtained using the QIIME script compute_core_microbiome.py.

All OTUs representing at least 0.25% of the reads on average in at least one of the three sponge species and water sample were considered for a more thorough phylogenetic analysis. Representative sequences of the OTUs were aligned using the SILVA online SINA alignment service (Pruesse*, et al.*, 2007). Each OTU was complemented with the two most closely related 16S rRNA gene sequences as determined by a BLAST search against the NCBI nucleotide database. More neighbours were included if the two nearest neighbours were both sponge or coral-derived until a neighbour of non-sponge/coral origin was reached. Neighbour sequences were downloaded from SILVA and together with the aligned OTUs imported in the ARB software package (Ludwig*, et al.*, 2004). Neighbour sequences > 800 bp were used to construct a Bayesian phylogenetic tree. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default program parameters were used, except allowing a minimum block length of 5 and gaps in 50% of positions. Phylogenetic trees were created by Bayesian analysis, using MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2001) at the Bioportal server (http://www.bioportal.uio.no). All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run, and their values were estimated from the data during the analysis. All Bayesian analyses were initiated with random starting trees and were run for 10^7 generations. The number of chains was set to four and Markov chains were sampled every 1000 iterations. Points prior to convergence were determined graphically and discarded. Calculated trees were imported into ARB, and short sequences obtained in this study were subsequently added by use of the ARB parsimony method without changing the tree topologies.

Quantitative PCR (qPCR)

Relative abundance of total bacterial and archaeal 16S rRNA genes was determined by qPCR amplification, using the BioRad CFX 384™ Real Time System (Bio-Rad Laboratories BV, Veenendaal, NL) with iQ SYBR® Green Supermix (Bio-Rad Laboratories BV). Reaction mixtures of 10 µL contained 5 µl iQ SYBR® Green Supermix, 250 nM of each primer for either bacterial 1369F/1492R (Suzuki*, et al.*, 2000) or archaeal 349F/806R (Takai & Horikoshi, 2000) assays (Table 2.1). A bacterial qPCR standard was generated by amplifying and cloning a *Rhodobacter*-like 16S rRNA gene (NCBI accession number:KJ453525) retrieved from *H. panicea*, using primers 27F/1492R (Table 2.1). An archaeal qPCR standard was generated by amplifying and cloning the 16S rRNA gene of *Methanosarcina mazei* using primers 21F/1492R (Table 2.1). For all runs, standard curves were generated by amplification of a serial dilution of the standard plasmid DNA template (in triplicate) with concentrations ranging from 10 ng/µl to 10^{-10} ng/µl. Amplification efficiencies for all reactions ranged from 91.5% to 100.5% with an r^2 value of 0.99 for standard curve regression. For the bacterial qPCR assay, the following cycling conditions were used: 95°C for 5 min, followed by 40 cycles of denaturation at 94.2°C for 30s, annealing at 53.8°C for 40s and extension and fluorescence reading at 72°C for 90s, followed by 72°C for 10 min. For the archaeal qPCR assay, the cycling conditions were the same except that the annealing temperature was 59°C. To test the specificity of the qPCR, after each run melting curve analysis was performed between 65 and 95°C with a heating increment rate of 0.5°C per 5s and a continuous fluorescence measurement to determine the presence of specific products. The PCR products were also subjected to standard agarose gel electrophoresis to verify that only amplicons of the expected size had been produced.

Molecular identification of sponge specimen

For taxonomic identification of the sponge specimen, partial 18S rRNA gene fragments from the sponges were amplified with PCR using the primer sets EukF and EukR (Medlin*, et al.*, 1988) (Table 2.1). Sequences were deposited under GenBank accession numbers KC899022 – KC899050.

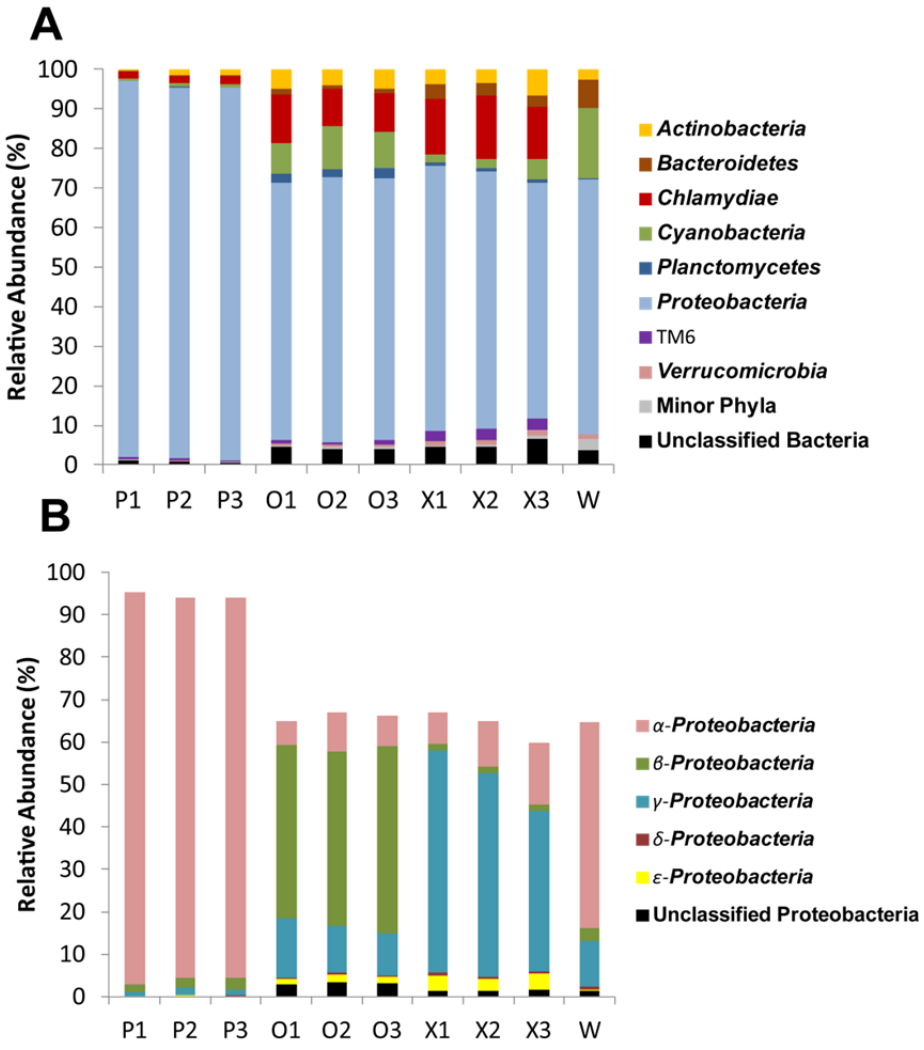

Figure 2.1. A Relative abundance of the dominant microbial phyla in sponges and sea water samples. Phyla with an average relative abundance lower than 1% in all sponge samples were grouped in the category "Minor Phyla". Sample codes indicate *H. panicea* (P1, P2, P3), *H. oculata* (O1, O2, O3), *H. xena* (X1, X2, X3) and seawater (W). **2.1.B** Relative abundance of proteobacterial classes.

Results

Composition of the microbial communities

Partial 18S rRNA gene sequencing data confirmed the taxonomy of the sponge hosts (Table S2.2). The microbial communities of triplicate specimens of the three North Sea sponges *H. oculata*, *H. xena* and *H. panicea* and of a seawater bacterioplankton sample were profiled for using 454 tag-pyrosequencing of PCR-amplified bacterial and archaeal 16S rRNA gene fragments. Trimming and quality filtering of the raw reads generated 84,625 high quality reads with an average length of 364.26 ± 67.5 bp. These sequences were clustered into 8239 operational taxonomic units (OTUs) at a similarity threshold of 97%. Removal of chimeric sequences reduced the number of OTUs to 6119. The minimum number of non-chimeric reads obtained for a sample was 4922 and the maximum was 10,583, with an average of 7969.6 ± 1922.7 reads. Rarefaction curves showed near saturation in *H. panicea*, and some levelling off in the two congeneric sponges *H. xena* and *H. oculata* (Figure S2.1). Good´s coverage indexes obtained for the three sponge species were 97.2% ± 0.2%, 86.1% ± 0.3% and 88.0 ± 0.1% for *H. panicea*, *H. xena* and *H. oculata* respectively, and 82.3% for the water sample. Representative sequences of each OTU were classified into the domains *Bacteria* (99.6% of the total data set) and *Archaea* (0.3%). A small proportion of sequences (0.1%) could not be classified to the domain level.

In total, 21 bacterial and archaeal phyla, including several candidate phyla, were detected in the data set. At phylum level (Figure 2.1.A), *Proteobacteria* dominated the community composition in all sponges. On average, this phylum contributed to 94.4% ± 0.7%, 65.9% ± 1.0% and 63.9% ± 3.7% of the total community in *H. panicea*, *H. oculata* and *H. xena*, respectively. A remarkable contribution was found for the phylum *Chlamydiae,* which was the second most abundant microbial phylum in the three sponges with relative abundances of 1.9% ± 0.02%, 10.4% ± 1.5% and 14.4% ± 1.4% in *H. panicea*, *H. oculata* and *H. xena*, respectively.

Other major phyla (defined as phyla with >1% of average relative abundance in any of the three sponge species) were *Cyanobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, Verrucomicrobia* and the candidate bacterial phylum TM6. Together these eight phyla represented on average >99% of the microbial community in the three sponge species

(in terms of relative abundance). The rest of the sponge microbial communities were composed by several phyla including *Firmicutes, Chloroflexi, Acidobacteria,* candidate bacterial phyla TM7 and OD1 and *Euryarchaeota* all detected at very low relative abundances (<0.3% in any sponge sample, Table S2.3). Although at the phylum level the three sponge species seemed to host microbial communities with a similar taxonomic composition, a species-specific pattern emerged after analysis at the class level. This was especially obvious for the phylogenetic affiliation of OTUs belonging to classes of *Proteobacteria*. Whereas *α-Proteobacteria* dominated *H. panicea* communities, *β-Proteobacteria* and *γ-Proteobacteria* were the two most abundant proteobacterial classes in *H. oculata* and *H. xena*, respectively (Figure 2.1.B).

OTU-level analysis of microbial community differences between the three sponge species

Statistical analysis of the microbial communities at the OTU level (Figure S2.2) confirmed that the three sponge species studied here hosted different microbial communities (ANOSIM, R=1, p<0.001). The predominant sponge microbiota, defined here as the set of OTUs that had relative abundances of at least 0.25% in at least one of the three sponge species, was examined in more detail (Figure 2.2A). Only 62 OTUs fulfilled this criterion, and their cumulative read abundances were 94.1% ± 0.68%, 72.7% ± 2.0% and 65.4% ± 1.5% in *H. panicea*, *H. xena* and *H. oculata*, respectively. The heat map derived from the relative abundance at the OTU level revealed a complex and species-specific pattern. Cluster analysis based on pairwise Bray-Curtis distances also indicated high intraspecific homogeneity (Figure 2.2.A). In addition, the two *Haliclona* species, which hosted distinct microbial communities, were more related to each other than to the *H. panicea* associated communities. The seawater microbial community clustered separately from all sponge communities. Notably, each sponge microbiome was clearly dominated by one highly abundant phylotype: OTU 2229 in *H. panicea* (80.5% ± 1.47% of the reads), OTU 7091 in *H. oculata* (37.2% ± 1.9%) and OTU 171 in *H. xena* (28.9% ± 5.4%). These three most abundant phylotypes were affiliated to *α-*, *β-* and *γ-Proteobacteria*, respectively, according to the RDP classifier and a Bayesian phylogram that included all the predominant OTUs (Figure 2.3.A, see also Figure S2.3). The remainder of predominant OTUs was taxonomically distributed

among several microbial phyla. Most of the 62 predominant OTUs were not detected or only at very low abundance in the seawater sample, with the exception of OTU 4050 (*α-Proteobacteria*) that was very abundant in the seawater (13.1%).

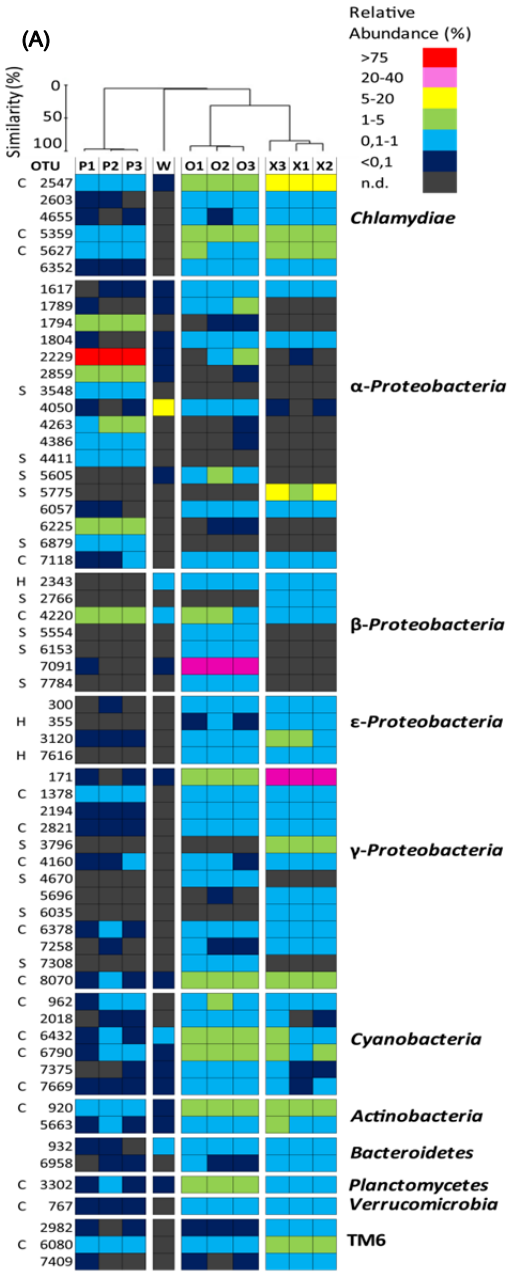

Figure 2.2.A Heat map and cluster analysis of the dominant bacterial OTUs at 97% similarities in three different North Sea sponges and seawater. The relative abundance of the most abundant OTUs (>0.25% of the total reads) in the different samples: *H. panicea* (P1, P2, P3*), H. oculata* (O1, O2, O3), *H. xena* (X1, X2, X3) and seawater (W) is shown. A range of colors was used to represent the relative abundance of the different OTUs (see legend). Samples were grouped using hierarchical clustering (complete linkage) based on the Bray-Curtis distance matrix calculated from the relative abundances of these OTUs. Capital letters in the left column indicate whether OTUs belong to the sponge common-OTUs group (C), are species-enriched (S), or are only found in the genus *Haliclona* (H). OTUs were grouped by their taxonomic classification at phylum level (class level in the case of *Proteobacteria*).

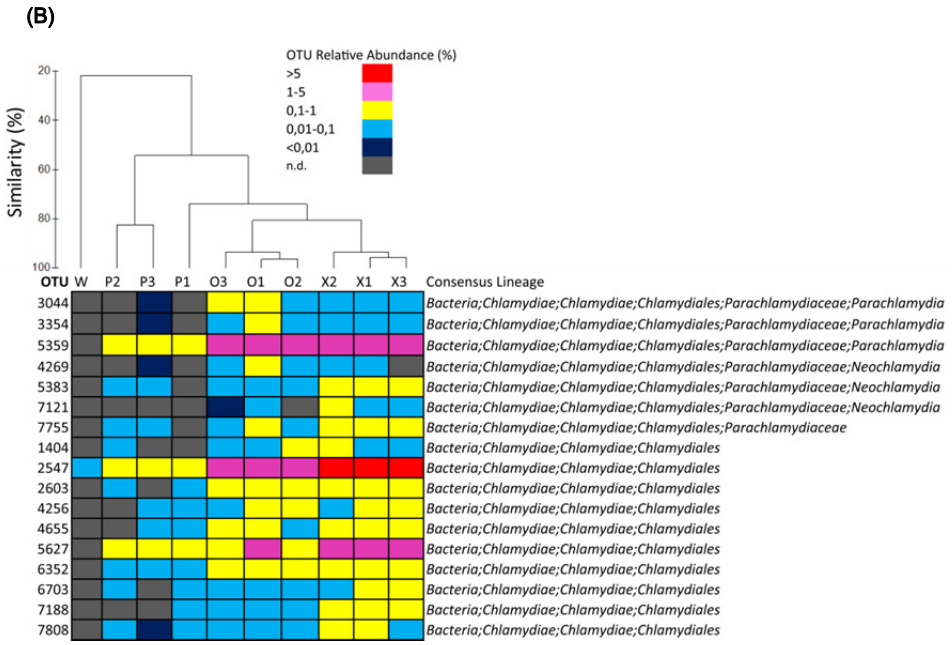

Figure 2.2.B Heat map of the most abundant OTUs affiliated to the phylum *Chlamydiae*. Each row denotes the relative abundance of the most abundant *Chlamydiae*-OTUs in the different samples. N.D. means not detected. A range of colors was used to indicate the relative abundance of the OTUs with respect to the total microbial community (see legend). Samples were grouped using a hierarchical clustering (complete linkage) based on the Bray-Curtis distance matrix calculated from the relative abundances of the represented OTUs. The taxonomic affiliations of the OTUs based on the Ribosomal Data Project (RDP) Classifier at a confidence threshold of 80% are shown.

Sponge species-enriched and common OTUs

Thirteen of the predominant OTUs were found to be species-enriched (Figure 2.2A, marked as 'S'), *i.e.* they were found in all three samples of a particular sponge species, but not found in any of the other sponge samples. None of the three most abundant OTUs mentioned previously (*i.e.* OTUs 2229, 7091 and 171) was found to be strictly species-specific. Especially "OTUs 2229" (the most dominant in *H. panicea*) and 171 (the most dominant in *H. xena*) were present in relatively high abundance in *H. oculata* (Figure 2.2A). On the other hand, the dominant OTU in *H. oculata*

(OTU 7091) was only found at very low relative abundance (<0.1%) in *H. panicea* and *H. xena* (Figure 2.2A). All species-enriched OTUs were taxonomically affiliated with the *Proteobacteria*. In the case of *H. panicea*, the three species-enriched OTUs were *α-Proteobacteria* (OTUs 3548, 4411 and 6879). In contrast, the two *Haliclona* species showed a more complex distribution with species-enriched OTUs in *α-, β-* and *γ-Proteobacteria* (Figure 2.2A). Finally, three predominant proteobacterial OTUs (2343, 355 and 7616) were *Haliclona*-specific, *i.e.*, they were found in all samples of the two *Haliclona* species but not in *Halichondria*. Two of these *Haliclona*-specific OTUs belonged to the *ε-Proteobacteria* (OTUs 355 and 7616).

A total of 22 common OTUs (*i.e.* OTUs found in all sponge samples) were detected in the sponge samples analyzed in this study. They accounted for 16.1% of the total number of non-chimeric reads of the North Sea sponges. Within the common OTUs, *Chlamydiae* represented the numerically most important taxonomic group, with 5 common OTUs and 37.6% of common OTUs reads (Figure 2.4, Table 2.2). The majority of the common OTUs (18) was also included in the set of predominant OTUs (Figure 2.2A). The *Chlamydia*-affiliated reads were distributed across 436 OTUs, which were distributed differentially among the three sponge species (ANOSIM, R=0.695, p=0.004). To allow a close examination, the most abundant OTUs affiliated to this phyla (those with >0.1% of abundance in any of the sponge samples) are displayed (Figure 2.2B). These 17 OTUs represented 76.6% ± 3.3%, 73.6% ± 0.5% and 77.5% ± 1.1% of the total number of reads affiliated taxonomically to the phylum *Chlamydiae* in *H. panicea*, *H. xena* and *H. oculata* respectively. Among them, three OTUs accounted for the major part of the reads (OTUs 2547, 5359 and 5627); the highest relative abundance was detected for the OTU 2547 in the sample X2 (7.9%) (Figure 2.2B). *Chlamydiae* were very scarce in the sea water sample (0.09% in total). Only one of the 17 abundant OTUs (2547) was found in the seawater, but at very low abundance (0.013%).

Presence of Archaea in the North Sea sponges

Only 39 OTUs were assigned to the *Archaea*, with a total relative read abundance of 0.035% ± 0.05%, 0.29% ± 0.19% and 0.13% ± 0.15% in *H. panicea*, *H. oculata* and *H. xena*, respectively (Table S2.3). Only one OTU (4056) was found in some of the sponge samples at relative abundances in the range of 0.1-0.2% (data not shown), which could be a consequence of seawater filtration by the sponges considering the high relative abundance of this OTU in the sea water (1.3%). This OTU was included in the Bayesian phylogram but it does not belong to a sponge-enriched cluster (Figure 2.4). This is congruent with the ratio of bacterial and archaeal 16S rRNA gene copy numbers as measured by qPCR, which amounted to 1673.6 ± 1038.5, 164.3 ± 16. 8 and 58.4 ± 11.2 in *H. panicea*, *H. oculata* and *H. xena*, respectively (Table S2.4 and Figure S2.4).

Discussion

It has been demonstrated before that different sponges from the same habitat harbour distinct microbial communities (Webster*, et al.*, 2010, Jackson*, et al.*, 2012, Cleary*, et al.*, 2013), indicating that host-specific factors play a role in structuring the sponge microbiota. This was also evident in the North Sea sponges studied here, where when compared to the high interspecific variation, intraspecific variation was small with the majority of the predominant microbes being conserved in all three individuals per species. The fact that each sponge species harbours a number of species-enriched microbial phylotypes suggests their importance in the ecology of the respective sponge host. Recent comparative metagenomic analysis of sponge microbiomes emphasized that taxonomically different, but functionally equivalent microbes and corresponding enzymes or biosynthetic pathways can provide similar symbiont functions in different sponge species (Fan*, et al.*, 2012, Ribes*, et al.*, 2012).

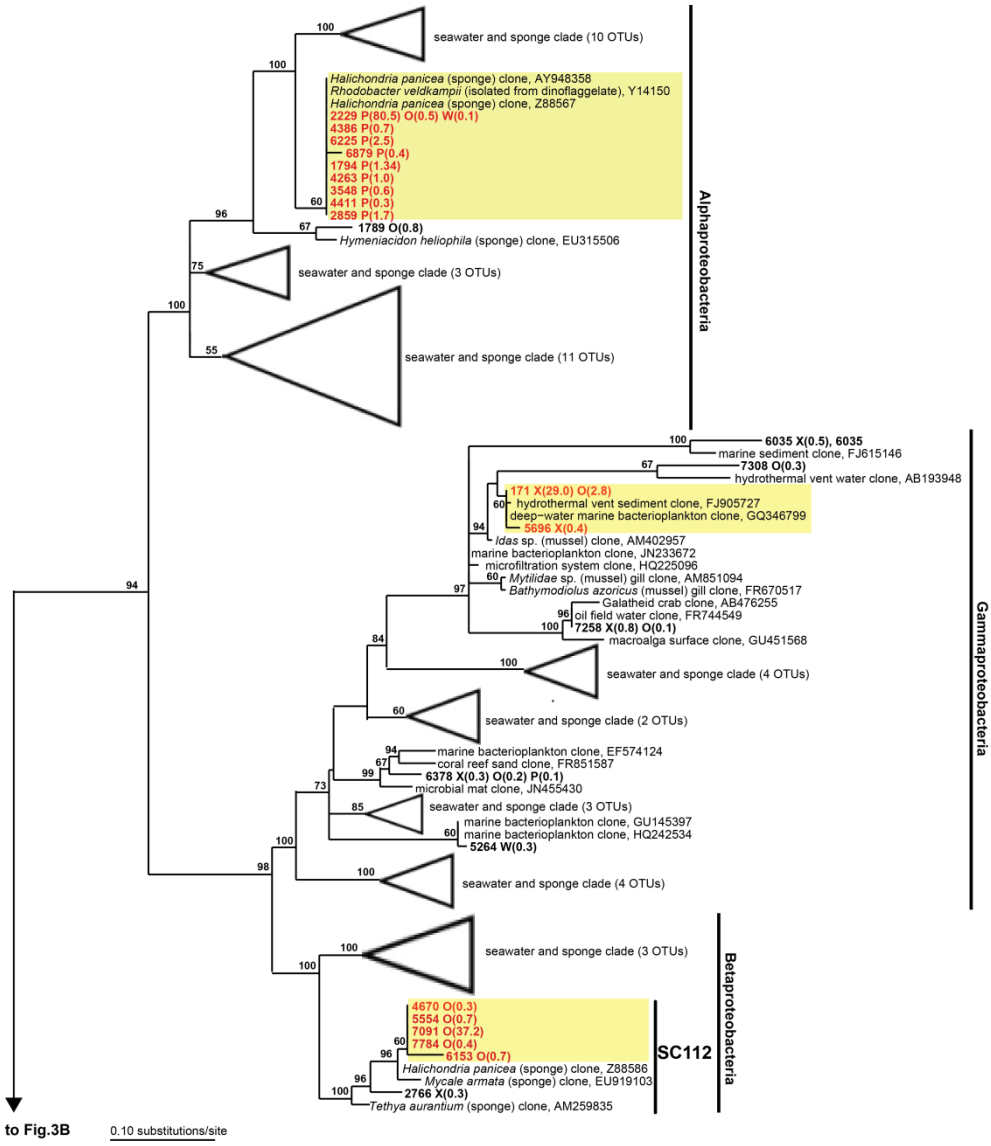

Figure 2.3A See caption (2.3A-B)

Figure 2.3.A-B Bayesian phylogram based on 16S rRNA gene sequences of OTUs representing more than 0.25% of the reads in *Halichondria panicea*, *Haliclona oculata*, *Haliclona xena* and seawater and their nearest neighbours. OTUs from this study are in bold and 'P' indicates that the OTU contains reads derived from *H. panicea* individuals, 'O' from *H.oculata*, 'X' from *H. xena*, and 'W' from seawater. Numbers in parentheses after the

sample type indication refer to the % of reads that are included in the corresponding OTU per sample (e.g. 6628 W(1.1) means that 1.1% of seawater-derived the reads are included in OTU6628 and that this OTU is not found in other samples). Abundances <0.1% are not shown. Yellow boxes with bold red indicate predominant OTUs cluster found in each North Sea sponge species. SC means sponge-enriched cluster and the numbering follows the denomination from Simister and co-workers (2012). The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values <50 are not indicated. The complete Bayesian phylogram is supplemented as Figure S2.3.

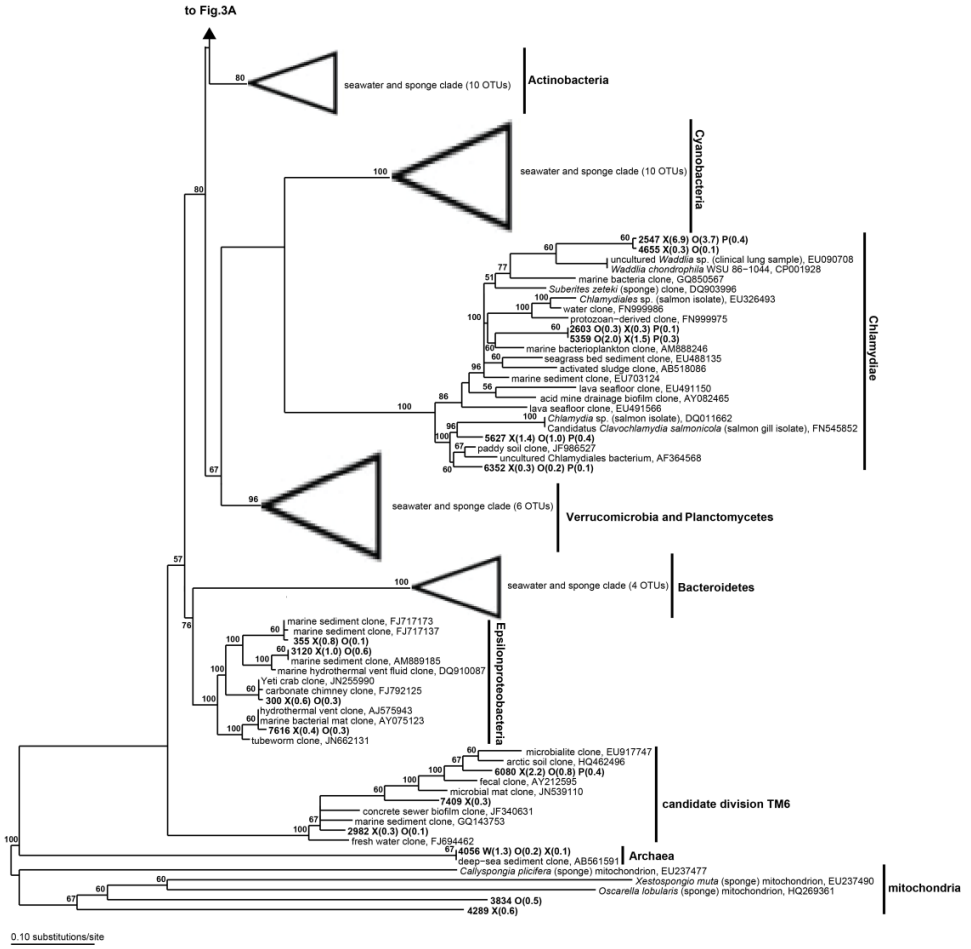

Figure 2.3.B See caption (2.3A-B)

Microbial diversity of *Halichondria panicea.*

Halichondria panicea has been identified as a LMA sponge based on electron microscopy (Althoff et al., 1998). There have been discrepancies in reports of predominant microbial populations in *H. panicea* (Wichels*, et al.*, 2006, Lee*, et al.*, 2009). Initially, *H. panicea* was reported to be dominated by a *Rhodobacter-like* α-proteobacterium based on 16S rRNA gene clone library analysis (Althoff*, et al.*, 1998). Using similar approaches, other reports found predominant symbionts of *H. panicea* affiliated with the α-proteobacterial genus *Roseobacter* (Wichels*, et al.*, 2006) or the phylum of *Actinobacteria* (Imhoff & Stöhr, 2003). We have now complemented the existing data using a deep sequencing approach and found that members of the *α-Proteobacteria* are the most predominant microbial populations in *H. panicea*. The dominance of *α-Proteobacteria* in *H. panicea* is striking with five closely related OTUs making up to 94.4% of the total sequence reads, and forming a sponge species-enriched clade based on Bayesian phylogenetic analysis (Figure 2.3A). OTU 2229 is the most predominant OTU in *H. panicea* with a relative abundance of 80.5% ± 1.47%, and is most closely related to *Rhodobacter veldkampii*, a facultative anaerobic purple non-sulfur bacterium (Hansen & Imhoff, 1985), thereby confirming the study of Althoff *et al.* (1998). To the best of our knowledge, the phyla *Chlamydiae, Planctomycetes* and *Verrucomicrobia* are described for the first time in *H. panicea.*

Microbial diversity of North Sea *Haliclona* spp.

One of the primary motivations to study microbial diversity in sponges is the increasing evidence that sponge symbionts play an important role in the synthesis of protective metabolites with great biotechnological interest (Piel, 2002, Fieseler*, et al.*, 2007, Hentschel*, et al.*, 2012). This is particularly relevant in the case of the genus *Haliclona*. To date, at least 190 secondary metabolites have been identified from sponges of the genus *Haliclona* exhibiting anti-fouling, antimicrobial, antifungal, antimalarial and cytotoxic activities (Yu*, et al.*, 2006). Many of these activities may actually be produced by their associated microbiota (Schippers*, et al.*, 2012). Thus the potential of sponges for producing bioactives is partly dependent on their microbial state. In high-microbial-abundance (HMA) sponges microbial cells can contribute up to 40% of the total sponge biomass (Taylor*, et al.*, 2007) and hence potentially harbour a larger arsenal of bacterial secondary

metabolic pathways. In turn, low-microbial-abundance (LMA) sponges have lower microbial abundance (comparable to concentrations in seawater), lower diversity and a different microbial profile as compared to high-microbial-abundance sponges (Hentschel*, et al.*, 2006, Kamke*, et al.*, 2010). Recently, Giles and co-workers (2013) suggested that the composition of microbial communities associated with LMA sponges is characterized by i) pre-dominance of a single large clade of either *Cyanobacteria* or *Proteobacteria*, and ii) by lower phylum level diversity compared to HMA sponges, and absence, or low-level presence of typical phyla often found in HMA sponges such as *Acidobacteria*, *Chloroflexi* and *Gemmatimonadetes* (Schmitt*, et al.*, 2011, Simister*, et al.*, 2012), and iii) a low number of representatives of so-called sponge-enriched clusters (i.e. phylogenetic groups highly enriched in sponges). Based on these criteria, *Haliclona oculata and Haliclona xena* investigated in this study could be defined as putative LMA sponges because of i) pre-dominance of one proteobacterial OTU ii) low abundance of *Acidobacteria, Chloroflexi* and *Gemmatimonadetes*, and (iii) presence of only a low number of sponge-enriched OTUs (Figure 2.3A). In addition, qPCR data showed that the 16S rRNA gene copies in the *Haliclona* species were in the same range as the LMA sponge *Halichondria panicea* (Table S2.4). This finding is consistent with other microbial diversity studies of *Haliclona* spp., where it was found that the sponge host is either dominated by a *γ-proteobacterium* (Kennedy*, et al.*, 2007, Sipkema*, et al.*, 2009, Erwin*, et al.*, 2011), a *δ-proteobacterium* (Lee*, et al.*, 2009) or an α-Proteobacterium (Khan*, et al.*, 2013). One of the largest sponge-enriched clusters (SC, *i.e.* phylogenetic groups highly enriched in sponges) with at least 100 sequences from 15 different sponge species from diverse geographic locations is affiliated with the *Betaproteobacteria* (Simister*, et al.*, 2012). In our study, we found that *H. oculata* hosted six OTUs (Figure 2.3A and Figure S2.3: 2766, 4670, 5554, 6153, 7091 and 7784) with nearest neighbours that all are derived from sponges. There are no apparent close cultured relatives, which make it difficult to speculate about the functional relevance to their sponge host (Thiel*, et al.*, 2007, Simister*, et al.*, 2012).

Common OTUs and putative functions

One of the highlights in our North Sea sponges study is the high relative abundance of *Chlamydiae* common in the three sponge species (Figure

2.2B). Chlamydia are a unique group of obligate intracellular bacteria including important pathogens of vertebrates and symbionts of free-living amoebae (Collingro*, et al.*, 2011).

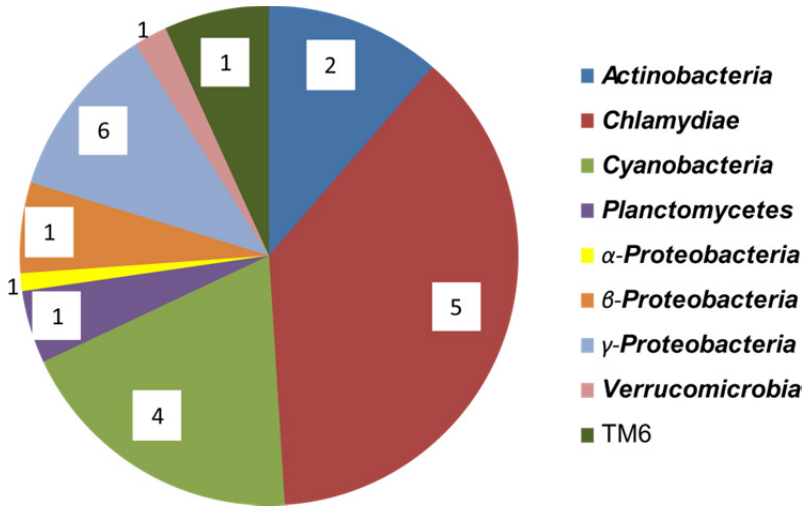

Figure 2.4 Taxonomic affiliation of common OTUs (% reads) of the three North Sea sponge species *Halichondria panicea*, *Haliclona oculata* and *Haliclona xena*. Total number of common OTUs included in each taxonomic group is indicated.

Despite ample molecular evidence for a large diversity and wide distribution of Chlamydia in nature (Collingro*, et al.*, 2005, Horn, 2008, Lagkouvardos*, et al.*, 2014), their ecology remains elusive. The presence of Chlamydia in sponges was first described for the marine sponge *Suberites zeteki* by 16S rRNA gene clone library analysis (Zhu*, et al.*, 2008). More recently, several studies revealed that at least ten other sponges, including one freshwater sponge (*Ephydatia fluviatilis*), harbour members of the C*hlamydiae* as part of their microbiota (Lee*, et al.*, 2011, Schmitt*, et al.*, 2012, Cleary*, et al.*, 2013, Costa*, et al.*, 2013, Wang*, et al.*, 2014). Our pyrosequencing study revealed seventeen OTUs with relative abundances by far exceeding those previously been reported in sponges (Table 2.2 and Figure 2.2.B). Comparison of chlamydial OTUs observed here with known environmental sequences (Table S2.5) revealed only low 16S rRNA gene sequence similarities ranging from 86% to 92%, which indicates that these may constitute novel *Chlamydiae* lineages, at least at genus level (see also Figure 2.3.B and Figure 2.2.B). This finding is in agreement with recent

large scale analysis of putative chlamydial proteins in metagenomic data sets, which revealed that the majority of novel chlamydial families comprise only sequences derived from marine environments, suggesting an association with marine hosts (Lagkouvardos*, et al.*, 2014).
OTUs belonging to the candidate phylum TM6 were previously found in all individuals of *Haliclona* (Gellius) sp. from a single location but not from the seawater (Sipkema*, et al.*, 2009), which suggested their role as symbiont in *Haliclona gellius.* Recent genome analysis of a TM6 representative confirmed its lifestyle as symbiont, albeit with no specific host (McLean*, et al.*, 2013), and analysis of 16S rRNA gene sequences suggested that members of TM6 are more widespread than previously thought (McLean*, et al.*, 2013).
For many other common OTUs from North Sea sponges it was found based on 16S rRNA sequences that they were related to genera commonly involved in the sulfur cycle, for example *Variovorax* sp.*, Tatlockia* sp. and *ɣ-proteobacteria* (Table S2.6). Based on their closest relatives, which were previously shown to be involved in sulfur cycle in bacterioplankton (GQ349502,(Walsh*, et al.*, 2009)), a microbial mat (JN454305, (Kirk Harris*, et al.*, 2013)) and mollusc gut (AY536218, (Duplessis*, et al.*, 2004)), it is tempting to speculate that these phylotypes found in our study might represent sulfide oxidizing bacteria (*i.e.* OTU 8070, OTU 4160 and OTU 6378) and contribute to the sulfur cycle that has been proposed to occur in sponges (Hoffmann*, et al.*, 2005).

Conclusion

The North Sea sponges *Halichondria panicea, Haliclona oculata* and *Haliclona xena* host species-specific bacterial communities, while archaea are present at only very low relative abundance. One α-proteobacterial *Rhodobacter*-like phylotype was the predominant symbiont in *H. panicea*, whereas the microbial communities of *H. xena* and *H. oculata* were dominated by a single β- and ɣ-proteobacterial phylotype, respectively. Several common phylotypes found in all three sponge species were found to belong to *Chlamydiae*, and were present at high relative abundance of up to 14.4% ± 1.4%, which is much higher than what has been reported for sponges to date. These *Chlamydiae*-affiliated phylotypes belong to three different families, but may represent novel genera as they are only 86-92% similar to known sequences.

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Supplementary Materials

Table S2.1. List of barcode sequences and ERS accession numbers used in this study. Sample codes indicate *H. panicea* (P1, P2, P3), *H. oculata* (O1, O2, O3), *H. xena* (X1, X2, X3) and sea water (W).

Sample	Barcode	Linker Primer	Accession Number
O1	AGACTATACT	CCTAYGGGRBGCASCAG	ERS179008
O2	AGCGTCGTCT	CCTAYGGGRBGCASCAG	ERS179009
O3	AGTACGCTAT	CCTAYGGGRBGCASCAG	ERS179010
P1	TAGAGACGAG	CCTAYGGGRBGCASCAG	ERS179002
P2	TCGTCGCTCG	CCTAYGGGRBGCASCAG	ERS179003
P3	ACATACGCGT	CCTAYGGGRBGCASCAG	ERS179004
X1	ACGCGAGTAT	CCTAYGGGRBGCASCAG	ERS179005
X2	ACTACTATGT	CCTAYGGGRBGCASCAG	ERS179006
X3	ACTGTACAGT	CCTAYGGGRBGCASCAG	ERS179007
W	TACTCTCGTG	CCTAYGGGRBGCASCAG	ERS179001

Table S2.2 Sponge individuals identity based on partial 18S rRNA gene. Nucleotide Blast was carried out on 12 August 2013 against non-redundant nucleotide database and only First Blast Hits not-related to our samples were shown

.

Sample Name	ID	NCBI Accession No.	First Nucleotide Blast Hit	Percentage Identity
HpeI	HpeI01	KC899022	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902238	99%
HpeI	HpeI02	KC899023	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902238	99%
HpeI	HpeI05	KC899024	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902239	99%
HpeII	HpeII06	KC899025	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902240	99%
HpeII	HpeII07	KC899026	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902238	99%
HpeII	HpeII08	KC899027	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902239	99%
HpeIII	HpeIII09	KC899028	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902240	99%
HpeIII	HpeIII10	KC899029	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902241	99%
HpeIII	HpeIII11	KC899030	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902238	99%
HpeIII	HpeIII12	KC899031	*Halichondria panicea* voucher BELUM<GBR>:Mc4070 small subunit 18S ribosomal RNA gene, partial sequence, KC902238	99%
HxeI	HxeI02	KC899032	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeI	HxeI03	KC899033	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeII	HxeII05	KC899034	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeII	HxeII06	KC899035	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeII	HxeII07	KC899036	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeIII	HxeIII08	KC899037	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	100%
HxeIII	HxeIII09	KC899038	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeIII	HxeIII10	KC899039	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HxeIII	HxeIII12	KC899040	*Haliclona sp.* OGL2003 18S rRNA (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate OGL2003, AJ703889	99%
HocI	HocI01	KC899041	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocI	HocI02	KC899042	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocII	HocII04	KC899043	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocII	HocII07	KC899045	*Haliclona oculata* voucher POR14116 18S ribosomal RNA gene, complete sequence, DQ927307	99%
HocII	HocII08	KC899046	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocIII	HocIII09	KC899047	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocIII	HocIII10	KC899048	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocIII	HocIII12	KC899049	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%
HocIII	HocIII13	KC899050	*Haliclona oculata* 18S ribosomal RNA gene, complete sequence, AY734450	99%

Table S2.3 Taxonomic composition of minor phyla in the samples (in % of read abundance). Sample codes indicate *H. panicea* (P1, P2, P3), *H. oculata* (O1, O2, O3), *H. xena* (X1, X2, X3) and sea water (W). n.d. means "not detected"

Taxonomy	P1	P2	P3	O1	O2	O3	X1	X2	X3	W
Unclassified Archaea	0.070	0.014	n.d.	0.078	0.306	0.294	0.041	0.058	0.240	1.525
Euryarchaeota	0.020	n.d.	n.d.	0.013	0.025	0.167	n.d.	n.d.	0.064	0.128
Unclassified Bacteria	0.020	n.d.	0.009	0.039	0.051	0.127	n.d.	0.029	0.128	0.538
Acidobacteria	n.d.	n.d.	n.d.	0.013	0.025	0.020	0.061	0.015	0.064	0.077
Chlorobi	n.d.	n.d.	n.d.	n.d.	n.d.	0,010	n.d.	0,044	n.d.	n.d.
Chloroflexi	0.141	n.d.	n.d.	0.013	0.013	n.d.	n.d.	n.d.	n.d.	0.461
Deferribacteres	n.d.	n.d.	n.d.	0.026	n.d.	0.020	0.020	0.029	n.d.	n.d.
WS3	n.d.	n.d.	n.d.	n.d.	n.d.	0.010	n.d.	n.d.	n.d.	0.013
Firmicutes	0.040	0.136	0.208	0.103	0.178	0.088	0.061	0.044	0.048	0.192
Lentisphaerae	n.d.	n.d.	n.d.	0.013	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nitrospira	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.029	0.032	0.026
OD1	n.d.	0.014	n.d.	0.026	0.038	0.020	0.020	0.029	0.064	0.038
Spirochaetes	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.020	0.015	n.d.	0.013
Synergistetes	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.013
TM7	0.020	n.d.	n.d.	0.026	0.025	0.049	0.102	0.073	0.128	0.013

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Table S2.4. Archaeal and bacterial 16S rRNA gene copy numbers associated with North Sea sponges and seawater estimated by quantitative PCR (qPCR). ND, "not determined".

	Archaeal 16S rRNA gene		Bacterial 16S rRNA gene		Ratio bacterial / archaeal 16S rRNA gene (±SD)
Sample	**Range**	**Average (±SD)**	**Range**	**Average (±SD)**	
HP	$2.1 \times 10^2 - 3.4 \times 10^2$	$259.2 \pm 7.4 \times 10^1$	$3.9 \times 10^3 - 3.7 \times 10^5$	$0.47 \pm 1.0 \times 10^5$	1673.6 ± 1038.5
HX	$1.3 \times 10^4 - 2.9 \times 10^4$	$19212.2 \pm 8.6 \times 10^3$	$8.7 \times 10^4 - 5.0 \times 10^5$	$2.5 \pm 1.9 \times 10^5$	164.3 ± 16.8
HO	$3.9 \times 10^3 - 8.6 \times 10^3$	$5848.0 \pm 2.5 \times 10^3$	$9.2 \times 10^4 - 5.9 \times 10^5$	$3.4 \pm 1.6 \times 10^5$	58.4 ± 11.2
Seawater	3.5×10^5	ND	2.4×10^6	ND	6.7

Table S2.5. Blastn search against non-redundant nucleotide database of all OTUs that contribute to >0.1% total reads. Nucleotide Blast was carried out on 24 January 2014 and only First Blast Hits identities are shown. Table is arranged by phylum and appearance as depicted in Bayesian phylogram (Figure S2.3A-2.3D)

OTU number and percentage of reads	Phylum/Class	First Blastn Hit Description and Accession Number	Similarity
8097 W(0.3)	*α-Proteobacteria*	Uncultured bacterium clone 1C226691, EU799128	99%
5820 W(0.3)	*α-Proteobacteria*	uncultured marine bacterium, clone LibI_C08, FR686215	97%
6628 W(1.1)	*α-Proteobacteria*	uncultured marine bacterium clone 16_09_01E02, FR685271	99%
156 W(11.5) O(0.1)	*α-Proteobacteria*	Uncultured bacterium clone OO.P4.IT.82.ab1, HQ821853	100%
2948 W(0.7)	*α-Proteobacteria*	Uncultured bacterium clone AND_GV0309_F6.5.2_7S0, JQ032338	99%
6409 W(0.4)	*α-Proteobacteria*	Uncultured bacterium clone cu1c-61, JN594637	99%
1804 O(0.6) X(0.2) W(0.1)	*α-Proteobacteria*	Uncultured alpha proteobacterium, clone 4734-27F, FR648148	100%
8027 W(0.3)	*α-Proteobacteria*	uncultured marine bacterium, clone LibK_A01, FR686302	98%
6557 W(0.5) X(0.1) O(0.1)	*α-Proteobacteria*	Uncultured bacterium clone PropaneSIP20-6-30, GU584816	99%
2598 W(1.27)	*α-Proteobacteria*	Uncultured bacterium clone N166e_782, JF451304	100%

Table S2.5. *Continued*

2229 P(80.5) O(0.5) W(0.1)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	100%
4386 P(0.7)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	94%
6225 P(2.5)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	96%
6879 P(0.4)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	98%
1794 P(1.34)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	97%
4263 P(1.0)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	95%
3548 P(0.6)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	99%
4411 P(0.3)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	98%
2859 P(1.7)	*α-Proteobacteria*	Uncultured bacterium clone sponge_clone10, AY948358	98%
1789 O(0.8)	*α-Proteobacteria*	Uncultured alpha proteobacterium clone YC499B15_M, AY701438	92%
5323 W(0.4)	*α-Proteobacteria*	Bacterium W13M53a, JQ042965	97%
7526 W(1.8)	*α-Proteobacteria*	*Erythrobacter sp*. CBMAI 1204, HQ433233	100%
7118 X(0.3) O(0.2) P(0.1)	*α-Proteobacteria*	Uncultured bacterium clone ELB16-030, DQ015802	98%
7195 W(0.6)	*α-Proteobacteria*	Uncultured marine bacterium clone MOLA_JAN08-5m-3, GU204520	98%
5475 W(0.3)	*α-Proteobacteria*	Uncultured bacterium clone DNA_36 270m, JN410162	99%
3525 W(0.6)	*α-Proteobacteria*	Uncultured marine bacterium clone MOLA_JUN08-5m-26, GU204674	99%
4050 W(13.1) O(0.3)	*α-Proteobacteria*	Uncultured bacterium clone DNA_56 270m, JN410213	99%

Table S2.5. *Continued*

6317 W(0.3)	*α-Proteobacteria*	Uncultured marine bacterium clone SHFB481, GU234900	97%
5189 W(1.5) O(0.1)	*α-Proteobacteria*	Uncultured alpha proteobacterium clone SHAS620, GQ349475	98%
2382 W(0.3)	*α-Proteobacteria*	Uncultured bacterium clone S25_1406, EF575062	99%
1617 X(0.5) O(0.2)	*α-Proteobacteria*	Uncultured bacterium clone Ca06 62, FR851534	99%
6057 X(0.5) O(0.2)	*α-Proteobacteria*	Uncultured bacterium clone EPR3968-O8a-Bc74, EU491741	99%
5775 X(5.5)	*α-Proteobacteria*	Uncultured bacterium clone W7-C10, EF471706	98%
5605 O(1.3)	*α-Proteobacteria*	Uncultured alpha proteobacterium clone DC22, EU919107	95%
6035 X(0.5), 6035	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone SI021806_100ZB, FJ615119	95%
7308 O(0.3)	*γ-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	99%
171 X(29.0) O(2.8)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone SGPW671, GQ346799	100%
5696 X(0.4)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone SGPW671, GQ346799	99%
7258 X(0.8) HO(0.1)	*γ-Proteobacteria*	Uncultured bacterium, clone IWNB014, FR744549	99%
4160 X(0.4) O(0.2) P(0.1)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone 9m05AISC05, EF629803	99%
2194 X(0.5) O(0.3) P(0.1)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone 9m05AISC06, EF629803	96%
2821 X(0.3) O(0.2) P(0.1)	*γ-Proteobacteria*	Uncultured bacterium clone LAgut-16S-P02, AY536218	97%
8070 X(1.6) O(1.5) P(0.2)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone SHAS719, GQ349532	97%
1378 X(0.3) O(0.2) P(0.1)	*γ-Proteobacteria*	Uncultured bacterium clone ncd2608f10c1, JF228258	100%

Table S2.5. *Continued*

192 W(0.4)	*γ-Proteobacteria*	Uncultured bacterium clone SN80, EU735671	96%
6378 X(0.3) O(0.2) P(0.1)	*γ-Proteobacteria*	Uncultured bacterium, clone Ca07 38, FR851587	94%
3438 W(0.3)	*γ-Proteobacteria*	Uncultured gamma proteobacterium clone Clip 49, HQ691970	99%
4376 W(0.5)	*γ-Proteobacteria*	Uncultured bacterium clone W1-22, FJ545516	100%
8040 W(0.3)	*γ-Proteobacteria*	uncultured marine bacterium, clone 16_03_02D08, FR684327	98%
5264 W(0.3)	*γ-Proteobacteria*	Uncultured SAR86 cluster bacterium clone M7CS04_10E05, HQ242534	99%
2153 W(1.0)	*γ-Proteobacteria*	Uncultured bacterium clone MF30b7, HQ230053	100%
328 W(0.6) X(0.1) O(0.1)	*γ-Proteobacteria*	Uncultured bacterium clone SI-BS120-215, JN172318	100%
3796 X(1.8)	*γ-Proteobacteria*	Uncultured bacterium clone HgCo23, EU236296	98%
2343 O(0.6) X(0.4) W(0.3)	*β-Proteobacteria*	Uncultured bacterium clone SI-BS110-78, JN172265	100%
6402 W(0.4)	*β-Proteobacteria*	Uncultured beta proteobacterium clone PI_RT99, AY580390	99%
4220 P(1.5) O(1.0) X(0.4) W(0.2)	*β-Proteobacteria*	*Variovorax sp.* DSH, JN561302	100%
602 W(0.1)	*β-Proteobacteria*	Uncultured beta proteobacterium, clone 4887, FR648297	99%
4670 O(0.3)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	98%
5554 O(0.7)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	94%
7091 O(37.2)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	97%
7784 O(0.4)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	99%
6153 O(0.7)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	95%

Table S2.5. *Continued*

2766 X(0.3)	*β-Proteobacteria*	Unidentified bacterium DNA for 16S rRNA (isolate HRV40), Z88592	97%
920 O(2.4) X(2.3) P(0.6) W(0.1)	*Actinobacteria*	Uncultured bacterium clone F08JOA, JF692438	100%
5663 X(0.7) O(0.6) P(0.1)	*Actinobacteria*	Uncultured actinobacterium isolate M38, AF544337	100%
3805 W(0.3)	*Actinobacteria*	Bacterium SCGC AAA071-N11, JF488663	100%
3676 W(1.0)	*Actinobacteria*	Uncultured bacterium clone cDNA_107 270m, JN410067	99%
2550 W(0.4)	*Cyanobacteria*	Uncultured bacterium clone SH100_100m_clone_105, HQ173805	99%
2716 W(0.6)	*Cyanobacteria*	Uncultured microorganism clone PK_Seq540_Edw_383, HM485252	98%
6432 O(2.4) X(0.7) P(0.1) W(0.1)	*Cyanobacteria*	Uncultured bacterium clone mdc40b10, AY537602	99%
962 O(0.9) X(0.2) P(0.2)	*Cyanobacteria*	Uncultured bacterium clone N414B_96, GU940948	99%
2018 O(0.3) HX(0.1)	*Cyanobacteria*	Uncultured bacterium clone N414B_96, GU940948	98%
7669 HO(0.5) X (0.1)	*Cyanobacteria*	Uncultured bacterium clone NSED2_28, JF495289	100%
3478 W(0.3)	*Cyanobacteria*	Uncultured bacterium clone pfbac5, HQ877727	97%
4977 W(1.4)	*Cyanobacteria*	Uncultured cyanobacterium clone SHWN_night2_16S_694, FJ745159	98%
7993 W(11.0) O(0.1)	*Cyanobacteria*	*Teleaulax acuta* chloroplast, AB471791	100%
6779 W(0.3)	*Cyanobacteria*	Uncultured bacterium clone JX72, JN004330	99%

Table S2.5. *Continued*

5362 W(0.3)	*Cyanobacteria*	Uncultured bacterium clone OO.P3.LT.31.ab1, HQ821713	99%
6790 O(2.7) X(1.3) P(0.2) W(0.1)	*Cyanobacteria*	Uncultured cyanobacterium clone 913-C6 (GOMB2), JF824768	100%
7375 O(0.7) X(0.1)	*Cyanobacteria*	Uncultured bacterium clone MoJ27, EU645250	100%
2547 X(6.9) O(3.7) P(0.4)	*Chlamydiae*	Uncultured *Verrucomicrobiales* bacterium, clone CT10B1025, AM888246	86%
4655 X(0.3) O(0.1)	*Chlamydiae*	Uncultured *Verrucomicrobiales* bacterium, clone CT10B1025, AM888246	86%
2603 O(0.3) X(0.3) P(0.1)	*Chlamydiae*	Uncultured *Verrucomicrobiales* bacterium, clone CT10B1025, AM888246	88%
5359 O(2.0) X(1.5) P(0.3)	*Chlamydiae*	Uncultured *Verrucomicrobiales* bacterium, clone CT10B1025, AM888246	90%
5627 X(1.4) O(1.0) HP(0.4)	*Chlamydiae*	Candidatus *Clavochlamydia salmonicola*, FN545852	91%
6352 X(0.3) O(0.2) P(0.1)	*Chlamydiae*	Uncultured *Chlamydiae* bacterium clone Paddy_40_5825, JF986527	92%
3302 O(1.7) X(0.5) P(0.1)	*Planctomycetes*	Uncultured planctomycete, clone IPI_257-353, FN822113	100%
3 W(0.4)	*Planctomycetes*	Uncultured bacterium clone QLS20-B19, AY940535	97%
767 X(0.8) O(0.2)	*Verrucomicrobia*	Uncultured *Verrucomicrobia* bacterium clone Pink_2C06, GQ483932	91%
7792 W(0.4)	*Verrucomicrobia*	Uncultured *Verrucomicrobia* bacterium clone DH131B17, JN672664	99%
4878 W(0.4)	*Bacteroidetes*	Uncultured bacterium clone SI-BU110-165, JN172434	100%
6958 X(0.5) O(0.1)	*Bacteroidetes*	Uncultured bacterium clone SGUS1082, FJ202066	96%

Table S2.5. *Continued*

3307 W(0.5)	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium clone SGSO650, GQ347886	99%
932 X(0.7) W(0.3) O(0.2)	*Bacteroidetes*	Bacterium SCGC AAA071-N05, JF488662	100%
355 X(0.8) O(0.1)	*ε-proteobacteria*	Uncultured bacterium clone F3_10.4_2, FJ717173	99%
3120 X(1.0) O(0.6)	*ε-proteobacteria*	Uncultured epsilon proteobacterium, AM889185	100%
300 X(0.6) O(0.3)	*ε-proteobacteria*	Uncultured epsilon proteobacterium clone Contig_0365__x2, JN255991	99%
7616 X(0.4) O(0.3)	*ε-proteobacteria*	Uncultured epsilon proteobacterium clone LF8GH1b46, JN662131	97%
6080 X(2.2) O(0.8) P(0.4)	*TM6*	Uncultured bacterium clone Crust2B09, EU917747	93%
7409 X(0.3)	*TM6*	Uncultured organism clone SBZP_5971, JN539128	90%
2982 X(0.3) O(0.1)	*TM6*	Uncultured candidate division TM6 bacterium clone JJB103, GQ143753	91%
4056 W(1.3) O(0.2) X(0.1)	*Archaea*	Uncultured archaeon, AB561591	99%
3834 O(0.5)	*Mitochondria*	Uncultured *Firmicutes* bacterium clone O5-89, GU955613	82%
4289 X(0.6)	*Mitochondria*	Callyspongia plicifera mitochondrion, complete genome, EU237477	96%

OTU	Taxonomy	O1	O2	O2	P1	P2	P3	X1	X2	X3	W
6080	Bacteria (TM6)	1.02	0.65	0.81	0.26	0.63	0.24	2.19	2.15	1.99	n.d.
920	Bacteria; *Actinobacteria; Actinobacteria; Actinomycetales*	2.03	2.34	2.66	0.30	0.89	0.75	1.69	1.81	3.33	0.04
3828	Bacteria; *Actinobacteria; Actinobacteria; Actinomycetales*	0.16	0.08	0.08	0.02	0.10	0.02	0.04	0.08	0.26	n.d.
2547	Bacteria; *Chlamydiae; Chlamydiae; Chlamydiales*	4.37	3.21	3.72	0.49	0.35	0.33	6.56	8.03	6.20	n.d.
5359	Bacteria; *Chlamydiae; Chlamydiae; Chlamydiales; Parachlamydiaceae; Parachlamydia*	2.38	1.54	1.77	0.02	0.30	0.41	1.44	1.50	1.50	n.d.
5627	Bacteria; *Chlamydiae; Chlamydiae; Chlamydiales*	1.16	0.93	1.16	0.35	0.41	0.49	1.28	1.69	1.24	n.d.
6221	Bacteria; *Chlamydiae; Chlamydiae; Chlamydiales; Parachlamydiaceae; Neochlamydia*	0.04	0.02	0.04	0.02	0.02	0.02	0.06	0.08	0.06	n.d.
6352	Bacteria; *Chlamydiae; Chlamydiae; Chlamydiales*	0.22	0.12	0.22	0.10	0.02	0.10	0.20	0.37	0.28	n.d.
962	Bacteria; *Cyanobacteria; Cyanobacteria;*	0.75	1,08	1.00	0.04	0.16	0.26	0.16	0.16	0.35	n.d.
6790	Bacteria; *Cyanobacteria; Cyanobacteria;* Family II; GpIIa	2.56	2.70	2.76	0.08	0.16	0.39	0.71	1.02	2.13	0.04
7669	Bacteria; *Cyanobacteria; Cyanobacteria;*	0.22	0.49	0.61	0.08	0.04	0.02	0.06	0.12	0.18	0.02
6432	Bacteria; *Cyanobacteria;*	1.67	3.25	2.44	0.08	0.14	0.06	0.55	0.39	0.95	0.12
3302	Bacteria; *Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae; Planctomyces*	1.85	1.36	1.83	0.08	0.22	0.06	0.47	0.53	0.49	n.d.
7118	Bacteria; *Proteobacteria; Alphaproteobacteria*	0.18	0.28	0.22	0.06	0.08	0.10	0.20	0.18	0.35	n.d.
4220	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Variovorax	1.20	1,04	0.89	1.12	1.48	1.69	0.33	0.51	0.04	0.16
2278	Bacteria; *Proteobacteria; Gammaproteobacteria*	0.16	0.08	0.14	0.02	0.02	0.02	0.06	0.12	0.18	n.d.
2821	Bacteria; *Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae*	0.33	0.16	0.30	0.04	0.06	0.04	0.26	0.28	0.26	n.d.
3489	Bacteria; *Proteobacteria; Gammaproteobacteria*	0.10	0,04	0.08	0.02	0.06	0.04	0.14	0.06	0.16	n.d.
4160	Bacteria; *Proteobacteria; Gammaproteobacteria*	0.33	0.08	0.10	0.04	0.06	0.08	0.43	0.35	0.45	n.d.
6378	Bacteria; *Proteobacteria; Gammaproteobacteria*	0.24	0,20	0.14	0.06	0.16	0.02	0.43	0.26	0.20	n.d.
8070	Bacteria; *Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Tatlockia*	1.85	1.44	1.16	0.06	0.39	0.02	1.63	1.56	1.48	n.d.
767	Bacteria; *Verrucomicrobia; Opitutae; Puniceicoccales; Puniceicoccaceae*	0.14	0.22	0.28	0.02	0.02	0.02	0.71	0.81	0.95	n.d.

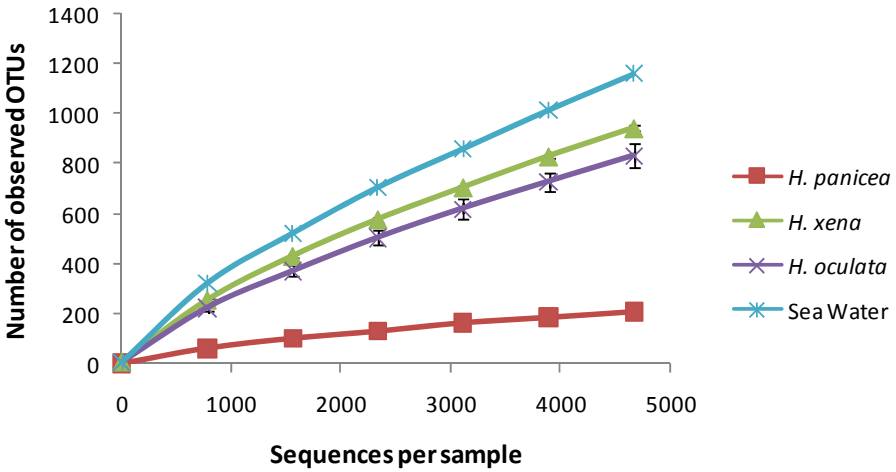

Figure S2.1. Rarefaction curves indicating the average observed number of operational taxonomic units (OTUs) for the different sample types. Errors bars show ± standard deviation.

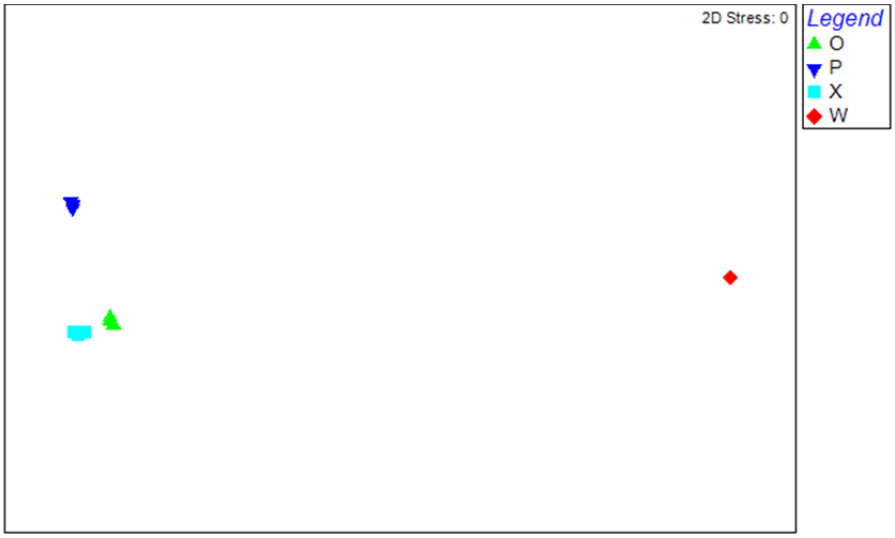

Figure S2.2. Nonmetric multidimensional scaling (NMDS) plot derived from Bray-Curtis similarity analysis based on abundances of assigned 97% OTUs of sponge and seawater samples.

Supplementary **Figure S2.3** is available upon request.

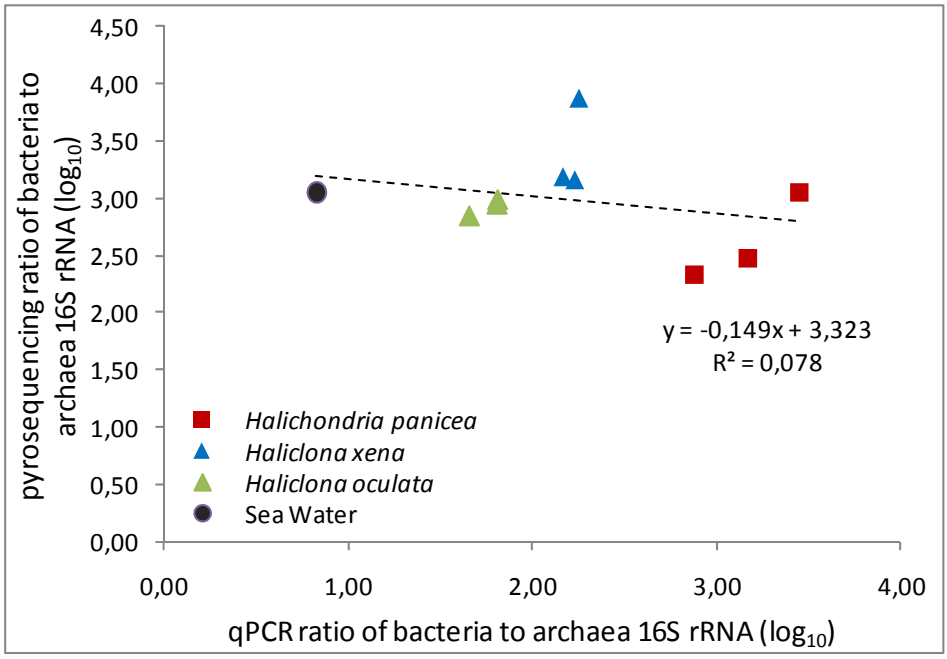

Figure S2.4 Scatter plot showing the correlation between bacteria/archaea ratios (Log10) inferred from amplicon pyrosequencing and measured by qPCR.

Chapter 3:

Molecular analysis of sponge-associated fungi

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Abstract

Fungi and other eukaryotes represent one of the last frontiers of microbial diversity that have not been thoroughly characterized in the sponge holobiont. In this study we employed pyrosequencing of 18S ribosomal RNA gene amplicons containing the V7 and V8 hypervariable regions to explore the fungal diversity of seven sponge species from the North Sea and the Mediterranean Sea. We found high diversity and novel fungal groups at the class level such as *Cystofilobasidiales*, *Leucosporidiales* and *Microstromatales*. The majority of fungal OTUs from sponges were found to be yeasts, such as *Malasseziales* spp., that have also been previously identified/isolated as parasites and pathogens in marine animals, and was never reported from any cultivation study. In contrast to the bacterial and archaeal populations associated with sponges, the existence of sponge-specific clusters (SSC) of fungi was found to be debatable and needs further investigation. In addition, other eukaryotes such as *Mesomycetozoea*, *Choanoflagellida* and *Cercozoa* were found to be constituents of the sponge microbial eukaryotic community, suggesting a higher eukaryotic diversity in the sponge holobiont than previously anticipated, possibly also providing a missing link in the study of sponge diseases. Thus, this study does not only provide additional insights in sponge-associated fungal diversity, but also into other eukaryotes that are present in sponges.

Keywords: sponge-associated fungi, pyrosequencing, sponge-eukaryote diversity, sponge holobiont, *Malasseziales* sp., *Mesomycetozoea*

Introduction

Fungi constitute a large proportion of microbial diversity on Earth (Hawksworth, 2001, Mueller & Schmit, 2007) and are considered as key players in terrestrial environments with respect to the decomposition of organic matter, nutrient recycling or as symbionts of plants or other fungi by improving host fitness (Rodriguez*, et al.*, 2004). Global fungal richness has been estimated between 1.5 to 1.6 million species (Hawksworth, 1991, Hawksworth, 2001), but despite extensive attempts to study and characterize fungi, their diversity remains underexplored. Most of our knowledge about evolution and ecology of fungi has been derived from cultured representatives of fungi from the terrestrial environment. In comparison, much less is known about marine fungal diversity and ecology. Marine fungi belong to a wide variety of families and have been estimated to contribute up to 0.6% of the total fungal diversity richness (Richards*, et al.*, 2012). The generally accepted definition of a marine fungus is broad and is based on the habitat as described by Kohlmeyer and Kohlmeyer (1979): “obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater and terrestrial milieus able to grow and possibly also sporulate in the marine environment”. Fungi are considered to play a role in marine ecosystems as saprotrophs, parasites, or symbionts (Hyde*, et al.*, 1998). Different habitats of marine fungi have been studied including deep-sea sediments (Singh*, et al.*, 2011), hydrothermal vents (Le Calvez*, et al.*, 2009), seawater (Kis-Papo*, et al.*, 2003) and anoxic regions of the deep part of oceans (Bass *et al.,* 2007). Marine fungi have also been described to be associated to marine plants and animals, such as sea fan (Toledo-Hernández*, et al.*, 2008), corals (Bentis*, et al.*, 2000, Amend*, et al.*, 2012) and algae (Loque*, et al.*, 2010). Marine sponges also provide a habitat for fungi. Whereas bacterial and archaeal diversity in sponges has been thoroughly characterized (Taylor*, et al.*, 2007, Simister*, et al.*, 2012), knowledge of fungal diversity in sponges has been lagging behind (Webster & Taylor, 2012). Indirect evidence of interactions between marine sponges and fungi was provided by the detection of fungal introns in the genomes of some marine sponge species, most probably acquired by horizontal gene transfer (Rot*, et al.*, 2006). Fungi have been repeatedly isolated from at least 27 sponge species (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006, Wang*, et*

al., 2008, Baker*, et al.*, 2009, Liu*, et al.*, 2010, Paz*, et al.*, 2010, Wiese*, et al.*, 2011, Passarini*, et al.*, 2012). However, the true diversity of sponge-associated fungi has been difficult to establish (Baker*, et al.*, 2009, Schippers*, et al.*, 2012). The reason is that designing specific PCR primers for the fungal 18S rRNA gene remains challenging since sponges and fungi are closely related from an evolutionary perspective (Borchiellini*, et al.*, 1998). This was demonstrated in a Hawaiian sponge study which picked up many sponge-derived sequences in their clone libraries (Gao*, et al.*, 2008). With the use of next-generation sequencing, it is now possible to overcome such interference of the host 18S rRNA gene by the sheer number of reads that is generated as shown in a recent study of a deep sea sponge (Wang*, et al.*, 2014). In addition, a recent study employing metagenomic analysis of another deep sea sponge provided new information regarding the presence of eukaryotes in sponges, including the detection of fungi (*Ascomycota* and *Basidiomycota*), protophytes (*Cryptophyta*, *Rhodophyta*, and *Viridiplantae*), protists (*Alveolata* and *Rhizaria*), protozoa (*Amoebozoa*, *Apusozoa*, *Mesomycetozoea*, *Euglenozoa*) and *Stramenopiles* (Li*, et al.*, 2014). The aim of this study was to determine the diversity of sponge-associated fungi as well as other eukaryotes associated with multiple shallow water sponges in two geographic regions with deep phylogenetic analysis of sponge-associated eukaryotes, further enhancing our knowledge on eukaryotic communities in sponges.

Materials and methods

Sample collection and processing

North Sea sponges *Halichondria panicea* (P1-P3)*, Haliclona xena* (X1-X3) and *Suberites massa* (M1-M3) were collected on December 3rd, 2008, from the Oosterschelde estuary, at the dive site Lokkersnol (N 51° 38’ 58.07”, E 3°53’ 5.11”E) by SCUBA diving at a depth of approximately 14 m. Mediterranean sponges *Aplysina aerophoba* (A1-A3)*, Petrosia ficiformis* (F1-F3)*, Axinella damicornis* (D1-D3) *and Axinella verrucosa* (V1-V3) were collected by SCUBA diving offshore L’Escala, Spain (N 42° 06’ 52.20”, E 03° 10’ 06.52”) at a depth of approximately 15 m on January 15th, 2012. Initial identification of sponges based on their morphology was done by Prof. Rene Wijjfels and Dr. Klaske Schippers for the North Sea sponges and by Dr. Detmer Sipkema and Prof M.J Uriz for the Mediterranean sponges. The identification was later confirmed using the 18S rRNA gene

sequence data. All sponge specimens from the North Sea and the Mediterranean Sea were collected in triplicate. Specimens were brought to the surface in ziplock plastic bags. The samples were immediately transported to the laboratory in excess of seawater and processed. Each sponge specimen was separately submerged and rinsed three times in a large volume of autoclaved artificial seawater (26.52 g NaCl, 2.45 g $MgCl_2$, 0.73 g KCl, 1.14 g $CaCl_2$ and 3.31 g $MgSO_4$/l) and kept at -80°C until further processing. Between 5 and 10 liters of seawater from both locations was collected and filtered immediately upon collection onto a 0.2 µm polycarbonate filter (GE Osmonics, Minnetonka, MN, USA).

DNA extraction, PCR amplification and sample preparation for pyrosequencing

North Sea sponges total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the tissue extraction protocol. Mediterranean sponges total DNA was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH) with the aid of a PreCellys® homogenizer (Bertin Technologies, France) following the manufacturers' protocol. For the seawater, the filters that were previously stored at -80°C were cut in pieces and submerged in lysis buffer (40 mM EDTA, 50 mM Tris, 0.75 M sucrose and pH 8.3) supplemented with lysozyme, proteinase K and sodium dodecyl sulfate, followed by the same DNA extraction protocol as the Mediterranean sponges. Amplification of partial 18S rRNA genes was performed using the GoTaq® Hot Start Polymerase kit (Promega GmbH, Mannheim, Germany) with the universal fungal primers FR1 and FF390 (Vainio & Hantula, 2000), which amplify a region of approximately 350 base pairs (bp) in length that includes the V7 and V8 hypervariable regions of the eukaryotic small-subunit (SSU) rRNA gene. This primer set has also previously been found to amplify 18S rRNA genes of other non-fungal eukaryotes such as *Choanoflagellida*, *Mesomycetozoa*, *Cnidaria* and *Porifera* sequences (Chemidlin Prévost-Bouré*, et al.*, 2011). Barcodes and linker sequences were added to the forward primer as described previously (Hamady*, et al.*, 2008). The PCR conditions were: initial denaturation (2 min at 95 ˚C) followed by 30 cycles of denaturation (30 s at 95 ˚C), primer annealing (45 s at 50 ˚C), primer extension (60 s at 72 ˚C), and a final extension (10 min at 72 ˚C). The final

PCR mixture (50 µl) contained 1x GoTaq® Green Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25u GoTaq® Hot Start Polymerase and 10 ng template DNA. PCR reactions were carried out in triplicate, pooled and cleaned using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany). Purified DNA concentrations were measured with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). An equimolar mixture with a final concentration of 1µg/ml PCR product was prepared, run on 1.25% (w/v) agarose gel and subsequently purified using the Milipore DNA Gel Extraction Kit (Milipore, Billerica, MA, USA). The pooled purified DNA was pyrosequenced on a 454 Roche platform at GATC Biotech, Konstanz, Germany. Pyrosequencing data was deposited at the European Bioinformatics Institute with accession numbers ERS255550 - ERS225575 (Table S3.2).

Sequence analysis, OTU richness and multivariate analysis

Pyrosequencing data was analysed using the QIIME 1.5.0 pipeline (Caporaso*, et al.*, 2010a). Low quality sequences were removed using default parameters, including i. reads with fewer than 200 or more than 1000 nucleotides; ii. reads with more than 6 ambiguous nucleotides, iii. homopolymer runs exceeding 6 bases, iv. reads with missing quality scores and reads with a mean quality score lower than 25; v. reads with mismatches in the primer sequence. Operational taxonomic units (OTUs) were identified at the 97% identity level using UCLUST v1.2.22 embedded in QIIME (Edgar, 2010). Representative sequences from the OTUs were aligned using PyNAST (Caporaso*, et al.*, 2010b) against the aligned Silva 104 core set. Taxonomic assignment of all OTUs was performed using the BLAST algorithm against the QIIME-compatible version of the Silva 104 release (Pruesse*, et al.*, 2007) as reference database. Possible chimeric OTUs were identified using QIIME's ChimeraSlayer and removed from the initially generated OTU list, producing a final set of 578 non-chimeric OTUs. Since the majority of reads was identified as sponge reads (107 OTUs), these were removed from the dataset prior to further analysis. 28 OTUs that represented a total of 35 reads that could not be classified to any taxonomic group were also excluded. The final 443 OTUs from the eukaryotic (non-sponge) dataset were considered for a more thorough analysis.

To estimate OTU richness, abundance data was first converted to incidence data (presence or absence). The presence-absence OTU matrix from sponge and seawater samples was used to calculate incidence-based species richness estimates using the 'Presence/Absence Data for Multiple Samples/Quadrats' option with default settings for eukaryotes with the SPADE program (Chao & Shen, 2013). The presence-absence OTU matrix was used because multiple genomic copies of eukaryotic 18S rRNA genes are often present (Amaral-Zettler*, et al.*, 2011).
Distance-based multivariate analyses were carried out with CANOCO 5 (ter Braak & Smilauer, 2002) using the same presence-absence OTU matrix to identify possible relations between geography or host and eukaryotic composition in sponge and seawater samples. A Non-Metric Dimensional Scaling (NMDS) plot was created to summarize the similarity in species (OTUs) composition between samples.

Phylogeny-based analysis of sponge-associated eukaryotes

For a more detailed phylogenetic analysis of the non-sponge eukaryotic OTUs from sponges we selected OTUs that fulfilled at least one of the following criteria: i) OTUs that had at least one sequence in a sponge sample and ii) OTUs that were present at a relative abundance of more than 0.25% of the reads in seawater. A total of 195 OTUs passed these criteria. Representative sequences of the OTUs were aligned using the SILVA online SINA alignment service (Pruesse*, et al.*, 2007). Each OTU was complemented with the two most closely related 18S rRNA gene sequences as determined by a BLAST search against the NCBI nucleotide database (9 October 2012). Nearest neighbour sequences and published 18S rRNA sponge-derived fungal sequences (Baker*, et al.*, 2009, Simister*, et al.*, 2012) were downloaded from the SILVA database (release 108) and together with aligned OTUs from our own dataset imported into the ARB software package (Ludwig*, et al.*, 2004). Nearest neighbour sequences and published sponge-derived fungal 18S rRNA sequences (87 OTU representative sequences) were used to construct a Bayesian phylogenetic tree. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default program parameters were used, except allowing a minimum block length of three and gaps in 50% of positions. Phylogenetic trees were created by

Bayesian analysis, using MrBayes v3.2 (Ronquist*, et al.*, 2012) at the freely available Bioportal server (www.bioportal.uio.no). All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run, and their values were estimated from the data during the analysis. All Bayesian analyses were initiated with random starting trees and were run for 10^7 generations. The number of chains was set to four and Markov chains were sampled every 1000 iterations. Points prior to convergence were determined graphically and discarded. Calculated trees were imported into ARB and short sequences obtained in this study were subsequently added by use of the ARB parsimony method without changing the tree topology.

Molecular identification of partial sponge 18S rRNA gene sequences

To check the identity of the sponge species, six OTUs that represented the highest number of reads per sponge species (i.e. OTU65: *Suberites massa*, OTU190: *Haliclona xena,* OTU319: *Aplysina aerophoba*, OTU320: *Petrosia ficiformis*, OTU333: *Axinella damicornis* and *Axinella verrucosa* and OTU495: *Halichondria panicea*) were compared with the non-redundant nucleotide database using the Blastn query (Table S3.1). A full length 18S rRNA gene amplicon of *H. panicea* (P1-P3) and *H. xena* (X1-X3) was recently published in another study (Naim*, et al.*, 2014) with accession numbers (KC899022-KC899040). I

Table 3.1 Number of non-sponge reads, observed OTUs and expected OTUs of seawater and sponge samples at a 97% sequence similarity threshold. Sponge *A. damicornis* 1, *A. damicornis* 3 and *A. verrucosa* 1 only contain reads from *Porifera* and were not shown in this table.

Sample Name	Abbrev.	Sponge taxonomic order	No. of total reads	No. of non-sponge reads[a]	non-sponge reads	
					Observed OTUs	Expected OTUs (Chao1)[b]
seawater North Sea	**WNS**		6613	6562	309	47898 ± 5422.9
H. panicea 1	**P1**	*Halichondrida*	12418	3	3	6 ± 4.3
H. panicea 2	**P2**	*Halichondrida*	8389	161	18	171 ± 74.2
H. panicea 3	**P3**	*Halichondrida*	7214	259	18	171 ± 74.2
H. xena 1	**X1**	*Haplosclerida*	3728	42	13	91 ± 45.1
H. xena 2	**X2**	*Haplosclerida*	4155	24	9	45 ± 25.5
H. xena 3	**X3**	*Haplosclerida*	5381	39	10	55 ± 30.0
S. massa 1	**M1**	*Hadromerida*	14265	57	21	231 ± 93.9
S. massa 2	**M2**	*Hadromerida*	19955	35	13	91 ± 45.1
S. massa 3	**M3**	*Hadromerida*	10961	20	16	136 ± 62.0
seawater Mediterranean Sea	**WMS**		2023	1935	54	1485 ± 393.1
A. aerophoba 1	**A1**	*Verongida*	14900	567	33	561 ± 186.7
A. aerophoba 2	**A2**	*Verongida*	15285	1757	38	741 ± 231.2
A. aerophoba 3	**A3**	*Verongida*	17472	410	13	91 ± 45.1
A. damicornis 2	**D2**	*Halichondrida*	15572	7	6	21 ± 13.5
A. verrucosa 2	**V2**	*Halichondrida*	13701	1	1	1 ± 0.0
A. verrucosa 3	**V3**	*Halichondrida*	130365	2	2	3 ± 2.0
P. ficiformis 1	**F1**	*Haplosclerida*	5704	2367	42	903 ± 268.9
P. ficiformis 2	**F2**	*Haplosclerida*	4623	2196	34	595 ± 195.3
P. ficiformis 3	**F3**	*Haplosclerida*	4462	2978	30	465 ± 161.6

a - non-sponge reads are defined as reads not classified as *Porifera*

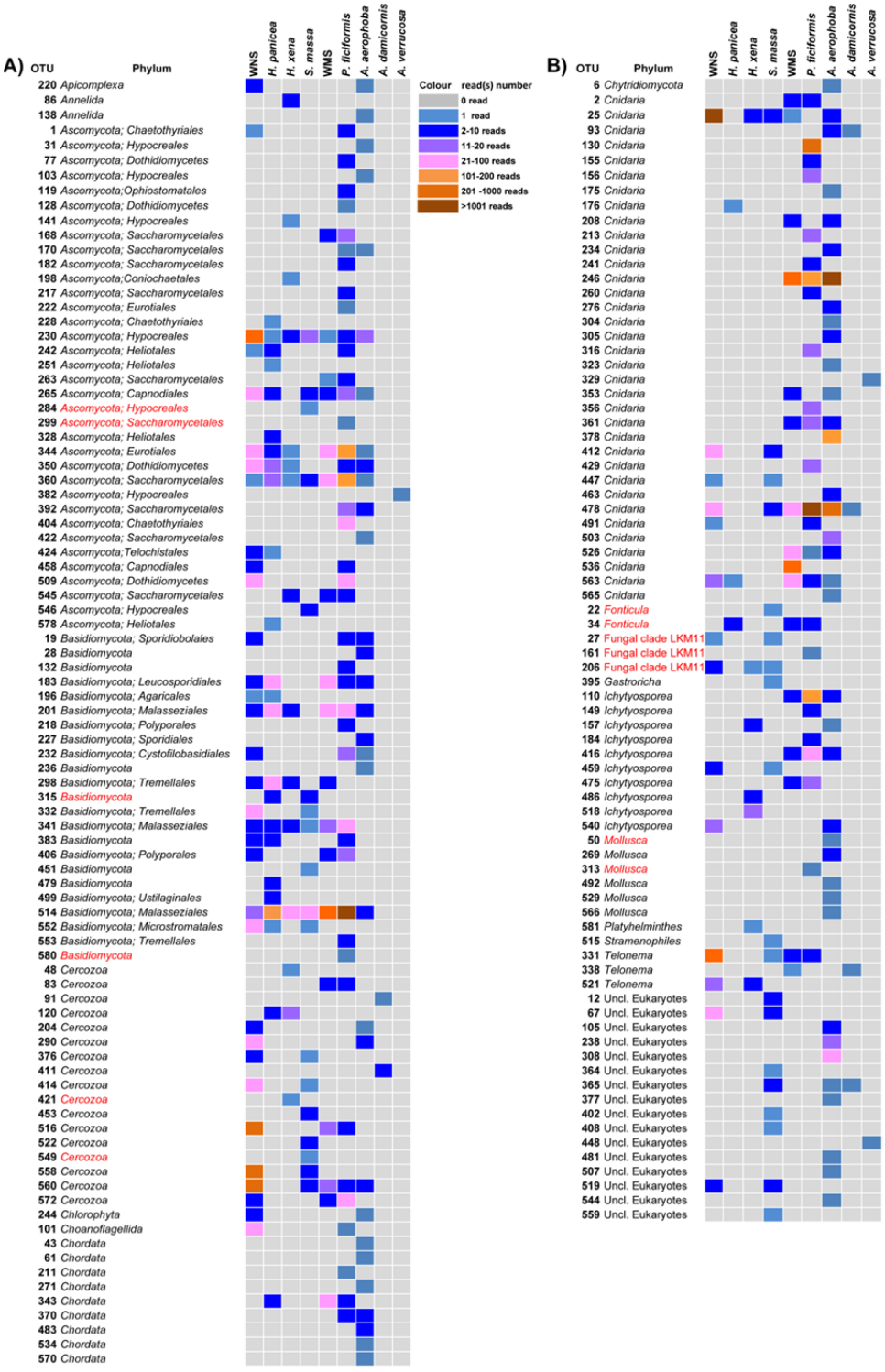
b - bias-corrected form for the Chao1; see Chao (2006)

Results

Community structure

After DNA sequence quality filtering, a total of 350,341 non-chimeric reads were retained. In total, 330,919 sequences (135 OTUs), contributing to nearly 93% of all reads, were either derived from sponges (330,884 reads) or unclassifiable (35 reads). These were subsequently removed from the data set, leaving 19,422 high-quality non-sponge eukaryotic sequences (443 OTUs). From the 443 classifiable non-sponge OTUs, 168 OTUs were retrieved from sponge specimen, whereas the other 275 were solely comprised of seawater-derived sequences (Figure 3.1.A and Figure 3.1.B). Members of 18 eukaryotic phyla and approximate phylum-level groups not formally recognized were detected in sponges and seawater including fungi (*Ascomycota, Basidiomycota, Chytridiomycota,* fungal clade LKM11), protists (*Apicomplexa, Cercozoa, Choanoflagellida, Mesomycetozoea*), algae (*Chlorophyta, Stramenopiles*) and other eukaryotes *(Annelida*, *Chordata, Cnidaria, Fonticula, Gastroricha, Mollusca, Platyhelminthes, and Telonema*) (Figure 3.2). All reads for Ad1, Ad3 and Av1 were sponge sequences, and no other eukaryotic sequences were obtained for these samples. Relative abundance of non-sponge eukaryotes is much higher in some of the tested sponges, most notably in *Petrosia ficiformis* and *Aplysina aerophoba*, which correspond to the known High Microbial Abundance (HMA) sponge species used (Hentschel*, et al.*, 2003). Higher eukaryotic richness (Chao1) was estimated from North Sea (47898 ± 5422.9) and Mediterranean seawater (1485 ± 393.1) in comparison to sponges, where predicted richness was between 1 ± 0.0 (V2) to 903 ± 268.9 (F1) in different sponge samples (Table 3.1).

Figure 3.1A-B Heatmap of non-sponge eukaryote OTUs from North Sea sponges and Mediterranean Sea sponges that are present either “only in sponges” or in “sponges and seawater” at phylum level. Samples were pooled per sponge species (*i.e*. P1+P2+P3, etc). Taxonomic affiliation is shown at phylum level except for fungi where order level taxonomy is also shown. Some OTUs are re-classified based on Bayesian analysis (in red, see also Figure 3.3). Figure 3.1A shows phyla *Apicomplexa*, *Annelida, Ascomycota, Basidiomycota, Cercozoa, Chlorophyta, Choanoflagellida* and *Chordata*, while 3.1B shows *Chytridiomycota*, *Cnidaria*, *Fonticula*, fungal clade LKM11, *Mesomycetozoa*, *Mollusca*, *Platyhelminthes*, *Stramenopiles*, *Telonema* and unclassified eukaryotes.


A)
OTU
Phylum
WNS
H. panicea
H. xena
S. massa
WMS
P. ficiformis
A. aerophoba
A. damicornis
A. verrucosa
220 Apicomplexa
86 Annelida
138 Annelida
1 Ascomycota; Chaetothyriales
31 Ascomycota; Hypocreales
77 Ascomycota; Dothidiomycetes
103 Ascomycota; Hypocreales
119 Ascomycota;Ophiostomatales
128 Ascomycota; Dothidiomycetes
141 Ascomycota; Hypocreales
168 Ascomycota; Saccharomycetales
170 Ascomycota; Saccharomycetales
182 Ascomycota; Saccharomycetales
198 Ascomycota;Coniochaetales
217 Ascomycota; Saccharomycetales
222 Ascomycota; Eurotiales
228 Ascomycota; Chaetothyriales
230 Ascomycota; Hypocreales
242 Ascomycota; Heliotales
251 Ascomycota; Heliotales
263 Ascomycota; Saccharomycetales
265 Ascomycota; Capnodiales
284 Ascomycota; Hypocreales
299 Ascomycota; Saccharomycetales
328 Ascomycota; Heliotales
344 Ascomycota; Eurotiales
350 Ascomycota; Dothidiomycetes
360 Ascomycota; Saccharomycetales
382 Ascomycota; Hypocreales
392 Ascomycota; Saccharomycetales
404 Ascomycota; Chaetothyriales
422 Ascomycota; Saccharomycetales
424 Ascomycota;Telochistales
458 Ascomycota; Capnodiales
509 Ascomycota; Dothidiomycetes
545 Ascomycota; Saccharomycetales
546 Ascomycota; Hypocreales
578 Ascomycota; Heliotales
19 Basidiomycota; Sporidiobolales
28 Basidiomycota
132 Basidiomycota
183 Basidiomycota; Leucosporidiales
196 Basidiomycota; Agaricales
201 Basidiomycota; Malasseziales
218 Basidiomycota; Polyporales
227 Basidiomycota; Sporidiales
232 Basidiomycota; Cystofilobasidiales
236 Basidiomycota
298 Basidiomycota; Tremellales
315 Basidiomycota
332 Basidiomycota; Tremellales
341 Basidiomycota; Malasseziales
383 Basidiomycota
406 Basidiomycota; Polyporales
451 Basidiomycota
479 Basidiomycota
499 Basidiomycota; Ustilaginales
514 Basidiomycota; Malasseziales
552 Basidiomycota; Microstromatales
553 Basidiomycota; Tremellales
580 Basidiomycota
48 Cercozoa
83 Cercozoa
91 Cercozoa
120 Cercozoa
204 Cercozoa
290 Cercozoa
376 Cercozoa
411 Cercozoa
414 Cercozoa
421 Cercozoa
453 Cercozoa
516 Cercozoa
522 Cercozoa
549 Cercozoa
558 Cercozoa
560 Cercozoa
572 Cercozoa
244 Chlorophyta
101 Choanoflagellida
43 Chordata
61 Chordata
211 Chordata
271 Chordata
343 Chordata
370 Chordata
483 Chordata
534 Chordata
570 Chordata
Colour
read(s) number
0 read
1 read
2-10 reads
11-20 reads
21-100 reads
101-200 reads
201 -1000 reads
>1001 reads
B)
OTU
Phylum
WNS
H. panicea
H. xena
S. massa
WMS
P. ficiformis
A. aerophoba
A. damicornis
A. verrucosa
6 Chytridiomycota
2 Cnidaria
25 Cnidaria
93 Cnidaria
130 Cnidaria
155 Cnidaria
156 Cnidaria
175 Cnidaria
176 Cnidaria
208 Cnidaria
213 Cnidaria
234 Cnidaria
241 Cnidaria
246 Cnidaria
260 Cnidaria
276 Cnidaria
304 Cnidaria
305 Cnidaria
316 Cnidaria
323 Cnidaria
329 Cnidaria
353 Cnidaria
356 Cnidaria
361 Cnidaria
378 Cnidaria
412 Cnidaria
429 Cnidaria
447 Cnidaria
463 Cnidaria
478 Cnidaria
491 Cnidaria
503 Cnidaria
526 Cnidaria
536 Cnidaria
563 Cnidaria
565 Cnidaria
22 Fonticula
34 Fonticula
27 Fungal clade LKM11
161 Fungal clade LKM11
206 Fungal clade LKM11
395 Gastroricha
110 Ichtyosporea
149 Ichtyosporea
157 Ichtyosporea
184 Ichtyosporea
416 Ichtyosporea
459 Ichtyosporea
475 Ichtyosporea
486 Ichtyosporea
518 Ichtyosporea
540 Ichtyosporea
50 Mollusca
269 Mollusca
313 Mollusca
492 Mollusca
529 Mollusca
566 Mollusca
581 Platyhelminthes
515 Stramenophiles
331 Telonema
338 Telonema
521 Telonema
12 Uncl. Eukaryotes
67 Uncl. Eukaryotes
105 Uncl. Eukaryotes
238 Uncl. Eukaryotes
308 Uncl. Eukaryotes
364 Uncl. Eukaryotes
365 Uncl. Eukaryotes
377 Uncl. Eukaryotes
402 Uncl. Eukaryotes
408 Uncl. Eukaryotes
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519 Uncl. Eukaryotes
544 Uncl. Eukaryotes
559 Uncl. Eukaryotes

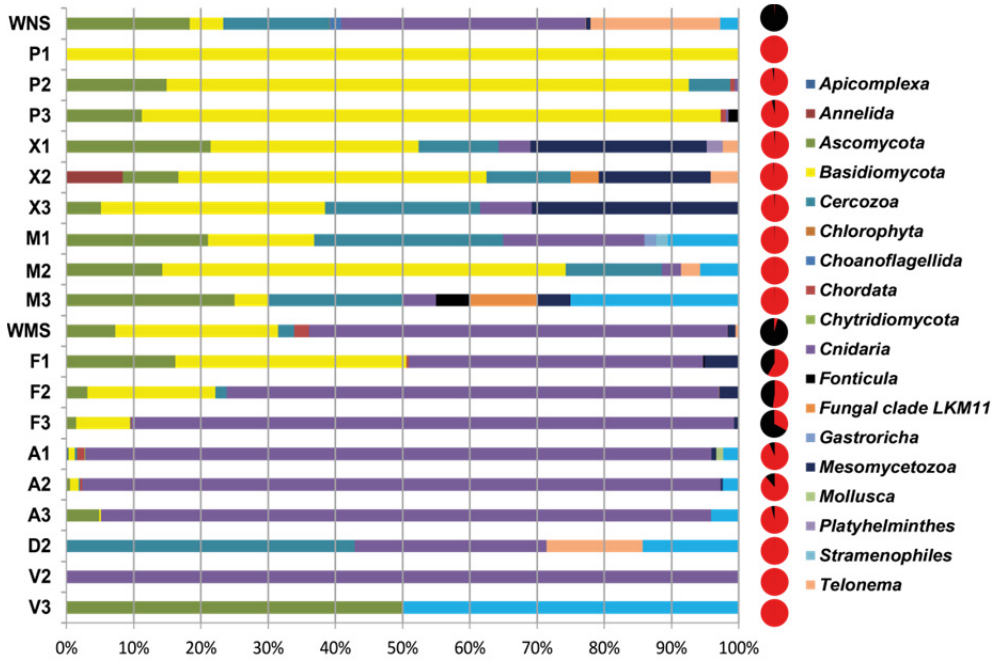


Figure 3.2 Taxonomic distribution of non-sponge eukaryotic reads that are present either only in sponges or in sponges and seawater at the phylum level using the QIIME-compatible version of the Silva 104 release (168 OTUs). OTUs with reads only from seawater are not shown. The resolution of taxonomic affiliation of operational taxonomic units (OTUs) was enhanced and corrected by importing these OTUs by parsimony in a Bayesian tree of related sequences (see Figure 3.3). Pie charts indicate percentage of non-sponge eukaryotes (black) and sponge (red) sequences per sample. WNS refers to North Sea seawater, 'P2-P3' refers to *H. panicea*, 'X2-X3' refers to *H. xena*, 'M1-M3' refers to *S. massa*, 'WMS' refers to Mediterranean Sea seawater, 'F1-F3' refers to *Petrosia ficiformis* and 'A1-A3' refers to *Aplysina aerophoba*, D2 refers to *Axinella damicornis* and V2-V3 refers to *Axinella verrucosa*.

Novel lineages of fungi in sponges and seawater

168 non-sponge OTUs obtained in this study were selected (based on criteria described in Materials and Methods) and characterized in more detail by Bayesian inference of phylogeny. 59 OTUs that were found either solely in sponges or both in sponges and seawater could be classified as true fungi (*Ascomycota*, *Basidiomycota* and *Chytridiomycota*, Figure 3.1.A and Figure 3.1.B) (Hibbett*, et al.*, 2007). In addition, we found a large number of yeast OTUs within both the *Ascomycota* (10 OTUs) and the *Basidiomycota* (10 OTUs) (Figure 3.F-3.H), with well-supported posterior probability values suggesting novel lineages at least at species level. Phylogenetic analysis (Figure 3.E to 3.H) also revealed a number of orders of fungi, namely *Cystofilobasidiales, Leucosporidiales, Microstromatales, Ophiostomatales, Sporidiales*, *Teloschistales* and *Ustilaginales,* which to our best knowledge have never been described in sponges before.

Effect of sponge host on associated eukaryotic communities

In order to assess overall similarity of eukaryotic communities associated with different sponges as well as the surrounding seawater, we performed unconstrained multivariate analysis using NMDS based on Bray-Curtis similarities. This analysis revealed that there was no clear distinction between eukaryotic communities in seawater and those from sponges, however, there was clear clustering of specimen from the same sponge species (Figure 3.4). In addition, there was no indication of geography-based clustering of sponge host-associated eukaryotic communities.

Discussion

Molecular analysis provides a different view of the eukaryotic communities associated with marine sponges than the cultivation-based studies reported up to now. The 18S rRNA gene targeted primers used in this study (FF390 and FR1) were chosen based on their presumed specificity for fungi (Vainio & Hantula, 2000). However, these primers have also previously been found to amplify the gene fragment from other eukaryotes (Hoshino & Morimoto, 2010, Chemidlin Prévost-Bouré*, et al.*, 2011). Hence, amplicons provide descriptive measures of both the fungal community and more generally the eukaryotic community associated with sponges. To this end, it has been shown that the FR1/FF390 primer set provides the best balance between

fungi-specificity and coverage for a short amplicon (tested in silico), and that it provides comparable and reproducible estimates of fungal relative abundance as compared to other primer sets in the literature such as primers to aplify amplicons ofITS1 and ITS2 (Mello*, et al.*, 2011, Krüger*, et al.*, 2012, Lindahl*, et al.*, 2013). Furthermore, it circumvents reproducibility limitations due to length polymorphism associated with the internal transcribed spacer region that is often used for fungal diversity studies (Chemidlin Prévost-Bouré*, et al.*, 2011).

Figure 3.3A-H Bayesian phylogram based on 16S rRNA gene sequences of 218 selected OTUs including i) their nearest neighbors, ii) 18S rRNA sequences published by Simister and co-workers (2012) and iii) Baker and co-workers (2009). Numbers in parentheses after the sample type indication refer to the absolute numbers of reads that were included in the corresponding OTU per sample (208 F(0/2/0) WMS (6) means that two reads of F2 and six reads of Mediterranean seawater are included in OTU208). Grey boxes indicate sponge-specific clusters (SSC) as defined by Simister and co-workers (2012). Yellow boxes represent yeasts, and putative novel yeasts identified from this study are shown in bold red . Taxonomic groups marked with an asterisk (*) indicate non-formal taxonomic classification (*i.e.* fungal environmental clade LKM11 is not a phylum, but a group assigned for environmental fungal sequences (see (Lara*, et al.*, 2010)). The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values of <50 are not indicated. Order level phylogeny is shown for the fungal phyla *Ascomycota* and *Basidiomycota*.

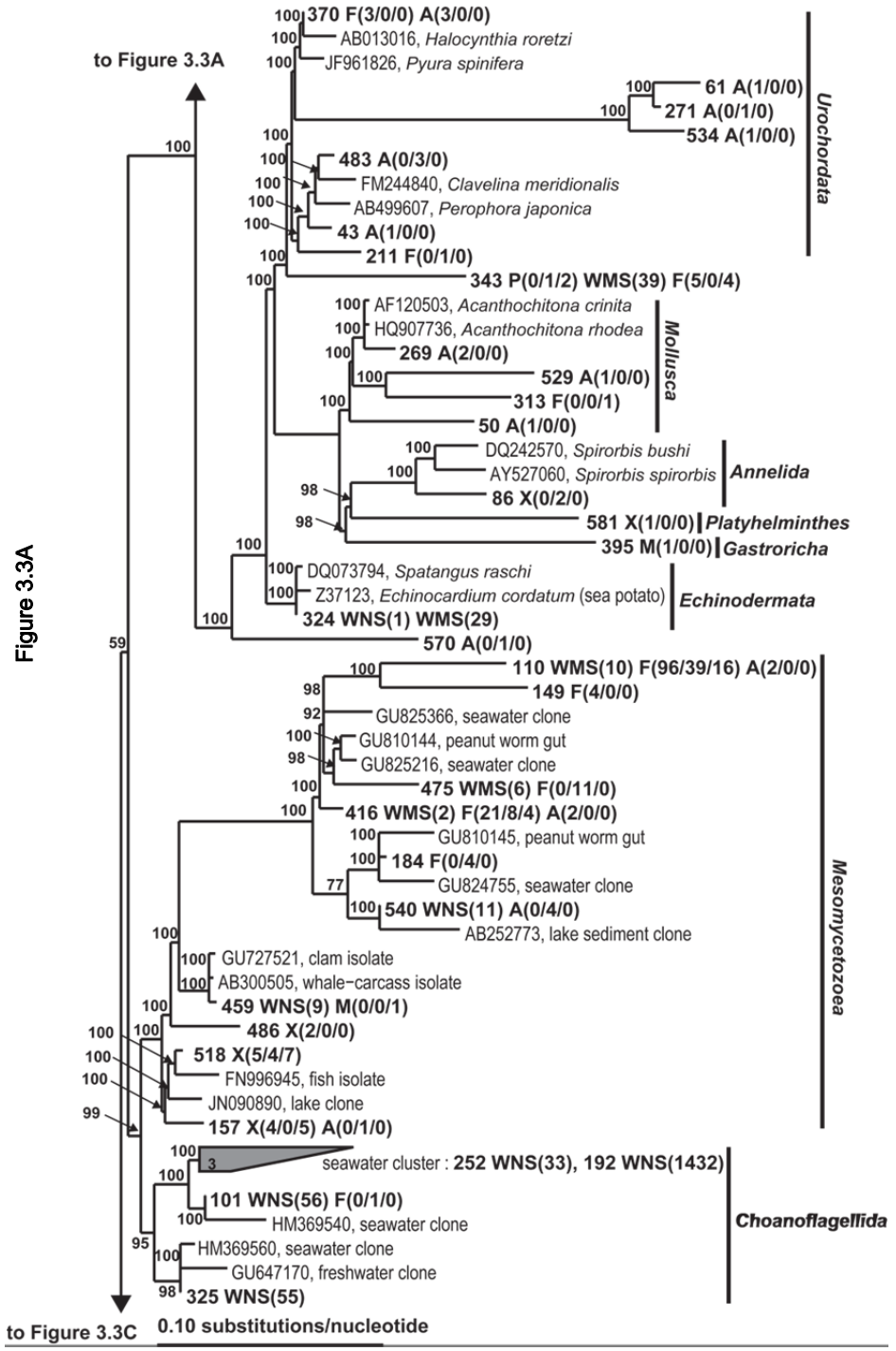

to Figure 3.3A
370 F(3/0/0) A(3/0/0)
AB013016, Halocynthia roretzi
JF961826, Pyura spinifera
61 A(1/0/0)
271 A(0/1/0)
534 A(1/0/0)
Urochordata
483 A(0/3/0)
FM244840, Clavelina meridionalis
AB499607, Perophora japonica
43 A(1/0/0)
211 F(0/1/0)
343 P(0/1/2) WMS(39) F(5/0/4)
AF120503, Acanthochitona crinita
HQ907736, Acanthochitona rhodea
269 A(2/0/0)
529 A(1/0/0)
313 F(0/0/1)
50 A(1/0/0)
Mollusca
DQ242570, Spirorbis bushi
AY527060, Spirorbis spirorbis
Annelida
86 X(0/2/0)
581 X(1/0/0)
Platyhelminthes
395 M(1/0/0)
Gastroricha
DQ073794, Spatangus raschi
Z37123, Echinocardium cordatum (sea potato)
Echinodermata
324 WNS(1) WMS(29)
570 A(0/1/0)
110 WMS(10) F(96/39/16) A(2/0/0)
149 F(4/0/0)
GU825366, seawater clone
GU810144, peanut worm gut
GU825216, seawater clone
475 WMS(6) F(0/11/0)
416 WMS(2) F(21/8/4) A(2/0/0)
GU810145, peanut worm gut
184 F(0/4/0)
GU824755, seawater clone
540 WNS(11) A(0/4/0)
AB252773, lake sediment clone
GU727521, clam isolate
AB300505, whale−carcass isolate
459 WNS(9) M(0/0/1)
486 X(2/0/0)
518 X(5/4/7)
FN996945, fish isolate
JN090890, lake clone
157 X(4/0/5) A(0/1/0)
Mesomycetozoea
3
seawater cluster : 252 WNS(33), 192 WNS(1432)
101 WNS(56) F(0/1/0)
HM369540, seawater clone
HM369560, seawater clone
GU647170, freshwater clone
325 WNS(55)
Choanoflagellida
to Figure 3.3C
0.10 substitutions/nucleotide

Figure 3.3A

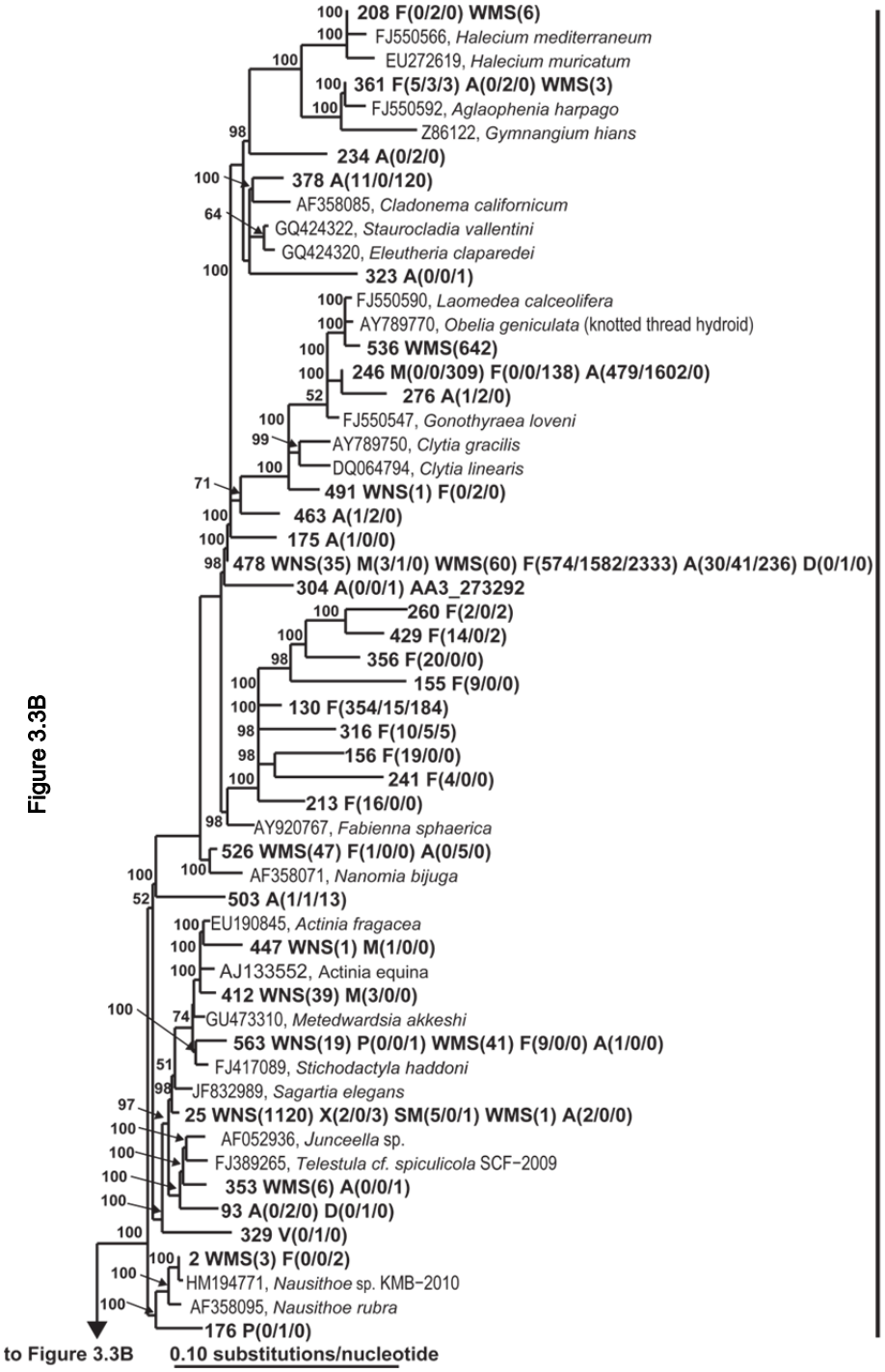

208 F(0/2/0) WMS(6)
FJ550566, Halecium mediterraneum
EU272619, Halecium muricatum
361 F(5/3/3) A(0/2/0) WMS(3)
FJ550592, Aglaophenia harpago
Z86122, Gymnangium hians
234 A(0/2/0)
378 A(11/0/120)
AF358085, Cladonema californicum
GQ424322, Staurocladia vallentini
GQ424320, Eleutheria claparedei
323 A(0/0/1)
FJ550590, Laomedea calceolifera
AY789770, Obelia geniculata (knotted thread hydroid)
536 WMS(642)
246 M(0/0/309) F(0/0/138) A(479/1602/0)
276 A(1/2/0)
FJ550547, Gonothyraea loveni
AY789750, Clytia gracilis
DQ064794, Clytia linearis
491 WNS(1) F(0/2/0)
463 A(1/2/0)
175 A(1/0/0)
478 WNS(35) M(3/1/0) WMS(60) F(574/1582/2333) A(30/41/236) D(0/1/0)
304 A(0/0/1) AA3_273292
260 F(2/0/2)
429 F(14/0/2)
356 F(20/0/0)
155 F(9/0/0)
130 F(354/15/184)
316 F(10/5/5)
156 F(19/0/0)
241 F(4/0/0)
213 F(16/0/0)
AY920767, Fabienna sphaerica
526 WMS(47) F(1/0/0) A(0/5/0)
AF358071, Nanomia bijuga
503 A(1/1/13)
EU190845, Actinia fragacea
447 WNS(1) M(1/0/0)
AJ133552, Actinia equina
412 WNS(39) M(3/0/0)
GU473310, Metedwardsia akkeshi
563 WNS(19) P(0/0/1) WMS(41) F(9/0/0) A(1/0/0)
FJ417089, Stichodactyla haddoni
JF832989, Sagartia elegans
25 WNS(1120) X(2/0/3) SM(5/0/1) WMS(1) A(2/0/0)
AF052936, Junceella sp.
FJ389265, Telestula cf. spiculicola SCF-2009
353 WMS(6) A(0/0/1)
93 A(0/2/0) D(0/1/0)
329 V(0/1/0)
2 WMS(3) F(0/0/2)
HM194771, Nausithoe sp. KMB-2010
AF358095, Nausithoe rubra
176 P(0/1/0)
to Figure 3.3B
0.10 substitutions/nucleotide
Cnidaria

Figure 3.3B

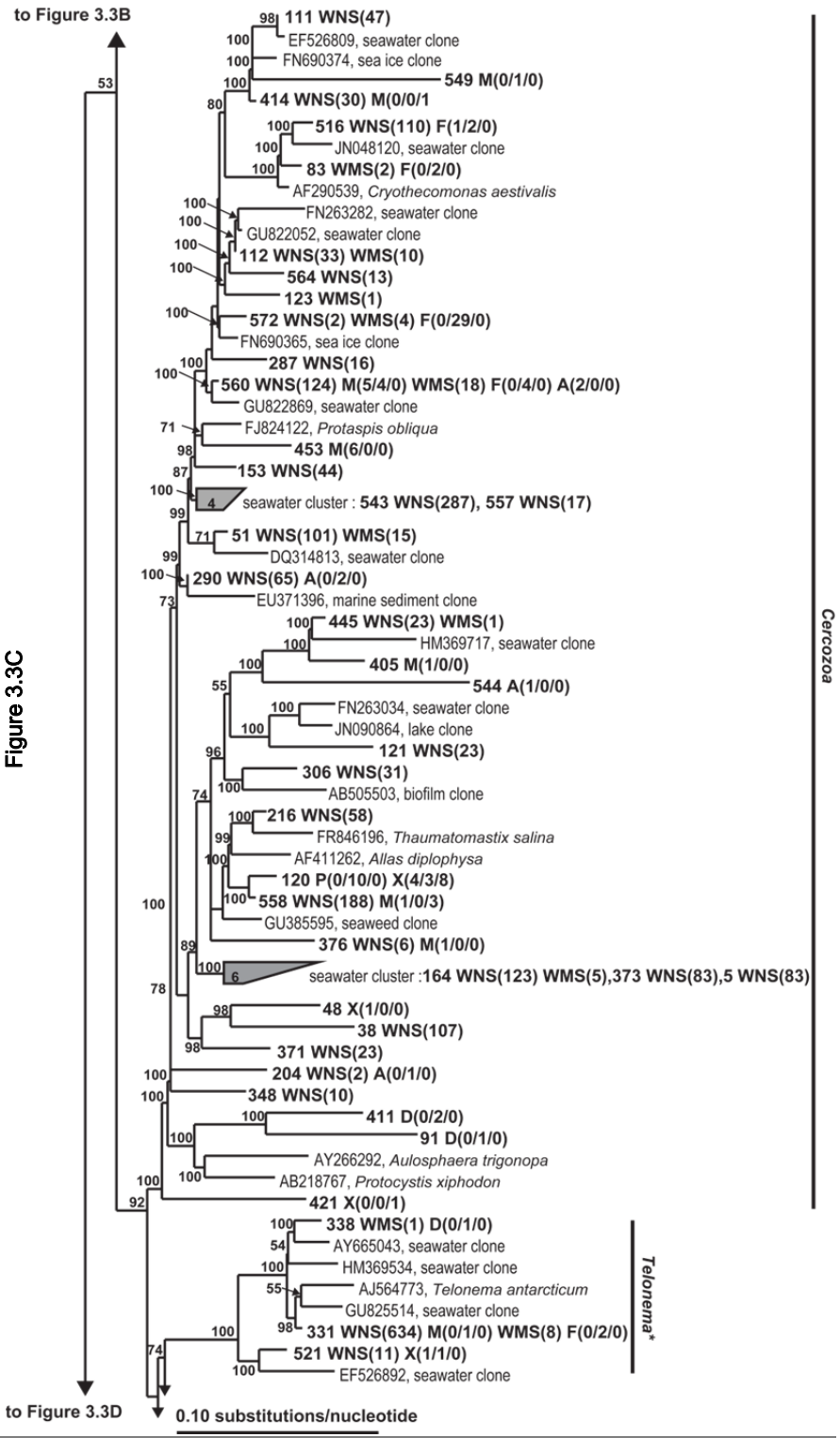

to Figure 3.3B
111 WNS(47)
EF526809, seawater clone
FN690374, sea ice clone
549 M(0/1/0)
414 WNS(30) M(0/0/1
516 WNS(110) F(1/2/0)
JN048120, seawater clone
83 WMS(2) F(0/2/0)
AF290539, Cryothecomonas aestivalis
FN263282, seawater clone
GU822052, seawater clone
112 WNS(33) WMS(10)
564 WNS(13)
123 WMS(1)
572 WNS(2) WMS(4) F(0/29/0)
FN690365, sea ice clone
287 WNS(16)
560 WNS(124) M(5/4/0) WMS(18) F(0/4/0) A(2/0/0)
GU822869, seawater clone
FJ824122, Protaspis obliqua
453 M(6/0/0)
153 WNS(44)
4 seawater cluster : 543 WNS(287), 557 WNS(17)
51 WNS(101) WMS(15)
DQ314813, seawater clone
290 WNS(65) A(0/2/0)
EU371396, marine sediment clone
445 WNS(23) WMS(1)
HM369717, seawater clone
405 M(1/0/0)
544 A(1/0/0)
FN263034, seawater clone
JN090864, lake clone
121 WNS(23)
306 WNS(31)
AB505503, biofilm clone
216 WNS(58)
FR846196, Thaumatomastix salina
AF411262, Allas diplophysa
120 P(0/10/0) X(4/3/8)
558 WNS(188) M(1/0/3)
GU385595, seaweed clone
376 WNS(6) M(1/0/0)
6 seawater cluster :164 WNS(123) WMS(5),373 WNS(83),5 WNS(83)
48 X(1/0/0)
38 WNS(107)
371 WNS(23)
204 WNS(2) A(0/1/0)
348 WNS(10)
411 D(0/2/0)
91 D(0/1/0)
AY266292, Aulosphaera trigonopa
AB218767, Protocystis xiphodon
421 X(0/0/1)
338 WMS(1) D(0/1/0)
AY665043, seawater clone
HM369534, seawater clone
AJ564773, Telonema antarcticum
GU825514, seawater clone
331 WNS(634) M(0/1/0) WMS(8) F(0/2/0)
521 WNS(11) X(1/1/0)
EF526892, seawater clone
Cercozoa
Telonema*
to Figure 3.3D
0.10 substitutions/nucleotide

Figure 3.3C

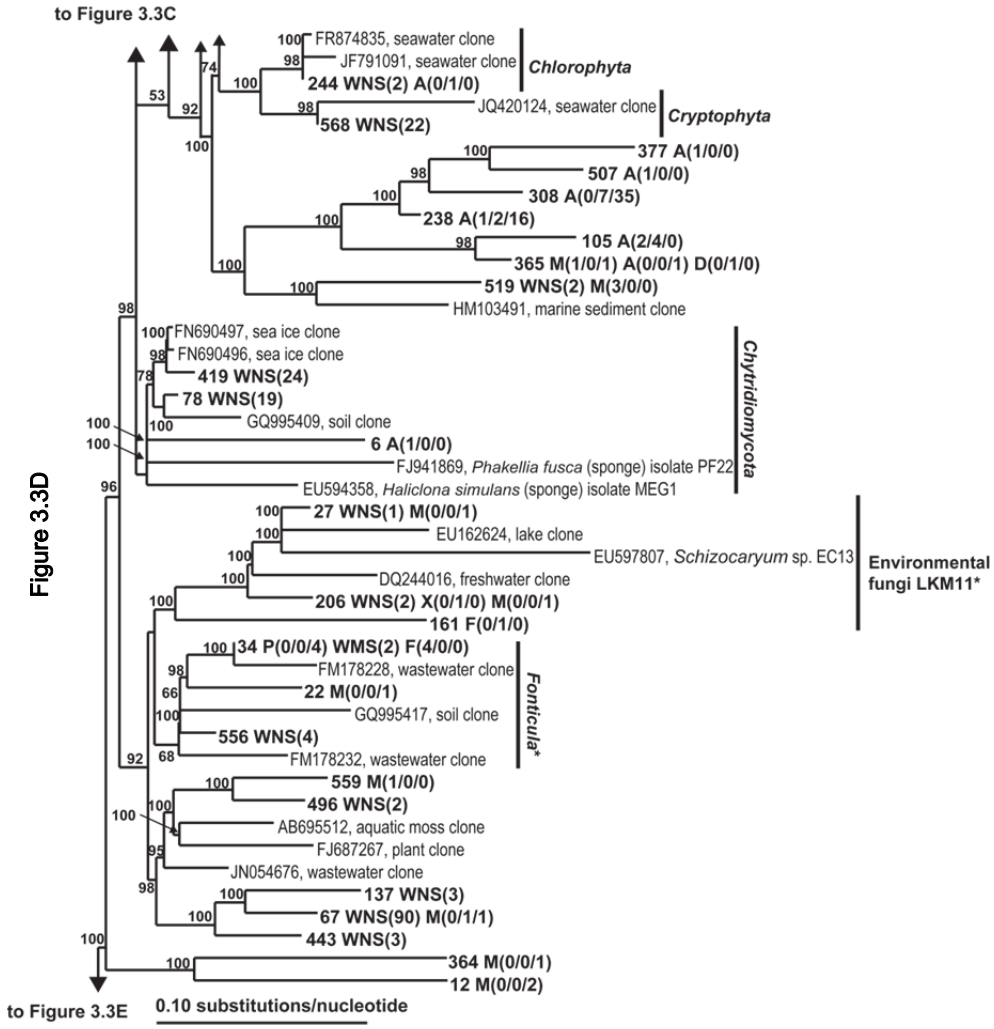
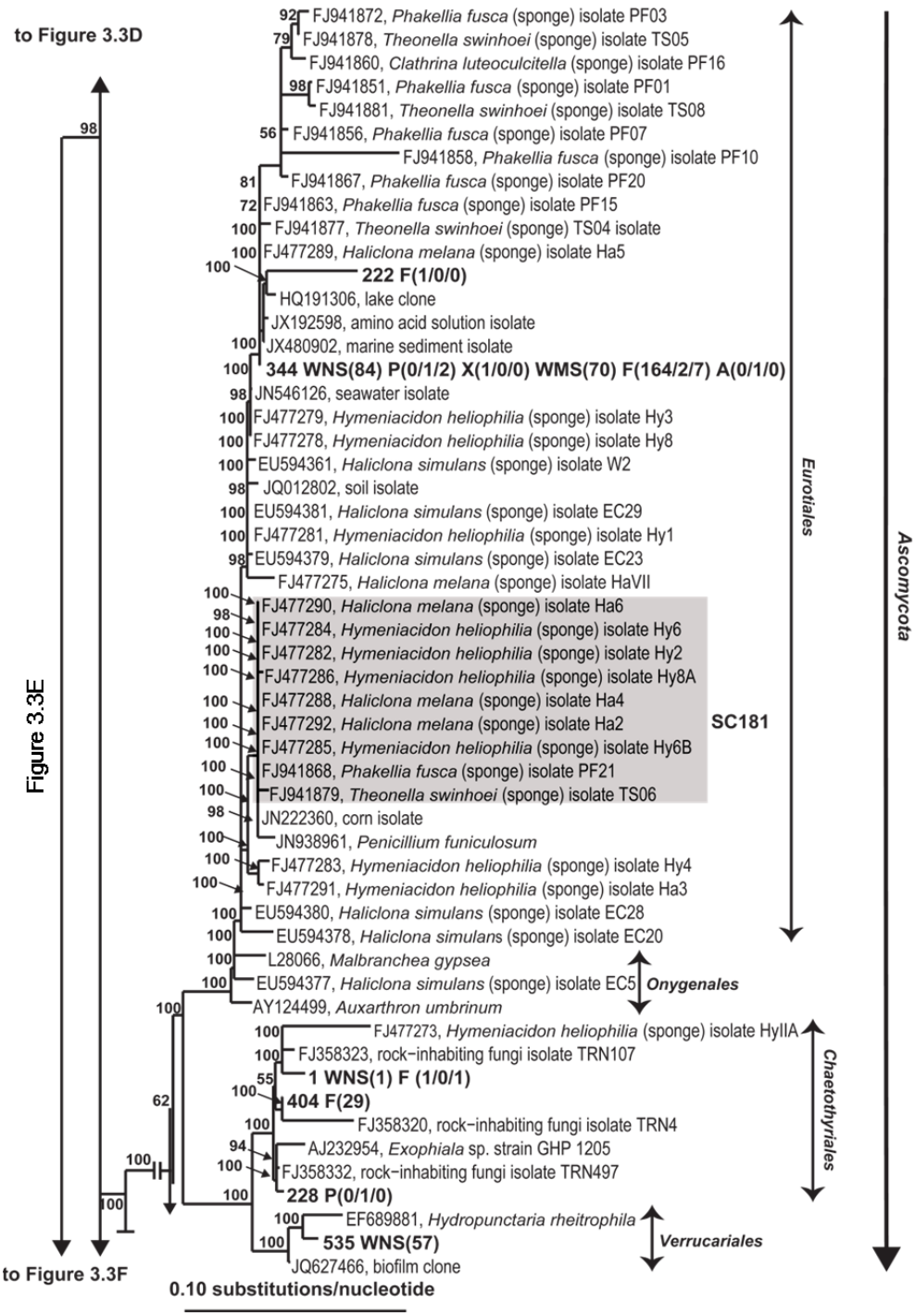
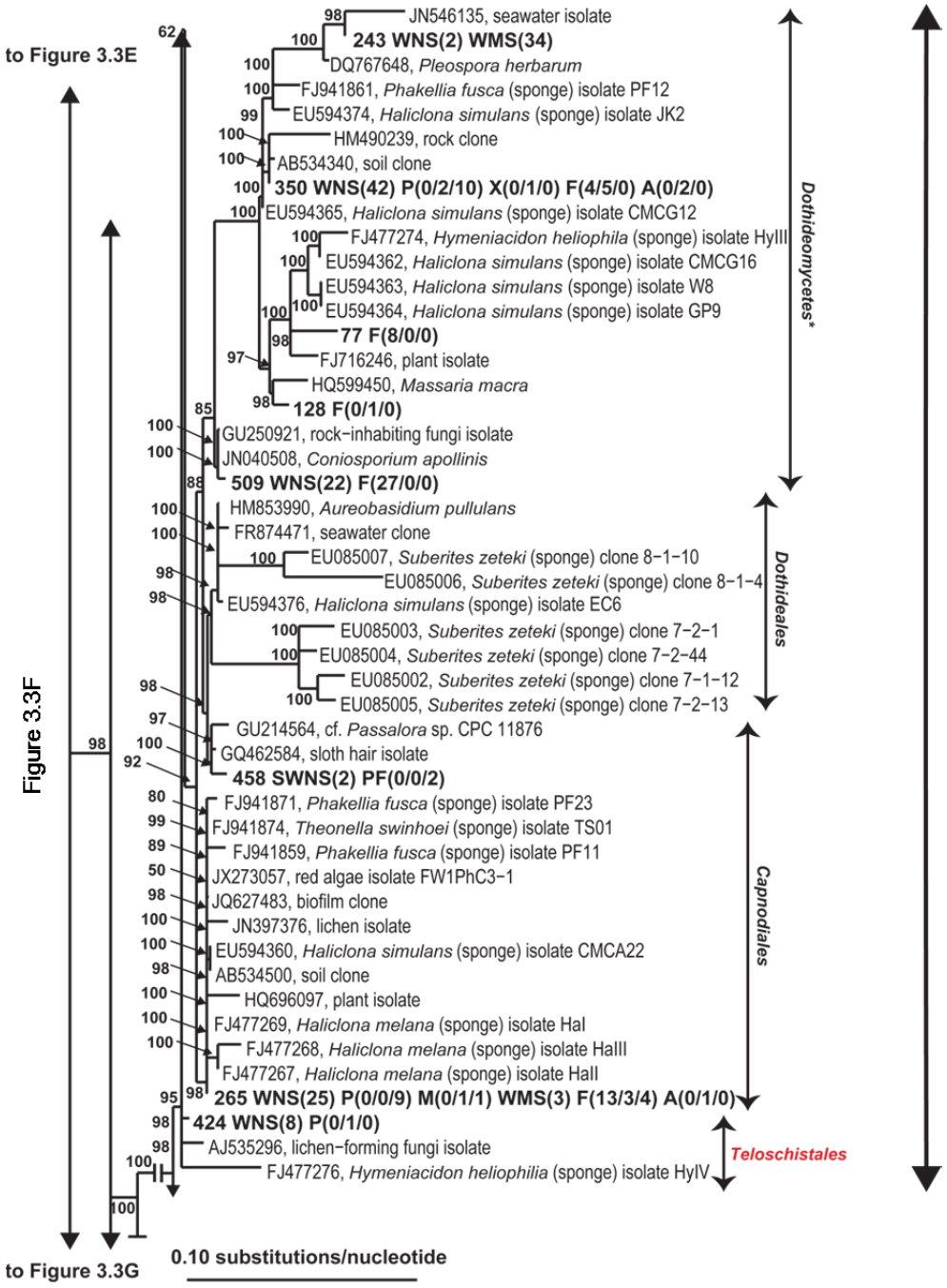

to Figure 3.3C
FR874835, seawater clone
JF791091, seawater clone
Chlorophyta
244 WNS(2) A(0/1/0)
JQ420124, seawater clone
Cryptophyta
568 WNS(22)
377 A(1/0/0)
507 A(1/0/0)
308 A(0/7/35)
238 A(1/2/16)
105 A(2/4/0)
365 M(1/0/1) A(0/0/1) D(0/1/0)
519 WNS(2) M(3/0/0)
HM103491, marine sediment clone
FN690497, sea ice clone
FN690496, sea ice clone
419 WNS(24)
78 WNS(19)
GQ995409, soil clone
6 A(1/0/0)
FJ941869, Phakellia fusca (sponge) isolate PF22
EU594358, Haliclona simulans (sponge) isolate MEG1
Chytridiomycota
27 WNS(1) M(0/0/1)
EU162624, lake clone
EU597807, Schizocaryum sp. EC13
DQ244016, freshwater clone
206 WNS(2) X(0/1/0) M(0/0/1)
161 F(0/1/0)
Environmental fungi LKM11*
34 P(0/0/4) WMS(2) F(4/0/0)
FM178228, wastewater clone
22 M(0/0/1)
GQ995417, soil clone
556 WNS(4)
FM178232, wastewater clone
Fonticula*
559 M(1/0/0)
496 WNS(2)
AB695512, aquatic moss clone
FJ687267, plant clone
JN054676, wastewater clone
137 WNS(3)
67 WNS(90) M(0/1/1)
443 WNS(3)
364 M(0/0/1)
12 M(0/0/2)
to Figure 3.3E
0.10 substitutions/nucleotide

Figure 3.3D


to Figure 3.3D
to Figure 3.3F
Figure 3.3E
FJ941872, Phakellia fusca (sponge) isolate PF03
FJ941878, Theonella swinhoei (sponge) isolate TS05
FJ941860, Clathrina luteoculcitella (sponge) isolate PF16
FJ941851, Phakellia fusca (sponge) isolate PF01
FJ941881, Theonella swinhoei (sponge) isolate TS08
FJ941856, Phakellia fusca (sponge) isolate PF07
FJ941858, Phakellia fusca (sponge) isolate PF10
FJ941867, Phakellia fusca (sponge) isolate PF20
FJ941863, Phakellia fusca (sponge) isolate PF15
FJ941877, Theonella swinhoei (sponge) TS04 isolate
FJ477289, Haliclona melana (sponge) isolate Ha5
222 F(1/0/0)
HQ191306, lake clone
JX192598, amino acid solution isolate
JX480902, marine sediment isolate
344 WNS(84) P(0/1/2) X(1/0/0) WMS(70) F(164/2/7) A(0/1/0)
JN546126, seawater isolate
FJ477279, Hymeniacidon heliophilia (sponge) isolate Hy3
FJ477278, Hymeniacidon heliophilia (sponge) isolate Hy8
EU594361, Haliclona simulans (sponge) isolate W2
JQ012802, soil isolate
EU594381, Haliclona simulans (sponge) isolate EC29
FJ477281, Hymeniacidon heliophilia (sponge) isolate Hy1
EU594379, Haliclona simulans (sponge) isolate EC23
FJ477275, Haliclona melana (sponge) isolate HaVII
FJ477290, Haliclona melana (sponge) isolate Ha6
FJ477284, Hymeniacidon heliophilia (sponge) isolate Hy6
FJ477282, Hymeniacidon heliophilia (sponge) isolate Hy2
FJ477286, Hymeniacidon heliophilia (sponge) isolate Hy8A
FJ477288, Haliclona melana (sponge) isolate Ha4
FJ477292, Haliclona melana (sponge) isolate Ha2
FJ477285, Hymeniacidon heliophilia (sponge) isolate Hy6B
FJ941868, Phakellia fusca (sponge) isolate PF21
FJ941879, Theonella swinhoei (sponge) isolate TS06
SC181
JN222360, corn isolate
JN938961, Penicillium funiculosum
FJ477283, Hymeniacidon heliophilia (sponge) isolate Hy4
FJ477291, Hymeniacidon heliophilia (sponge) isolate Ha3
EU594380, Haliclona simulans (sponge) isolate EC28
EU594378, Haliclona simulans (sponge) isolate EC20
L28066, Malbranchea gypsea
EU594377, Haliclona simulans (sponge) isolate EC5
Onygenales
AY124499, Auxarthron umbrinum
FJ477273, Hymeniacidon heliophilia (sponge) isolate HyIIA
FJ358323, rock−inhabiting fungi isolate TRN107
1 WNS(1) F (1/0/1)
404 F(29)
FJ358320, rock−inhabiting fungi isolate TRN4
AJ232954, Exophiala sp. strain GHP 1205
FJ358332, rock−inhabiting fungi isolate TRN497
228 P(0/1/0)
Chaetothyriales
EF689881, Hydropunctaria rheitrophila
535 WNS(57)
JQ627466, biofilm clone
Verrucariales
Eurotiales
Ascomycota
0.10 substitutions/nucleotide


to Figure 3.3E
JN546135, seawater isolate
243 WNS(2) WMS(34)
DQ767648, Pleospora herbarum
FJ941861, Phakellia fusca (sponge) isolate PF12
EU594374, Haliclona simulans (sponge) isolate JK2
HM490239, rock clone
AB534340, soil clone
350 WNS(42) P(0/2/10) X(0/1/0) F(4/5/0) A(0/2/0)
EU594365, Haliclona simulans (sponge) isolate CMCG12
FJ477274, Hymeniacidon heliophila (sponge) isolate HyIII
EU594362, Haliclona simulans (sponge) isolate CMCG16
EU594363, Haliclona simulans (sponge) isolate W8
EU594364, Haliclona simulans (sponge) isolate GP9
77 F(8/0/0)
FJ716246, plant isolate
HQ599450, Massaria macra
128 F(0/1/0)
GU250921, rock-inhabiting fungi isolate
JN040508, Coniosporium apollinis
509 WNS(22) F(27/0/0)
HM853990, Aureobasidium pullulans
FR874471, seawater clone
EU085007, Suberites zeteki (sponge) clone 8-1-10
EU085006, Suberites zeteki (sponge) clone 8-1-4
EU594376, Haliclona simulans (sponge) isolate EC6
EU085003, Suberites zeteki (sponge) clone 7-2-1
EU085004, Suberites zeteki (sponge) clone 7-2-44
EU085002, Suberites zeteki (sponge) clone 7-1-12
EU085005, Suberites zeteki (sponge) clone 7-2-13
GU214564, cf. Passalora sp. CPC 11876
GQ462584, sloth hair isolate
458 SWNS(2) PF(0/0/2)
FJ941871, Phakellia fusca (sponge) isolate PF23
FJ941874, Theonella swinhoei (sponge) isolate TS01
FJ941859, Phakellia fusca (sponge) isolate PF11
JX273057, red algae isolate FW1PhC3-1
JQ627483, biofilm clone
JN397376, lichen isolate
EU594360, Haliclona simulans (sponge) isolate CMCA22
AB534500, soil clone
HQ696097, plant isolate
FJ477269, Haliclona melana (sponge) isolate HaI
FJ477268, Haliclona melana (sponge) isolate HaIII
FJ477267, Haliclona melana (sponge) isolate HaII
265 WNS(25) P(0/0/9) M(0/1/1) WMS(3) F(13/3/4) A(0/1/0)
424 WNS(8) P(0/1/0)
AJ535296, lichen-forming fungi isolate
FJ477276, Hymeniacidon heliophilia (sponge) isolate HyIV
Dothideomycetes*
Dothideales
Capnodiales
Teloschistales
Figure 3.3F
to Figure 3.3G
0.10 substitutions/nucleotide

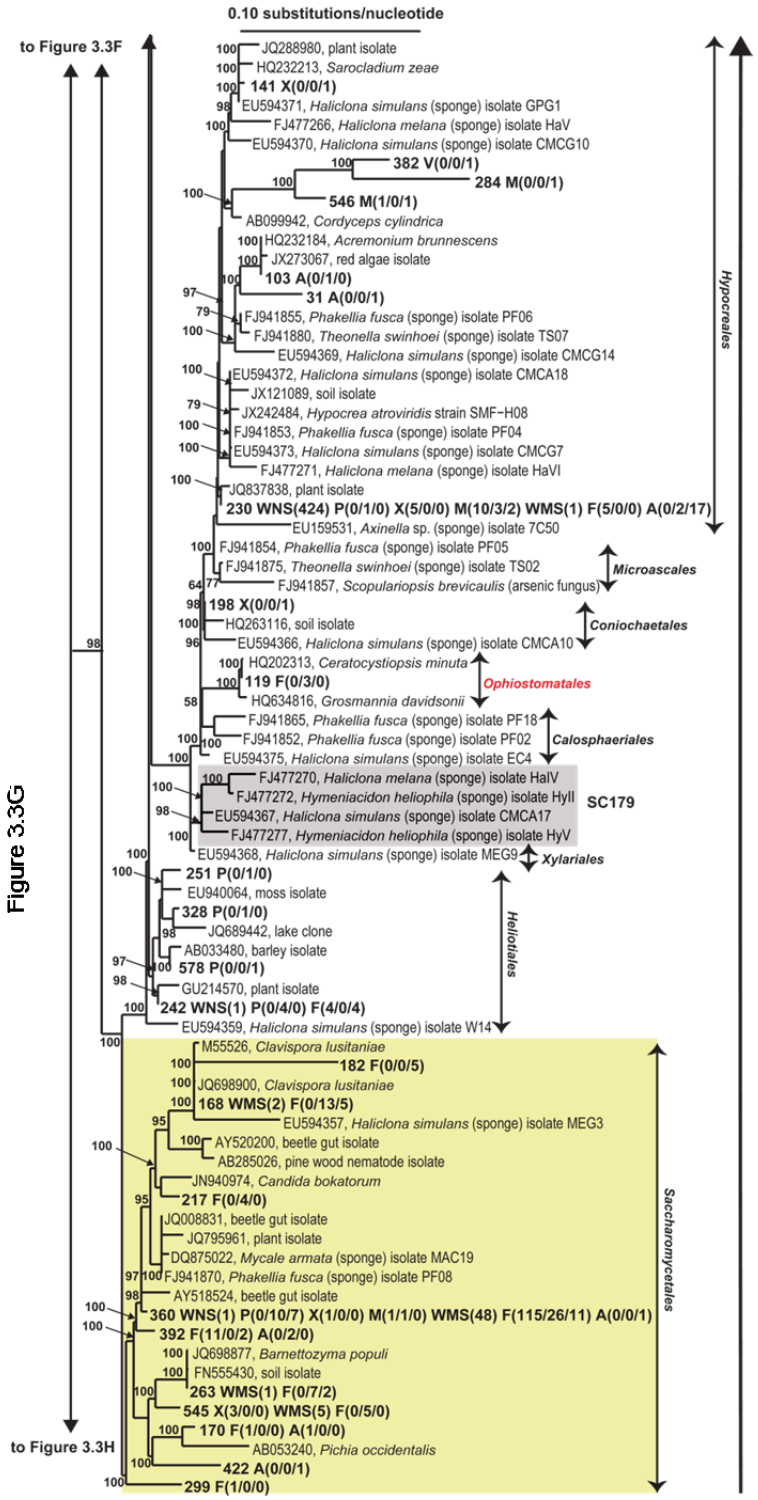

0.10 substitutions/nucleotide
to Figure 3.3F
JQ288980, plant isolate
HQ232213, Sarocladium zeae
141 X(0/0/1)
EU594371, Haliclona simulans (sponge) isolate GPG1
FJ477266, Haliclona melana (sponge) isolate HaV
EU594370, Haliclona simulans (sponge) isolate CMCG10
382 V(0/0/1)
284 M(0/0/1)
546 M(1/0/1)
AB099942, Cordyceps cylindrica
HQ232184, Acremonium brunnescens
JX273067, red algae isolate
103 A(0/1/0)
31 A(0/0/1)
FJ941855, Phakellia fusca (sponge) isolate PF06
FJ941880, Theonella swinhoei (sponge) isolate TS07
EU594369, Haliclona simulans (sponge) isolate CMCG14
EU594372, Haliclona simulans (sponge) isolate CMCA18
JX121089, soil isolate
JX242484, Hypocrea atroviridis strain SMF−H08
FJ941853, Phakellia fusca (sponge) isolate PF04
EU594373, Haliclona simulans (sponge) isolate CMCG7
FJ477271, Haliclona melana (sponge) isolate HaVI
JQ837838, plant isolate
230 WNS(424) P(0/1/0) X(5/0/0) M(10/3/2) WMS(1) F(5/0/0) A(0/2/17)
EU159531, Axinella sp. (sponge) isolate 7C50
FJ941854, Phakellia fusca (sponge) isolate PF05
FJ941875, Theonella swinhoei (sponge) isolate TS02
FJ941857, Scopulariopsis brevicaulis (arsenic fungus)
198 X(0/0/1)
HQ263116, soil isolate
EU594366, Haliclona simulans (sponge) isolate CMCA10
HQ202313, Ceratocystiopsis minuta
119 F(0/3/0)
HQ634816, Grosmannia davidsonii
FJ941865, Phakellia fusca (sponge) isolate PF18
FJ941852, Phakellia fusca (sponge) isolate PF02
EU594375, Haliclona simulans (sponge) isolate EC4
FJ477270, Haliclona melana (sponge) isolate HaIV
FJ477272, Hymeniacidon heliophila (sponge) isolate HyII
EU594367, Haliclona simulans (sponge) isolate CMCA17
FJ477277, Hymeniacidon heliophila (sponge) isolate HyV
EU594368, Haliclona simulans (sponge) isolate MEG9
251 P(0/1/0)
EU940064, moss isolate
328 P(0/1/0)
JQ689442, lake clone
AB033480, barley isolate
578 P(0/0/1)
GU214570, plant isolate
242 WNS(1) P(0/4/0) F(4/0/4)
EU594359, Haliclona simulans (sponge) isolate W14
M55526, Clavispora lusitaniae
182 F(0/0/5)
JQ698900, Clavispora lusitaniae
168 WMS(2) F(0/13/5)
EU594357, Haliclona simulans (sponge) isolate MEG3
AY520200, beetle gut isolate
AB285026, pine wood nematode isolate
JN940974, Candida bokatorum
217 F(0/4/0)
JQ008831, beetle gut isolate
JQ795961, plant isolate
DQ875022, Mycale armata (sponge) isolate MAC19
FJ941870, Phakellia fusca (sponge) isolate PF08
AY518524, beetle gut isolate
360 WNS(1) P(0/10/7) X(1/0/0) M(1/1/0) WMS(48) F(115/26/11) A(0/0/1)
392 F(11/0/2) A(0/2/0)
JQ698877, Barnettozyma populi
FN555430, soil isolate
263 WMS(1) F(0/7/2)
545 X(3/0/0) WMS(5) F(0/5/0)
170 F(1/0/0) A(1/0/0)
AB053240, Pichia occidentalis
422 A(0/0/1)
299 F(1/0/0)
to Figure 3.3H
Hypocreales
Microascales
Coniochaetales
Ophiostomatales
Calosphaeriales
SC179
Xylariales
Heliotiales
Saccharomycetales

Figure 3.3G

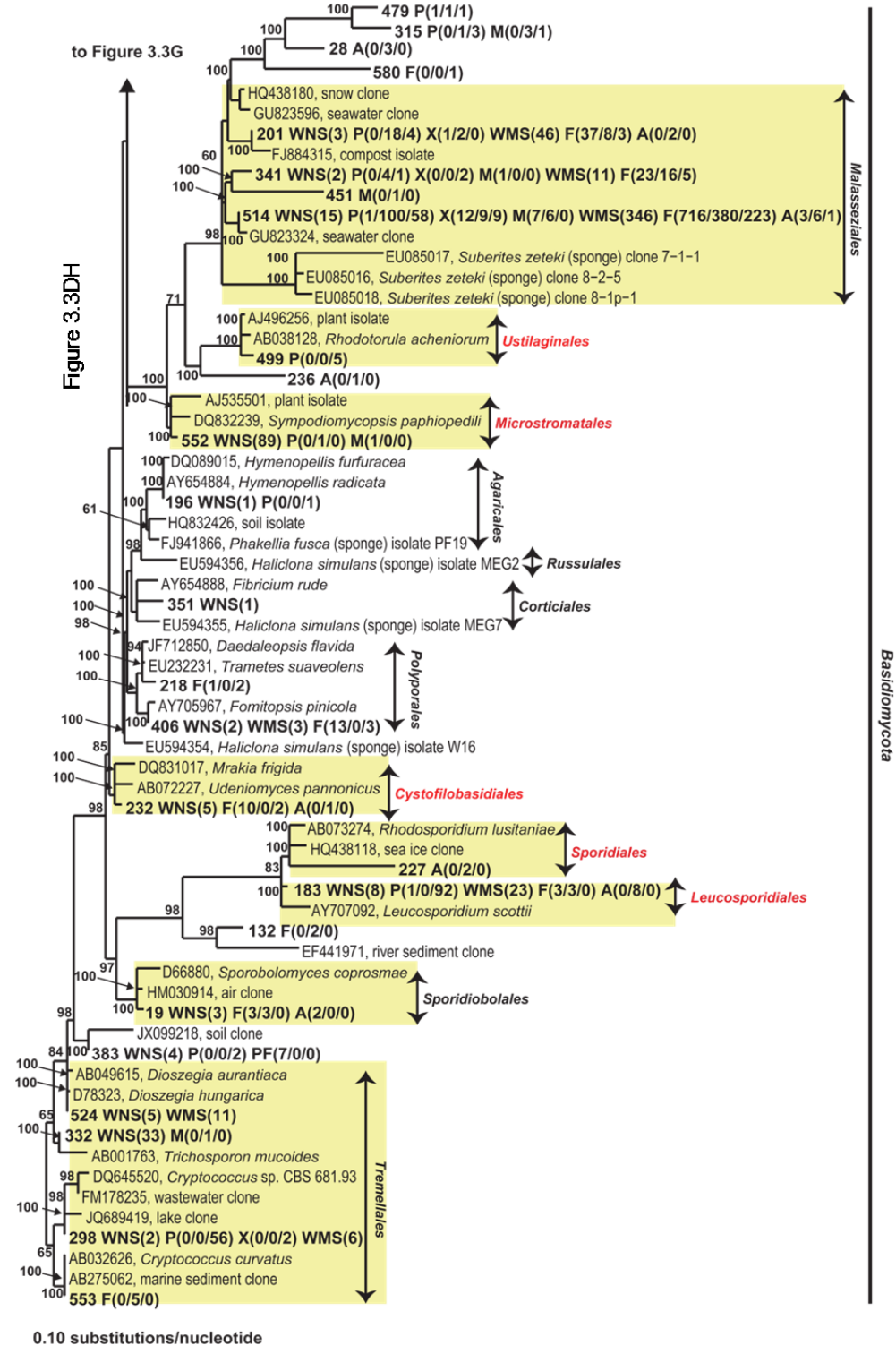


Figure 3.3DH
to Figure 3.3G
479 P(1/1/1)
315 P(0/1/3) M(0/3/1)
28 A(0/3/0)
580 F(0/0/1)
HQ438180, snow clone
GU823596, seawater clone
201 WNS(3) P(0/18/4) X(1/2/0) WMS(46) F(37/8/3) A(0/2/0)
FJ884315, compost isolate
341 WNS(2) P(0/4/1) X(0/0/2) M(1/0/0) WMS(11) F(23/16/5)
451 M(0/1/0)
514 WNS(15) P(1/100/58) X(12/9/9) M(7/6/0) WMS(346) F(716/380/223) A(3/6/1)
GU823324, seawater clone
EU085017, Suberites zeteki (sponge) clone 7−1−1
EU085016, Suberites zeteki (sponge) clone 8−2−5
EU085018, Suberites zeteki (sponge) clone 8−1p−1
Malasseziales
AJ496256, plant isolate
AB038128, Rhodotorula acheniorum
Ustilaginales
499 P(0/0/5)
236 A(0/1/0)
AJ535501, plant isolate
DQ832239, Sympodiomycopsis paphiopedili
Microstromatales
552 WNS(89) P(0/1/0) M(1/0/0)
DQ089015, Hymenopellis furfuracea
AY654884, Hymenopellis radicata
196 WNS(1) P(0/0/1)
HQ832426, soil isolate
FJ941866, Phakellia fusca (sponge) isolate PF19
Agaricales
EU594356, Haliclona simulans (sponge) isolate MEG2
Russulales
AY654888, Fibricium rude
351 WNS(1)
EU594355, Haliclona simulans (sponge) isolate MEG7
Corticiales
JF712850, Daedaleopsis flavida
EU232231, Trametes suaveolens
218 F(1/0/2)
AY705967, Fomitopsis pinicola
406 WNS(2) WMS(3) F(13/0/3)
Polyporales
EU594354, Haliclona simulans (sponge) isolate W16
DQ831017, Mrakia frigida
AB072227, Udeniomyces pannonicus
232 WNS(5) F(10/0/2) A(0/1/0)
Cystofilobasidiales
AB073274, Rhodosporidium lusitaniae
HQ438118, sea ice clone
227 A(0/2/0)
Sporidiales
183 WNS(8) P(1/0/92) WMS(23) F(3/3/0) A(0/8/0)
Leucosporidiales
AY707092, Leucosporidium scottii
132 F(0/2/0)
EF441971, river sediment clone
D66880, Sporobolomyces coprosmae
HM030914, air clone
19 WNS(3) F(3/3/0) A(2/0/0)
Sporidiobolales
JX099218, soil clone
383 WNS(4) P(0/0/2) PF(7/0/0)
AB049615, Dioszegia aurantiaca
D78323, Dioszegia hungarica
524 WNS(5) WMS(11)
332 WNS(33) M(0/1/0)
AB001763, Trichosporon mucoides
DQ645520, Cryptococcus sp. CBS 681.93
FM178235, wastewater clone
JQ689419, lake clone
298 WNS(2) P(0/0/56) X(0/0/2) WMS(6)
AB032626, Cryptococcus curvatus
AB275062, marine sediment clone
553 F(0/5/0)
Tremellales
Basidiomycota
0.10 substitutions/nucleotide

Eukaryotic diversity

The presence of eukaryotes other than fungi in sponges has been previously demonstrated. These studies reported protists (*Alveolata*, *Mesomycetozoea, Rhizaria*), protozoans (*Amoebozoa*, *Apusozoa* and *Euglenozoa*), protophytes (*Cryptophyta*, *Rhodophyta*, *Viridiplantae*), *Cnidaria, Annelida* and *Stramenopiles* (Webster*, et al.*, 2004, Sipkema & Blanch, 2010, Li*, et al.*, 2014, Wang*, et al.*, 2014). One of the highlights of the present study was the detection of taxonomic lineages within the eukaryotic community of shallow water sponges that were previously only reported for deep-sea sponges (Li*, et al.*, 2014, Wang*, et al.*, 2014). In particular this concerns the class of *Mesomycetozoea* (previously known as *Ichtyosporea* (Mendoza*, et al.*, 2002) and the phylum *Cercozoa* (supergroup *Rhizaria*), which have been recorded in high numbers (31.0% and 15.0%, respectively) of the total 18S rRNA reads, from a deep sea sponge *Lamellomorpha* sp. by whole metagenome sequencing (Li*, et al.*, 2014). Both of these taxa are fungus-like organisms, the taxonomy of which was proposed based solely on molecular phylogenetic analyses rather than on morphological characteristics (Ragan*, et al.*, 1996, Cavalier-Smith, 1997). *Mesomycetozoea* is a distinct class of protists near to the point where fungi and animals diverged (Ragan*, et al.*, 1996, Lord*, et al.*, 2012). The lifestyle of *Mesomycetozoea* is commonly parasitic and it has been shown to be the etiological agent of disease in fish, in which resistant cysts are formed in major organs and subsequently kill fish either because infected fish are unable to take up oxygen due to the large numbers of cysts in the gills or because vital organs become infected (Ragan*, et al.*, 1996, Hershberger*, et al.*, 2008, Andreou*, et al.*, 2012). They have also been found in other marine organisms, such as mussel (Takishita*, et al.*, 2008). Of at least 30 known genera of *Mesomycetozoea* none is known to be free-living, and only a few (at least 5 genera) representatives have been cultured *in vitro* (Glockling*, et al.*, 2012). Members of the *Cercozoa* on the other hand are both free-living and parasitic. Parasitic cercozoans can infect plants, invertebrate animals and other protists, whereas free-living *Cercozoa* can feed on bacteria, fungi, algae, other protozoa or even microscopic animals (Bass & Cavalier-Smith, 2004, Chantangsi*, et al.*, 2010). Higher richness of cercozoan OTUs detected from seawater in comparison to sponges may be caused by the much lower sequence depth of non-sponge reads in sponges than in seawater samples, but could

sugges that only some are retained in the sponge holobiont. This could potentially be a breakthrough in the study of sponge-related diseases as numerous studies on sponge disease have failed to identify the etiological agent (Luter*, et al.*, 2010a, Luter*, et al.*, 2010b, Angermeier*, et al.*, 2011). Furthermore, discovery of lower fungi (*i.e.* fungus-like organisms not included in the kingdom Fungi (see (Taylor*, et al.*, 2000, Hibbett*, et al.*, 2007)) and other fungal-like organisms such *Cercozoa, Fonticula, Mesomycetozoea,* and *Telonema* (Figure 3.C-3.D) underlined the previously underestimated diversity of microbial eukaryotes within sponges. Similarly, we also found several OTUs (*i.e*. OTU130, OTU246 and OTU478) with high numbers of reads in sponges assigned to the phylum *Cnidaria* (*i.e.* sea anemones, corals, jellyfish, Figure 3.3.A), suggesting that sponges filter the larvae of these animals. Our findings helped to close the gap of knowledge on environmental eukaryotic diversity as pointed out in several other eukaryotic diversity studies (Stoeck*, et al.*, 2010, Amaral-Zettler*, et al.*, 2011, Edgcomb*, et al.*, 2011).

Sponge-associated fungi

In general, culture-dependent approaches to obtain fungi from sponges almost always recovered fungal strains that belong to the orders *Pleosporales, Hypocreales* and *Eurotiales* (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006, Wang*, et al.*, 2008), all of which are filamentous fungi. The presence of filamentous fungi in sponges was confirmed for two South China Sea sponges by using clone libraries, which showed that fungal orders such as *Dothideomycetes*, *Eurotiomycetes*, *Pezicomycetes* and *Sordariomycetes* dominated the fungal communities (Jin*, et al.*, 2014). Similarly, in our culture-independent approach *Pleosporales* (OTU350 and OTU509), *Hypocreales* (OTU230) and *Eurotiales* (OTU344) were detected in the majority of sponge species (5 out of 7, see Figure 3.1.A). However, our approach revealed a larger diversity of sponge-associated fungi, especially for the phylum *Basidiomycota* (Figure 3.3.H) than previously reported (Gao*, et al.*, 2008, Baker*, et al.*, 2009, Vaca*, et al.*, 2012). This could be related to difficulties in culturing them (Hunt*, et al.*, 2004). To date, at least 30 orders of sponge-associated fungi have been discovered (Yu*, et al.*, 2012). This includes 22 orders of *Ascomycota* and eight orders of *Basidiomycota*. Our culture-independent effort added two additional orders that belong to the phylum *Ascomycota* (*Ophiostomatales* and

Teloschistales, Figure 3.3.F and Figure 3.3.G) and five additional orders of yeasts (*Cystofilobasidiales, Leucosporidiales, Microstromatales, Sporidiales*, and *Ustilaginales*) within the phylum *Basidiomycota* (Figure 3.3H). Previously, culture-independent studies of corals (Amend*, et al.*, 2012) and the Hawaiian sponges *Suberites zeteki* and *Mycale armata* (Gao*, et al.*, 2008) found high diversity of yeasts from the order *Malasseziales,* suggesting their role as possible symbiont in marine invertebrates. It should be noted that Gao an co-workers (2008) used internal transcribed spacer (ITS) primers that are selective towards *Basidiomycota,* while Amend and co-workers (2012) employed 26S rRNA primers that are commonly used to study yeasts (Kurtzman & Robnett, 1998). Since different molecular markers were employed, a direct comparison is not possible, but the fact that we also detected *Malasseziales* targeting the 18S rRNA gene suggests that they are not uncommon members of the sponge holobiont. This is further supported by another study where vertical transmission of an unidentified yeast has been demonstrated in the marine sponge *Chondrilla sp.,* which points towards a symbiotic relationship between the yeast and the sponge host (Maldonado*, et al.*, 2005). *Malasseziales* were also detected in the deep sea environment (water and sediments) using a culture-independent approach (Jebaraj*, et al.*, 2010, Edgcomb*, et al.*, 2011, Singh*, et al.*, 2011), indicating they are indeed ubiquitous in marine environments. However, to the best of our knowledge these *Malasseziales* were never detected by cultivation. Considering relative paucity in molecular sponge-associated fungal diversity data and the slowly evolving nature of the 18S rRNA gene, these novel lineages indicate that higher diversity of marine fungi is yet to be explored.

One of the great unknowns of fungal diversity in sponges is whether sponge-specific fungal clusters exist (*i.e.* fungi that are only found in sponges and not in other environments) in a similar fashion as sponge-specific bacterial clusters and sponge-specific archaeal clusters (Taylor*, et al.*, 2007, Simister*, et al.*, 2012). The first sponge-specific clusters (SSC) of fungal assemblages were proposed by Simister and co-workers (2012), of which after a closer examination it is apparent that each cluster is derived from cultivable fungal assemblages (SC181, Figure 3.E and SC179, Figure 3.G). It could be that cultivation media used in those studies (Ding*, et al.*, 2010, Rozas*, et al.*, 2011) enriched certain fungal groups, especially

filamentous fungi. This phenomenon was also observed for bacteria cultured from sponges, where bacteria that are present in low numbers in the sponge microbiota were selectively enriched by the cultivation medium (Schippers*, et al.*, 2012)., . From our culture-independent study, we did not find any evidence of sponge-specific fungi clusters, and until a large-scale cultivation-independent study of sponge-associated fungi is carried out for many different sponges across the globe, the presence of fungal SSC remains debatable.

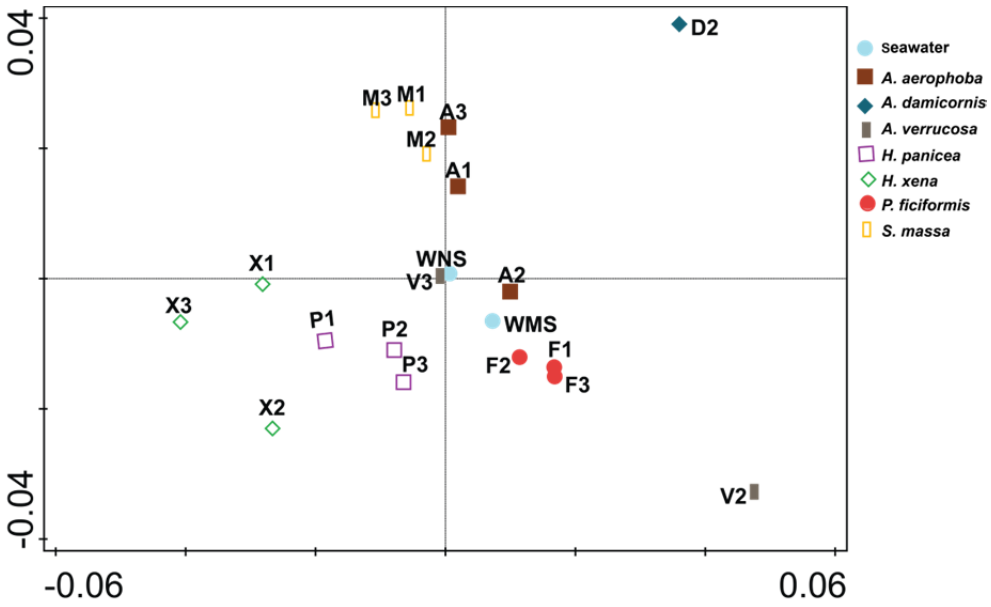


Figure 3.4 Non-metric multidimensional scaling (NMDS) plot derived from Bray-Curtis similarity analysis based on presence or absence OTU matrix consisting of 168 OTUs that are that are present either only in sponges or in sponges and seawater

Ecological perspective on sponge-associated fungi

Our work demonstrates the presence of fungi in several different sponge species using a culture-independent approach. Other studies have consistently shown associations of fungi with sponges (Kohlmeyer & Volkmann-Kohlmeyer, 1990, Gao*, et al.*, 2008, Proksch*, et al.*, 2008, Baker*, et al.*, 2009, Ding*, et al.*, 2010, Liu*, et al.*, 2010, Li*, et al.*, 2014, Wang*, et al.*,

2014), which could indicate that functional relationships exist between fungi and sponges. Despite a gap of knowledge about the fungal life cycle in sponges and other environmental fungi (Jones, 2011, Richards*, et al.*, 2012), it is enticing to speculate about the role of sponge-associated fungi. They are potentially parasites or pathogens as many sponge-associated fungi are closely related to fungal pathogens in marine animals. For instance, *Metchniskowia spp.,* which were found in *H. simulans* (Baker*, et al.*, 2009) and Antarctic sponges (Vaca*, et al.*, 2012), are responsible for the infection and mortality of krill and prawns (Donachie & Zdanowski, 1998, Chen*, et al.*, 2007). In our own study, we found the fungal orders *Malasseziales, Saccharomycetales, Capnodiales* and *Heliotales* that are closely related to fungi causing diseases in animals and plants *(i.e.* OTU77 and OTU509,) (Suh*, et al.*, 2006, Walker*, et al.*, 2011, Ohm*, et al.*, 2012, Saunders*, et al.*, 2012). Despite the relatedness to pathogenic fungi, no clear cases of fungi as the etiological agent in sponge diseases have been reported. On the other hand sponges may solely serve as a reservoir for (pathogenic) marine fungi once they (or their spores) are trapped by the sponge through its efficient water filtration system. For example, *Aspergillus sydowii*, a pathogen of sea fans was also isolated from the marine sponge *Spongia obscura* (Ein-Gil*, et al.*, 2009).

Sponge-associated fungi account for the highest number of novel bioactive compounds reported from marine environment (Saleem*, et al.*, 2007). The bioactive compounds that are produced by sponge-associated fungi are different from their terrestrial counterparts suggesting they are enriched in marine sponges (Proksch*, et al.*, 2003, Bugni & Ireland, 2004, Konig*, et al.*, 2006). It was postulated that sponges recruit fungi to participate in the indirect chemical defense system due to their sessile nature (Silliman & Newell, 2003, Namikoshi & Xu, 2009, Rao*, et al.*, 2012). Fungal genera that are common producers of bioactives in the terrestrial environment, such as *Acremonium, Fusarium*, and *Trichoderma (order: Hypocreales)*, *Aspergillus and Penicillium (order: Eurotiales)*, and *Phoma (order: Pleosporales)* were easily recovered from sponges by a culture-dependent approach and were found to produce novel secondary metabolites (Chu*, et al.*, 2011, Liu*, et al.*, 2011, Martins*, et al.*, 2011, Wiese*, et al.*, 2011). Also in our study, we detected *Eurotiales*, *Hypocreales* and *Pleosporales* indicating that these fungal orders could play similar role in the sponge holobiont.

Conclusion

Ecology and diversity of sponge-associated fungi has been primarily understudied because of the difficulty in amplifying non-sponge fungal targets with a culture-independent approach. The advent of next-generation sequencing allowed us to take a closer look at their distribution and diversity in sponges. Our work has provided additional evidence with respect to the importance of *Malassezia*les in the marine environment, particularly in sponges as it was detected in the majority of the sponge species studied (5 out of 7). Future studies should combine efforts to study this particular fungal order from different marine hosts and focus on the biological relationship with their host. Additional evidence on other eukaryotes that are present in sponges such as *Mesomycetozoa* and *Cercozoa* is presented in this study and contributes to our understanding of the sponge holobiont.

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Supplementary Material

Table S3.1. Blastn query of selected representative OTUs with highest number of sponge (Porifera) reads from pyrosequencing data against the non-redundant nucleotide database (25 April 2014). Full length sequences of 18S rRNA genes of *S. massa* are not present in the NCBI database.

OTU	Sponge species and taxonomic order	Nearest neighbours and accession numbers	Identity	Nearest neighbours taxonomic order
65	*S. massa, Hadromerida*	*Suberites aurantiacus* voucher MNRJ15701, KC902192	98%	*Hadromerida*
		Suberites sp. 0PHG1226-Q, KC902212	98%	*Hadromerida*
		Suberites sp. 0M9H2772-G , KC902367	98%	*Hadromerida*
		Myxilla incrustans voucher BELUM:Mc5139, KC902097	100%	*Poecilosclerida*
		Desmoxya pelagiae voucher BELUM:Mc7764, KC902064	100%	*Halichondrida*
190	*H. xena, Haplosclerida*	*Haliclona* sp. OGL2003, AJ703889	99%	*Haplosclerida*
		Haliclona xena clone Hxel02 , KC899032	98%	*Haplosclerida*
		Haliclona oculata , AY734450	98%	*Haplosclerida*
		Haliclona sp. BC7, KF176618	99%	*Haplosclerida*
		Haliclona fascigera voucher 0CDN9808-J, KC902346	97%	*Haplosclerida*
319	*A. aerophoba, Verongida*	*Aplysina aerophoba* , AY591799	99%	*Verongida*
		Aplysina fistularis voucher SI06x138, KC902387	100%	*Verongida*
		Pseudoceratina sp. USNM 1204854, KC902318	100%	*Verongida*
		Pseudoceratinidae sp. BMOO-07061, KC902301	100%	*Verongida*
		Aplysina cauliformis voucher USNM_1204849, KC902201	100%	*Verongida*
320	*P. ficiformis, Haplosclerida*	*Petrosia ficiformis voucher MCZ:DNA105722*, JX945623	97%	*Haplosclerida*
		Petrosia strongylata voucher 0CDN9767-Z, KC902222	89%	*Haplosclerida*
		Petrosia aff. hartmani USNM 1204818 voucher USNM_1204818, KC902101	89%	*Haplosclerida*
		Cladocroce sp. 0CDN9562-C, KC902202	89%	*Haplosclerida*
		Calyx sp. NIWAKD1132, DQ927313	89%	*Haplosclerida*
333	*A. damicornis, Halichondrida*	*Axinella damicornis* voucher BELUM:Mc4987, KC902335	99%	*Halichondrida*
		Axinella corrugata voucher USNM_1133842, KC902226	99%	*Halichondrida*
		Axinella corrugata, AY737637	99%	*Halichondrida*
		Axinella sp. YQ-2012 isolate S2, JX915785	99%	*Halichondrida*
		Axinella verrucosa, GQ466050	99%	*Halichondrida*
333	*A. verrucosa, Halichondrida*	*Axinella verrucosa*, GQ466050	99%	*Halichondrida*
		Axinella damicornis voucher BELUM:Mc4987, KC902335	99%	*Halichondrida*
		Axinella corrugata voucher USNM_1133842, KC902226	99%	*Halichondrida*
		Axinella corrugata, AY737637	99%	*Halichondrida*
		Axinella sp. YQ-2012 isolate S2, JX915785	99%	*Halichondrida*
495	*H. panicea, Halichondrida*	*Halichondria panicea voucher BELUM:Mc407*, KC902238	100%	*Halichondrida*
		Halichondria panicea clone Hpel01, KC899022	99%	*Halichondrida*
		Demospongiae sp. BMOO-07060, KC902270	99%	*Halichondrida*
		Halichondria bowerbanki voucher BELUM:Mc4003, KC902247	99%	*Halichondrida*
		Hymeniacidon heliophila voucher 0M9G1074-H, KC901957	99%	*Halichondrida*

Table S3.2. List of barcode sequences and ERS accession numbers used in this study. Sample codes indicate *H. panicea* (P1-P3), *H. xena* (X1-X3), *S. massa* (M1-M3), *P. ficiformis* (F1-F3), *A. aerophoba* (A1-A3), *A. damicornis* (D1-D3), *A. verrucosa* (V1-V3) and sea water (WNS & WMS).

Sample	Barcode	Linker Primer	ERS Accession Number
WNS	AACGGCTT	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225559
P1	AACCTTGG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225550
P2	AACGAACG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225551
P3	AACGAAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225552
X1	AACGATCC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225553
X2	AACGATGG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225554
X3	AACGCCAT	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225555
M1	AACGTACC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225556
M2	AACGTAGG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225557
M3	AACGTTCG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225558
WMS	AAGCTAGG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225571
F1	AAGCATCC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225560
F2	AAGCATGG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225561
F3	AAGCCGAA	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225562
A1	AACGTTGC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225563
A2	AAGCAACG	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225564
A3	AAGCAAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225575
D1	AAGCCGTT	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225565
D2	AAGCGCAA	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225566
D3	AAGCGCTT	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225567
V1	AAGCGGAT	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225568
V2	AAGCGGTA	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225569
V3	AAGCTACC	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ERS225570

Chapter 4:

Diversity of polyketide synthase-encoding genes from marine sponge-derived fungi

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Abstract

Fungi from the sponge holobiont are extensively studied for their secondary metabolite production, but remain one of the last frontiers to be thoroughly explored in microbial ecology of sponges. Among the well-known metabolites from sponge-derived fungi are trichoharzin, gymnastatins, penicillinide and citrinide. Polyketide-based compounds are the largest class of secondary metabolites produced by fungi. In this study, we have isolated genetically diverse fungal assemblages from the genera *Penicillium*, *Alternaria* and *Rhodotorula* from the marine sponges *Petrosia ficiformis*, *Corticium candelabrum* and *Aplysina aerophoba*. A PCR-based approach was used to amplify and identify genes that code for polyketide synthases (PKS) and polyketide-synthase-nonribosomal peptide synthase (PKS-NRPS) hybrids from the cultivable fraction of sponge-derived fungi. High diversity of fungal PKS genes was found for *Penicillium* and *Alternaria*, highlighting the genetic potential of sponge-derived fungi for the production of novel bioactives.

Keywords: sponge-derived fungi, polyketide synthase (PKS), PKS-NRPS, *Penicillium* sp., *Alternaria* sp., *Rhodotorula* sp.

Introduction

Fungi from the marine environment are among the least known fungi. The definition of marine fungi according to Kohlmeyer and Kohlmeyer (1979) includes obligate marine fungi, which grow and sporulate exclusively in the marine or estuarine habitat, and facultative marine fungi, which grow equally well in marine, freshwater, and terrestrial environments. Marine fungi are prolific producers of a structurally and chemically diverse range of secondary metabolites (Bugni & Ireland, 2004, Saleem*, et al.*, 2007). These secondary metabolites may represent new drugs to fight cancer, novel antibiotics, cholesterol-lowering drugs and other pharmaceutically relevant compounds (Bugni & Ireland, 2004, Galm*, et al.*, 2005, Sharma & Sharma, 2014). Fungal secondary metabolites in general include polyketides, non-ribosomal peptides, terpenes and indole alkaloids (Keller*, et al.*, 2005, Mayer*, et al.*, 2007, Agarwal & Moore, 2014). The marine sponge holobiont is one of the marine habitats that has been extensively studied for the diversity of associated microorganisms from ecological and biotechnological perspectives, but the knowledge about fungi in sponges has lagged behind (Wang, 2006, Taylor*, et al.*, 2007, Naim*, et al.*, 2015). Historically, identification of sponge-associated fungi largely depended on fungal morphology (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006), but a combination of a morphological and a molecular approach is becoming the new standard practice (Wiese*, et al.*, 2011). Fungi commonly isolated from sponges belong to the phylum *Ascomycota*, and include members of genera such as *Aspergillus, Cladosporium, Fusarium, Penicillium* and *Trichoderma*, which are also commonly found in terrestrial environments (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006, Ding*, et al.*, 2010, Liu*, et al.*, 2010, Zhou*, et al.*, 2011, Schippers*, et al.*, 2012, Yu*, et al.*, 2012). Members of these genera were shown to possess prolific antifungal, antimicrobial and anti-tumor activity (Liu*, et al.*, 2010, Paz*, et al.*, 2010). The ecological role of fungi associated with sponges is yet to be fully established since no mycelial growth has been observed in sponges (Proksch*, et al.*, 2010). However, it has been hypothesized that secondary metabolites synthesised by sponge-associated fungi may play a role in the sponge's chemical defence (Jin*, et al.*, 2014, Wang*, et al.*, 2014). Polyketides represent the largest group of fungal secondary metabolites and are synthesized by a multidomain protein called type I polyketide synthase (PKS) (Keller*, et al.*,

2005). While type II and type III PKS are also found in fungi, they are mostly known from bacteria and plants, respectively (Shen, 2003, Yu*, et al.*, 2012). The fungal PKS type I protein module is related to eukaryotic fatty-acid synthases, which contain similar domain structures with different catalytic activities known as β-ketoacyl synthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP) domain. The only differences are that ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains that are required for ketone reduction in fatty acids, are not present in all fungal PKS enzymes (Keller*, et al.*, 2005, Evans*, et al.*, 2011). Other multidomain, multimodular enzymes responsible for the production of fungal secondary metabolites are non-ribosomal peptide synthetases (NRPS), which catalyze the production of non-ribosomal peptides derived from both proteinogenic and non-proteinogenic amino acids (Keller*, et al.*, 2005, Anke*, et al.*, 2009). PKS and NRPS domains can also fuse to form PKS-NRPS hybrids that lead to compounds with a large structural diversity (Boettger*, et al.*, 2012, Boettger & Hertweck, 2013).
With this study we aim to assess PKS and PKS-NRPS hybrid diversity in fungi isolated from marine sponges. To this end, we employed a PCR-based approach using two sets of degenerate primers targeting conserved regions of KS domain and KS-AT trans domain encoding nucleotide sequences, which have been previously used to identify different types of PKS genes (Bingle*, et al.*, 1999, Kroken*, et al.*, 2003, Amnuaykanjanasin*, et al.*, 2005).

Material and Methods

Sample collection

Three different species of marine sponges from the Mediterranean Sea were used as starting material for the fungal cultivation study. *Petrosia ficiformis* (Pf1) and *Corticium candelabrum* (CC1) were collected on June 5th 2008 from Punta de Santa Ana, Blanes (41° 40' 33'' N, 2° 48' 10" E) at the depth of 10 m. *Aplysina aerophoba* (AA3) was collected on January 15th 2012 from Cala Montgo (42° 06' 52.20" N, 03° 10' 06.52" E) at a depth of 12 m. Specimens were brought to the surface in plastic ziplock bags. The sponge samples were rinsed three times with sterile artificial seawater (ASW) and were cut into pieces of approximately 0.1 cm^3. Subsequently they were homogenized with mortar and pesttle, and two tissue volumes of

sterile artificial seawater were added to obtain a homogeneous cell suspension. The cell suspension was divided in aliquots of 1.2 ml and mixed with 0.6 ml 50% sterile glycerol in ASW. The samples were frozen until –20°C before they were stored at –80°C.

Fungal cultivation and morphological identification

Artificial seawater (ASW) was prepared by dissolving 33 g Instant Ocean reef crystals (Aquarium Systems, Sarrebourg, France) in 1 L of distilled water for usage in all media except Marine medium. The cell suspensions were plated on Mucin Agar (Muc) medium (1 g mucin dissolved ; 15g agar; 1 L ASW), Mueller Hinton medium (MH) (2 g Beef extract; 17.5 acid hydrolysate of casein; 1.5 g starch; 1 L ASW; 15 g agar) and Marine medium (MM) (Difco™ Marine agar 2216, 1 L of distilled water). All medium was supplemented with 1 mL trace metal solution (Sipkema *et al.*, 2011) and the pH was between 7.0 to 7.5. All culture media were supplemented with antibiotics (antibacterial) with different modes of action (Table 4.1). The plates were sealed and incubated in the dark for 39 - 80 days at 15 °C. Colonies with appearance similar to filamentous fungi and yeasts were collected and transferred to a new Petri dish containing Potato Dextrose Agar (PDA Sigma-Aldrich, Steinheim, Germany) prepared with artificial seawater (ASW). The purity of the isolates and the fungal cell morphology was further examined using light microscopy with methylene blue staining. The morphological characterization (visual and microscopic) of the colonies was made after 7 days of growth on PDA medium at 25 °C. The macroscopic variables analyzed included colony diameter, obverse and reverse colony colour, and the presence or absence of exudates, while microscopic examination was based on conidiophores and spores formation. Pure isolates were first stored on PDA agar (prepared with ASW) slant cultures at 4 ºC for 3 months and then the spores were collected and transferred to 1 ml PDA liquid cultures (prepared with ASW) mixed with 0.6 ml 50% sterile glycerol and stored at –80°C.

Extraction of fungal genomic DNA and 18S rRNA gene amplification

After cultivation on PDA medium for 7 days, fungal spores and mycelium were collected as material for DNA extraction. The extraction of DNA was performed using the MP® FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to manufacturer's protocol with a minor modification; the pellet was washed twice instead of once during the washing step (step 12). The concentration of DNA was checked using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) while its integrity was determined by 1% (w/v) agarose gel using the SYBR® Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA). The primer pair EUKF/EUKR (Medlin*, et al.*, 1988) was used to amplify near full length fragments of the small-subunit 18S rRNA gene (Table 4.2). The 50 µl PCR reaction solution consisted of of nuclease free water containing 0.2 mM of each dNTP, 1X Green GoTaq® buffer (Promega, Leiden, The Netherlands), 2.5 U GoTaq® Polymerase, 10 pmol of each primer, 2.5 mM $MgCl_2$ and 20 ng fungal genomic DNA template. When the PCR products were weak or absent, 1µl of a 500 mg/ml solution of Bovine Serum Albumin (BSA) was added to the PCR reaction or the DNA template was diluted (1:10). The PCR conditions were: initial denaturation of 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 40 s and elongation at 72 °C for 1min and 30 s. The reaction was terminated with the final elongation of 5 min at 72 °C. The product was checked for correct size (~1800 bp) on a 1% (w/v) agarose gel supplemented with SYBR® Safe DNA Gel Stain (Invitrogen GmbH, Darmstadt, Germany) run at 100 V for 30 min. Finally, the PCR products were sequenced by GATC Biotech (Konstanz, Germany) using primer EUKF. Partial 18S rRNA gene sequences were deposited at the NCBI Genbank under accession numbers KF703449 – KF703493.

Genomic fingerprinting using RAPD-PCR

The genotypes of *Penicillium* spp. were assessed using two random amplified polymorphic DNA (RAPD) primers, M13 (Stenlid*, et al.*, 1994) and OPD10 (Roslan*, et al.*, 2009) (Table 4.2). The 50 µl PCR reaction solution consisted of nuclease free water containing 0.2 mM of each dNTP, 1X Green GoTaq® Flexi buffer (Promega), 2.5 U HotStart GoTaq® Flexi Polymerase (Promega), 10 pmol of either primer M13 or OD10, 2.5 mM

$MgCl_2$ and 20 ng fungal genomic DNA as template. The PCR conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 84 °C for 30 s, annealing at 46 °C for 1 min (M13 primer) or at 30 °C for 1 min (OPD10 primer) and elongation at 72 °C for 2 min. The reaction was terminated with a final elongation of 7 min at 72 °C. The product was checked on a 2% (w/v) agarose gel run at 100 V for 60 min. The 1 kb plus molecular marker (Fermentas GmbH, St. Leon-Rot, Germany) was used as molecular weight standard. The resulting banding patterns were analysed using Bionumerics 3.5 (Applied Maths, Sint-Martens-Latum, Belgium) to define bands and subsequently similarity indices (SI's). Prior to analysis, gel images were normalized using the size markers. Levels of similarity between fingerprints were calculated according to the Dice coefficient (Carriço*, et al.*, 2005) by applying the unweighted pair group method with mathematical averages (UPGMA) using the similarity matrix generated.

Table 4.1 Description of cultivation media used to grow fungal isolates, presence or absence of fungal reducing PKS and PKS/NRPS hybrid genes and colony morphology. A complete morphological characterization of fungal isolates is presented in Figure 4.1.A-B.

Medium Culture [a]	Isolate	Sponge species	Morphological characterization (microscopic)	Reducing PKS genes [b]	PKS/NRPS hybrid genes [c]	Colony Morphology [d]
MH + Erythromycin	**F01**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Erythromycin	**F02**	*A. aerophoba*	*Penicillium sp.*	+	-	M1
MH + Erythromycin	**F03**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Erythromycin	**F04**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Erythromycin	**F45**	*A. aerophoba*	Yeast	-	-	M3
MH + Chloramphenicol	**F13**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Chloramphenicol	**F14**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Chloramphenicol	**F15**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MH + Chloramphenicol	**F39**	*A. aerophoba*	Yeast	-	-	M3
MH + Chloramphenicol	**F40**	*A. aerophoba*	Yeast	-	-	M3
MH + Chloramphenicol	**F41**	*A. aerophoba*	Yeast	-	-	M3
MH + Poly B+ Penicillin	**F20**	*P. ficiformis*	*Alternaria sp.*	+	-	M2
Mucin + Chloramphenicol	**F06**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
Mucin + Chloramphenicol	**F07**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
Mucin + Chloramphenicol	**F37**	*A. aerophoba*	Yeast	-	-	M3
Mucin + Chloramphenicol	**F38**	*A. aerophoba*	Yeast	-	-	M3
Mucin + Poly B+ Linezolid	**F08**	*P. ficiformis*	*Penicillium sp.*	-	+	M1
Mucin + Poly B+ Linezolid	**F09**	*P. ficiformis*	*Penicillium sp.*	-	+	M1
Mucin + Poly B+ Linezolid	**F10**	*P. ficiformis*	*Penicillium sp.*	-	+	M1
Mucin + Poly B+ Penicillin	**F12**	*C. candelabrum*	*Penicillium sp.*	+	+	M1
Mucin + Kanamycin	**F11**	*P. ficiformis*	*Alternaria sp.*	-	-	M2
MM + Cefotaxine	**F05**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Cefotaxine	**F16**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Cefotaxine	**F17**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Cefotaxine	**F18**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Cefotaxine	**F44**	*A. aerophoba*	Yeast	-	-	M3
MM + Sulfo + trimethoprim	**F19**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Chloramphenicol	**F21**	*A. aerophoba*	*Penicillium sp.*	-	+	M1
MM + Chloramphenicol	**F22**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Chloramphenicol	**F23**	*A. aerophoba*	*Penicillium sp.*	-	+	M1
MM + Rifamycin	**F24**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F25**	*A. aerophoba*	*Penicillium sp.*	-	+	M1
MM + Rifamycin	**F26**	*A. aerophoba*	*Penicillium sp.*	-	+	M1
MM + Rifamycin	**F27**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F28**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F29**	*A. aerophoba*	*Penicillium sp.*	-	+	M1
MM + Rifamycin	**F30**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F31**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F32**	*A. aerophoba*	*Penicillium sp.*	+	+	M1
MM + Rifamycin	**F42**	*A. aerophoba*	Yeast	-	-	M3
MM + Rifamycin	**F43**	*A. aerophoba*	Yeast	-	-	M3
MM + Poly B+ Penicillin	**F33**	*C. candelabrum*	*Penicillium sp.*	+	+	M1
MM + Poly B+ Penicillin	**F34**	*C. candelabrum*	*Penicillium sp.*	+	+	M1
MM + Poly B+ Penicillin	**F35**	*C. candelabrum*	*Penicillium sp.*	-	+	M1
MM + Poly B+ Penicillin	**F36**	*C. candelabrum*	*Penicillium sp.*	+	+	M1

a MH= Muller Hinton Medium; MM=Marine Medium; Poly B= Polymyxin B; Sulfo= Sulfomethoxazole

b Screened with KA series primers,KAF1/KAF2 and KAR1/KAR2 (Amnuaykanjanasin *et al*., 2005)

c Screened with XKS1/XKS2 primers (Amnuaykanjanasin *et al*., 2005)

d Numbers indicate different morphological characteristics (Figure 4.1A).

Table 4.2 Primers used in this study

Primer	Sequence (5′-3′)[a]	Target	Annealing temperature (°C)	Use	Reference
EukF	AAC CTG GTT GAT CCT GCC AGT	eukaryotes 18S rRNA gene	55	clone libraries	Medlin *et al.*, 1988
EukR	TGA TCC TTC TGC AGG TTC ACC TAC	eukaryotes 18S rRNA gene	55	clone libraries	Medlin *et al.*, 1988
M13	TTA TGT AAA CGA CGG CCA GT	minisatellite DNA	46	RAPD-PCR	Stenlid *et al*., 1994
OPD10	GTG ATC GCA G	genomic DNA	30	RAPD-PCR	Roslan *et al*., 2009
KAF1	GAR KSI CAY GGI ACI GGI AC	KS domain	55	clone libraries	Amnuaykanjanasin *et al*., 2005
KAF2	GAR GCI CAY GCI ACI TCI AC	KS domain	55	clone libraries	Amnuaykanjanasin *et al*., 2005
KAR1	CCA YTG IGC ICC RTG ICC IGA RAA	AT domain	55	clone libraries	Amnuaykanjanasin *et al*., 2005
KAR2	CCA YTG IGC ICC YTG ICC IGT RAA	AT domain	55	clone libraries	Amnuaykanjanasin *et al*., 2005
XKS1	TTY GAY GCI BCI TTY TTY RA	KS domain	55	clone libraries	Nicholson *et al*., 2000
XKS2	CRT TIG YIC CIC YDA AIC CAA A	KS domain	55	clone libraries	Amnuaykanjanasin *et al*., 2005

[a]Degenerate bases and inosine are described as followings: B = C/G/T; D = A/G/T; I = inosine; K = G/T; M= A/C; R = A/G; S = C/G; and Y = C/T

Screening for PKS and PKS/NRPS hybrid genes by PCR

Gene fragments encoding the conserved KS domain and the KS-AT interdomain region (referred to as KS-AT) were amplified from extracted fungal genomic DNA using PCR with the degenerate KA series primers as described previously (Amnuaykanjanasin*, et al.*, 2005, Amnuaykanjanasin*, et al.*, 2009). The forward primer KAF1/KAF2 was paired with the reverse primer KAR1 or KAR2 for PCR amplification of these PKS gene fragments (Table 4.2). Each reaction consisted of approximately 1 µg of fungal genomic DNA, 1x Colourless GoTaq® Flexi buffer (Promega), 2.5 U HotStart GoTaq® DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and nuclease-free water up to 50 µl. The PCR conditions were: 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C, elongation for 2 min at 72 °C and a final elongation for 7 min at 72 °C. PCR screening for nucleotide sequences encoding the conserved KS domain of polyketide synthase-nonribosomal peptide synthase (PKS/NRPS) hybrids was

performed using the primer combination of XKS1/XKS2 (Amnuaykanjanasin*, et al.*, 2005). The same PCR conditions as for the KA series primers were used. PCR amplicons were visualized using a 1% (w/v) agarose gel gel supplemented with SYBR® Safe DNA Gel Stain (Invitrogen GmbH, Darmstadt, Germany) run at 100 V for 30 min. PCR reactions with expected amplicon size (700-800 bp for KAF/KAR primers and 1.1–1.2 kb for XKS1/XKS2) were purified using the Gene Jet PCR Purification kit (Thermo Fisher Scientific®, Dreieich, Germany) following the manufacturer's instructions and then cloned using the pGEM®-T Easy Vector System I (Promega). Clones were screened for the presence and size of the insert using T7-Sp6 primers, and those with the correct size were sequenced at GATC Biotech (Constance, Germany) using either the forward primer KAF1 or XKS1.

Phylogenetic analysis of fungal 18S rRNA

Sequence data of 18S rRNA genes were edited with Vector NTI Advance® 10 Software (Invitrogen, Carlsbad, CA, USA); ambiguous regions of 5'-end and 3'-end were trimmed based on the chromatograms. Sequences were checked for potential chimera using KeyDNA tools software (http://KeyDNAtools.com). Nearest neighbours were determined by comparison to the NCBI Genbank nucleotide (nr/nt) database using BLASTn searches (August 21, 2013). 18S rRNA sequences of our isolates and their nearest neighbours were imported into the ARB program (Ludwig*, et al.*, 2004), aligned using the FastAlign function of the alignment editor implemented in the ARB program and refined manually. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default Gblocks program parameters were used, except allowing a minimum block length of 2 and gaps in 50% of the positions. Phylogeny was inferred using MrBayes version 3.2 (Ronquist*, et al.*, 2012), assuming the GTR (general time reversible) phylogenetic model with 6 substitution rate parameters, gamma-distributed rates across sites and default settings of the program. 10,000,000 tree generations were calculated and sampled every 1000th generation. Points prior to convergence were determined graphically and discarded. The consensus tree was imported and edited with the ARB program.

18S rRNA gene sequences obtained for *Aplysina aerophoba* (AA3) in the framework of a culture-independent survey using next-generation

sequencing as described in Chapter 3 of this thesis was used for comparison. To identify sequence similarity between fungal isolates from *A. aerophoba* (AA3) and OTUs from the pyrosequencing, 18S rRNA gene sequences of fungal isolates from *A. aerophoba* (AA3) were blasted against a database containing the pyrosequencing OTUs using a locally installed version of Blast2-2-29 (Zhang*, et al.*, 2000).

Phylogenetic analysis of fungal PKS gene

Sequence data of cloned PKS encoding genes were processed by identifying vector sequences using the NCBI VecSreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) and manually removing vector sequences. Putative introns and amino acid sequences were predicted using gene prediction programme AUGUSTUS (Stanke & Morgenstern, 2005, Stanke*, et al.*, 2008), freely available online (http://bioinf.uni-greifswald.de/augustus/submission.php) with *Aspergillus terreus* as model organism. Closest relative search for the fungal PKS sequences was done using blastx against the NCBI GenBank non-redundant protein database with the standard genetic code translation table. Fungal PKS amino acid sequences obtained in this study as well as their nearest relatives determined by BLASTx were aligned using the MAFFT multiple sequence alignment tool (Katoh*, et al.*, 2005) available online (http://mafft.cbrc.jp/alignment/server/). Gblocks version 0.91b (Castresana, 2000) was used to systematically remove ambiguous regions of the alignment with default Gblocks parameters, except allowing minimum block length of 5 and gaps in 50% of the positions. Protein phylogeny was inferred using MrBayes version 3.2 (Ronquist*, et al.*, 2012) using the Cyberinfrastructure for Phylogenetic Research (CIPRES) (Miller*, et al.*, 2010). The parameters were set using the Whelan and Goldman amino acid replacement matrix (Whelan & Goldman, 2001), gamma-shaped rate variation with a proportion of invariable sites, the approximation of the continuous gamma distribution rate was set at 4, and otherwise the default settings of MrBayes were used. 10,000,000 tree generations were calculated and sampled every 1000th generation. Points prior to convergence were determined graphically and discarded. The consensus tree was imported and edited with the ARB program.

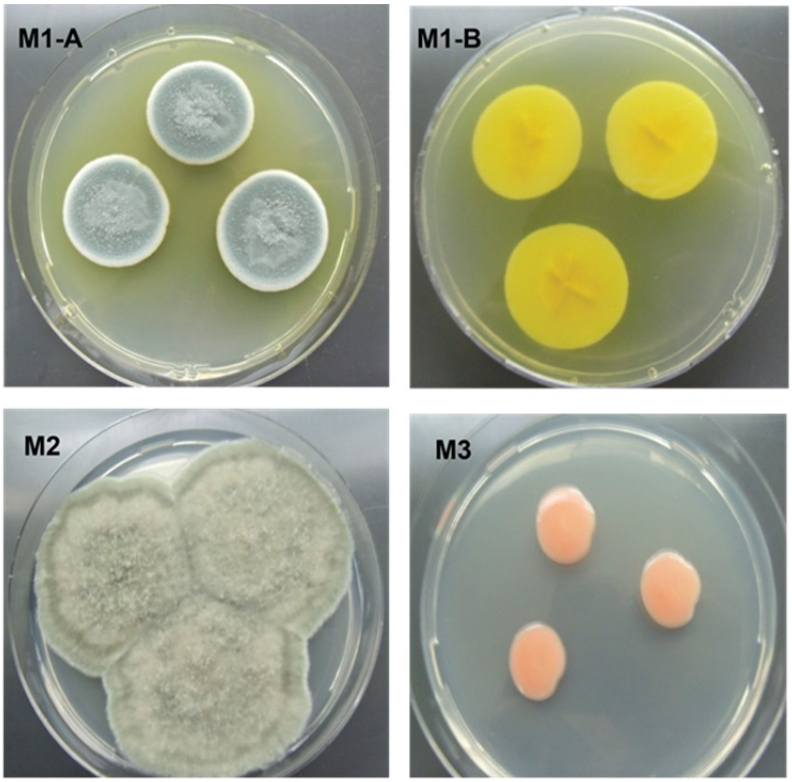

Figure 4.1.A Colony morphology of sponge isolates based on visual examination. Colony morphologies shown here are from M1-A: front view of isolate F10, M1-B: back view of isolate F10 (*Penicillium* sp.), M2: isolate F11 (*Alternaria* sp.) and M3: isolate F37 (yeast).

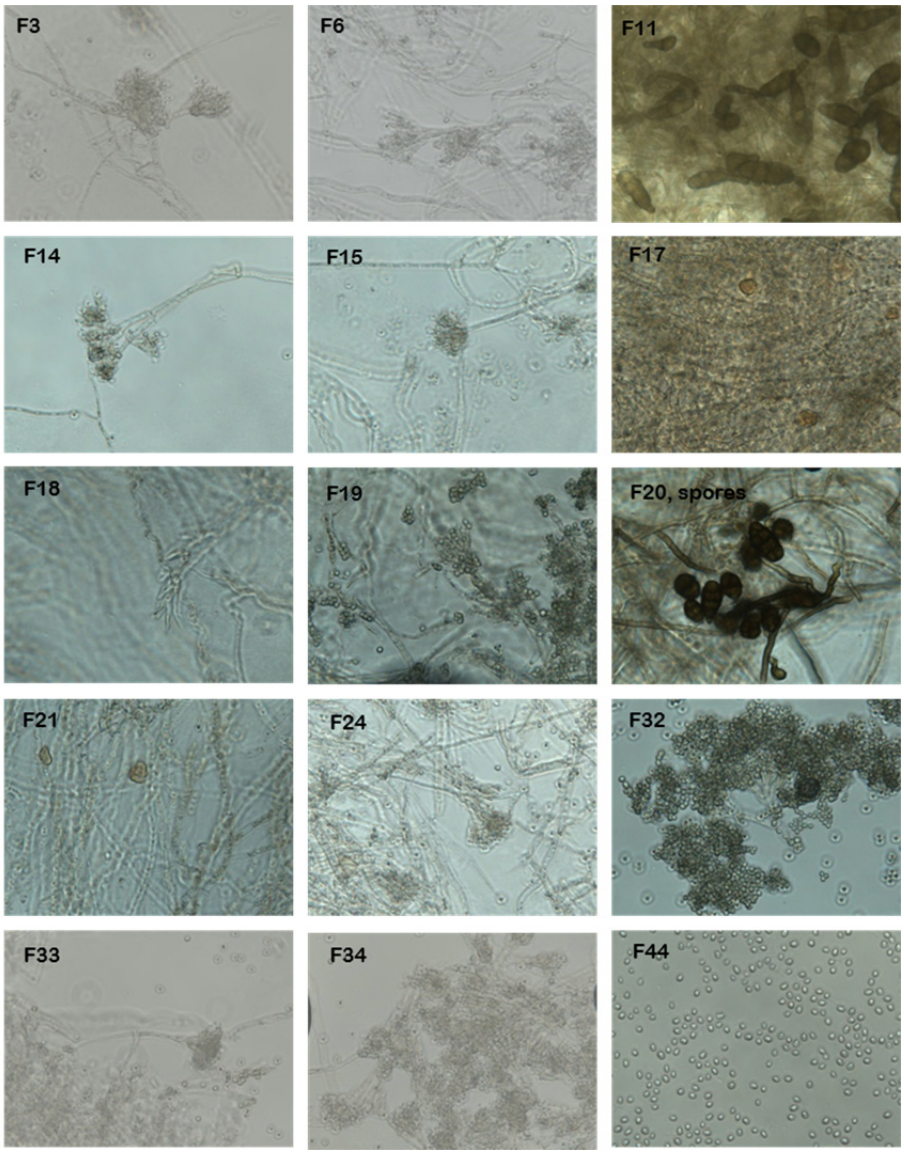

Figure 4.1.B Structural morphology (conidiophores and spores) of different fungal isolates examined using light microscopy with methylene blue staining. Colony morphologies shown here are from isolate F03, F06, F11, F14, F15, F17, F18, F19, F20, F21, F24, F32, F33, F34 and F44. All pictures depicted are *Penicillium* spp. except isolate F11 and F20 which are *Alternaria* spp., and isolate F44 which represented the yeast. A complete description of different fungal isolates is presented in Table 4.1. Basic colony morphology of fungal isolates from sponges is presented in Figure 4.1A.

Results

Identification of fungal isolates

Three different media supplemented with ten different antibiotics were used to grow fungal colonies from three Mediterranean sponges (*A. aerophoba*, *C. candelabrum* and *P. ficiformis*). From Mueller-Hinton agar (MH), 12 fungal isolates were recovered; five isolates from MH agar containing Erythromycin, six isolates from MH agar containing Chloroamphenicol and one isolate from MH agar containing Polymyxin B + Penicillin. Next, 9 isolates were recovered using Mucin agar; four isolates were recovered from Mucin agar containing Chloroamphenicol, three from Mucin agar containing Polymyxin B + Linezolid and one each from Mucin agar containing Polymyxin B + Penicillin and mucin agar containing Kanamycin. Finally, 24 fungal isolates were recovered using marine medium (MM); 11 of them isolated from MM agar containing Rifamycin, five of them isolated from MM agar containing Cefotaxine, four isolates from MM agar containing Polymyxin B + Penicillin, three isolates from MM agar containing Chloroamphenicol and one isolate from MM agar containing Sulfomethoxazole + Trimethoprim (Table 4.1). Of these 45 isolates, 26 isolates with morphological characteristics of filamentous fungi and nine yeast isolates were obtained from *A. aerophoba* (Figure 4.1.A-B and Table 4.1). Five filamentous fungi isolates were from *P. ficiformis* and from *C. candelabrum* (Table 4.1). By integrating the morphological characteristics (Figure 4.1.A and Figure 4.1.B) and molecular characterization based on 18S rRNA gene sequences, it was shown that members of the genus *Penicillium* dominated the cultivable fraction of sponge-derived fungi with 34 out of 36 filamentous fungal isolates. The genus *Alternaria* was represented by 2 isolates (isolate F11 and F20), recovered from Mucin agar containing Kanamycin and MH agar containing Polymyxin B + Penicillin, respectively. Yeast isolates appeared on PDA agar (the medium that was used for subcultures) as round colonies with salmon orange pigment (Figure 4.1.A) and resembled ovoid cells when checked under the microscope (Figure 4.1.B). From the phylogenetic tree (Figure 4.2), it was confirmed that 34 isolates were affiliated to the genus *Penicillium*, genus *Alternaria* (two isolates), and the yeasts were affiliated to the genus *Rhodotorula* (nine isolates).

Figure 4.2 Bayesian phylogram based on 18S rRNA gene sequences of sponge-derived fungi and their nearest neighbours. Fungal phylum and order level taxonomy are also indicated. The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values <50 are not indicated.

Filamentous fungi from this study were closely related to known plant endophytes such as *Eupenicillium javanicum*, *Penicilllium solitum*, *Penicillium chrysogenum*, *Penicillium tardum*, *Penicillium freii* and *Alternaria* sp. (Figure 4.2). Queries of the18S rRNA genes of the cultured isolates against the non-redundant nucleotide NCBI database using Blastn gave similarity values between 94% to 98%. For filamentous fungi isolates F10, F17, F21, F24, F 26, F27, F29, F30, F33, F36 and yeast isolates F38, F41, F43 and F45 similarity was lower than 97%, indicating that they are putative new fungal species (Table 4.3). The low similarity of our sponge-derived fungal isolates to known sequences is also reflected in their position in the phylogenetic tree (Figure 4.2), where they are grouped in several distinctive clusters separate from other known species of *Penicillium*, *Alternaria* and *Rhodotorula*.

Comparison of 18S rRNA gene sequences of fungal isolates recovered from *Aplysina aerophoba* (AA3) with data from a previous pyrosequencing study (Chapter 3) did not reveal any similarities above 97%.

Microdiversity of *Penicillium* spp.

RAPD analysis using primer set OPD10 indicated genetic variability among *Penicillium* isolates based on cluster analysis (Figure 4.3), with one to seven bands generated per isolate. The M13 primer produced more monomorphic fragments than OPD10, leading to a number of isolates with the same profile (*i.e.* isolate F1 to F4, F16 to F18, F23 to F25, F28 to F30 and F34 to F36) (Figure S4.1). Therefore only the OPD10-based banding patterns were used for further analysis. The apparent low diversity based on 18S rRNA of *Penicillium* species was offset by genotypic diversity among isolates based on RAPD profile clustering. For instance, at 60% similarity, it was observed that F16, F18, F17 and F21 isolates formed a separate cluster from F23, F24 and F25 (Figure 4.3). There was no clear correlation of the RAPD clustering pattern (Figure 4.3) when compared to the 18S rRNA gene phylogeny (Figure 4.2), polyketide synthase (PKS) genes (Figure 4.4) and number of introns (Table 4.4).

Table 4.3 Fungal isolates from this study and their nearest neighbors determined by 18S rRNA gene sequence similarities using Blastn against NCBI Genbank (nr/nt) database.

Isolate	Nearest isolates and NCBI accession number	Similarity	Source
F01	*Penicillium tardum* strain KCTC16051 18S rRNA gene, AF245233	97%	plant endophyte
F02	*Penicillium freii* (IBT 3464) 18S rRNA gene, AJ005446	96%	plant endophyte
F03	*Penicillium* sp. D-1 18S rRNA gene, JF950269	98%	soil
F04	*Penicillium freii* (IBT 3464) 18S rRNA gene, AJ005446	97%	plant endophyte
F05	*Penicillium solitum* strain 20-01 18S rRNA gene, JN642222	98%	plant endophyte
F06	*Penicillium tardum* strain KCTC16051 18S rRNA gene, AF245233	97%	plant endophyte
F07	*Penicillium* sp. D-1 18S rRNA gene, JF950269	98%	soil
F08	*Eupenicillium javanicum* isolate AFTOL-ID 429 18S rRNA gene, EF413620	97%	plant endophyte
F09	*Eupenicillium javanicum* isolate AFTOL-ID 429 18S rRNA gene, EF413620	97%	plant endophyte
F10	*Eupenicillium javanicum* isolate AFTOL-ID 429 18S rRNA gene, EF413620	96%	plant endophyte
F11	*Alternaria* sp. CPCC 480546 18S rRNA gene, EU827604	98%	plant endophyte
F12	*Penicillium solitum* strain 20-01 18S rRNA gene, JN642222	98%	plant endophyte
F13	*Penicillium freii* (IBT 3464) 18S rRNA gene, AJ005446	98%	plant endophyte
F14	*Penicillium* sp. D-1 18S rRNA gene, JF950269	98%	soil
F15	*Penicillium* sp. CPCC 480008 18S rRNA gene, AB074658	97%	plant endophyte
F16	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F17	*Penicillium tardum* strain KCTC16051 18S rRNA gene, AF245233	96%	plant endophyte
F18	*Penicillium freii* (IBT 3464) 18S rRNA gene, AJ005446	96%	plant endophyte
F19	*Penicillium solitum* strain 20-01 18S rRNA gene, JN642222	97%	plant endophyte
F20	*Alternaria* sp. CPCC 480546 18S rRNA gene, EU827604	97%	plant endophyte
F21	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F22	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F23	*Penicillium* sp. D-1 18S rRNA gene, JF950269	98%	soil
F24	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F25	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F26	*Penicillium* sp. D-1 18S rRNA gene, JF950269	94%	soil
F27	*Penicillium* sp. D-1 18S rRNA gene, JF950269	94%	soil
F28	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F29	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F30	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F31	*Penicillium* sp. D-1 18S rRNA gene, JF950269	98%	soil
F32	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F33	*Penicillium* sp. D-1 18S rRNA gene, JF950269	96%	soil
F34	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F35	*Penicillium* sp. D-1 18S rRNA gene, JF950269	97%	soil
F36	*Penicillium tardum* strain KCTC16051 18S rRNA gene, AF245233	96%	plant endophyte
F37	Uncultured *Pucciniomycotina* clone D0810_54_M 18S rRNA gene, EU647158	98%	groundwater sediment
F38	Uncultured *Pucciniomycotina* clone D0810_54_M 18S rRNA gene, EU647159	96%	sediment
F39	Uncultured fungus clone nco86h05c1 18S rRNA gene, KC674623	97%	human skin
F40	Uncultured Pucciniomycotina clone D0810_54_M 18S rRNA gene, EU647159	98%	groundwater sediment
F41	Uncultured Pucciniomycotina clone D0810_54_M 18S rRNA gene, EU647159	96%	groundwater sediment
F42	Uncultured Pucciniomycotina clone D0810_54_M 18S rRNA gene, EU647159	97%	groundwater sediment
F43	*Rhodotorula mucilaginosa* AFTOL-ID 1548 18S rRNA gene, DQ832199	96%	human pathogen
F44	*Rhodotorula mucilaginosa* AFTOL-ID 1548 18S rRNA gene, DQ832199	97%	human pathogen
F45	Uncultured *Pucciniomycotina* clone D0810_54_M 18S rRNA gene, EU647159	96%	groundwater sediment

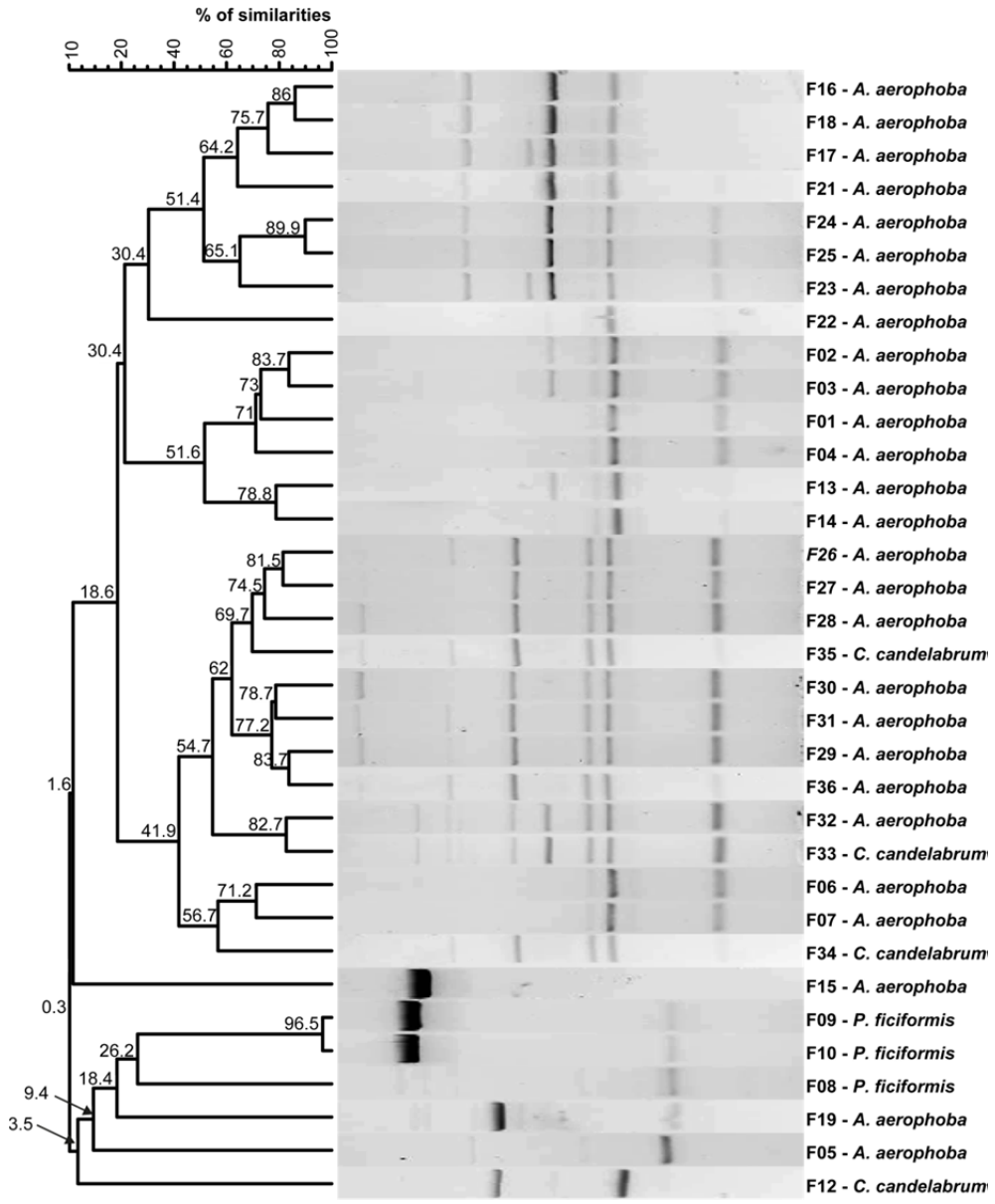

Figure 4.3 UPGMA dendrogram generated from random amplified polymorphic DNA (RAPD) profiles of 34 *Penicillium* spp. isolates obtained using primer OPD10, and constructed from a genetic similarity matrix using the Dice coefficient (Dice, 1945). The numbers along the branches are percentages of similarity calculated from the Dice similarity coefficient for cluster analysis.

Screening of fungal isolates for PKS genes and PKS-NRPS hybrid genes

All 45 isolates in this study were tested for the presence of PKS genes. For amplification of KS-AT domain encoding sequences, the combination of the forward primers (KAF1/KAF2) and reverse primers (KAR1/KAR2) was used, but successful amplification (expected band size of *ca*. 700-800 bp) was obtained only with the primer pair KAF1/KAR2 for 26 isolates (Table 4.1). PCR products were cloned, and annotation of resulting sequences using BlastX compared against NCBI non-redundant protein database gave 39 putative PKS sequences (Table 4.4 and Table S4.1). The XKS1/XKS2 primer set for amplification of KS domain encoding sequences associated with putative PKS-NRPS hybrid genes yielded products of the expected band size of *ca*. 1.1-1.2 kb for 33 isolates (Table 4.1). Comparison against NCBI non-redundant protein database using BlastX yielded 71 putative PKS and PKS-NRPS hybrid sequences (Table 4.4 and Table S4.1). BlastX identity score varied from 41% to 100% with known PKS sequences (Table S4.1). PKS or PKS-NRPS hybrid genes were not detected from yeast isolates. Putative introns were detected in 62 nucleotide sequences: four from sequences obtained with KAF1/KAR2 primers and 58 introns obtained from XKS1/XKS2 primers (Table 4.4).

Phylogenetic analysis of PKS sequences comprising the ketosynthase (KS) conserved region and the KS-AT inter-domain region was conducted in order to relate the genetic potential of sponge-derived fungi to known polyketide chemistries. Based on the Bayesian inference of the protein phylogeny, two isolates (F09 and F15) formed a separate cluster with a high posterior probability (pp) value of 100%, closely related to LovB type polyketide architecture. This LovB type polyketide, also referred to as reducing PKS clade II with domain architecture of KS-AT-DH-(ME)-KR-PP-(CON)-AMP-PP, is responsible for the production of lovastatin/citrinin nonaketide (Cox*, et al.*, 2009, Boettger & Hertweck, 2013). The majority of the PKS sequences was classified as reducing PKS clade I. Reducing PKS clade I proteins possess the domain architecture of KS-AT-DH-(ME)-ER-KR-PP that is responsible for the production of, for example, compactin and lovastatin, both used as cholesterol-lowering agents (Cox*, et al.*, 2009). Sequences retrieved from three isolates (F06, F12 and F34) were closely related to another type of PKS known as PKS-NRPS hybrid with the motif KS-AT-DH-(ME)-KR-PP-(CON)-AMP-PP-C-A-PCP, commonly involved in

the production of compounds such as cyclopiazonic acid, cytochalasans (reviewed extensively in (Scherlach*, et al.*, 2010)), fusarin, equisetin and fusaridione (Fisch, 2013). Several fungal isolates (*i.e.* isolate F05, F19, F34) were found to harbour more than one type of PKSs, and corresponding sequences were found in several clades in the Bayesian phylogenetic tree (Figure 4.4, see also Table 4.4).

Figure 4.4. A-B. Bayesian phylogram (unrooted) based on KS gene sequences calculated by Bayesian inference using Wheelan and Goldman amino acid replacement matrix, gamma-shaped rate variation with proportion of invariable sites and with approximation of the continuous gamma rate set at 4). Nearest neighbours with known compounds produced or related to symbiosis or pathogenesis are indicated. Numbers above or below nodes indicate Bayesian posterior probability values. Nodes with PP values <50 are not indicated.

Table 4.4 Putative PKS fragments (71 clones) of sponge-derived fungi and their closest match in the NCBI Genbank non-redundant protein database based on amino acid sequence similarity.

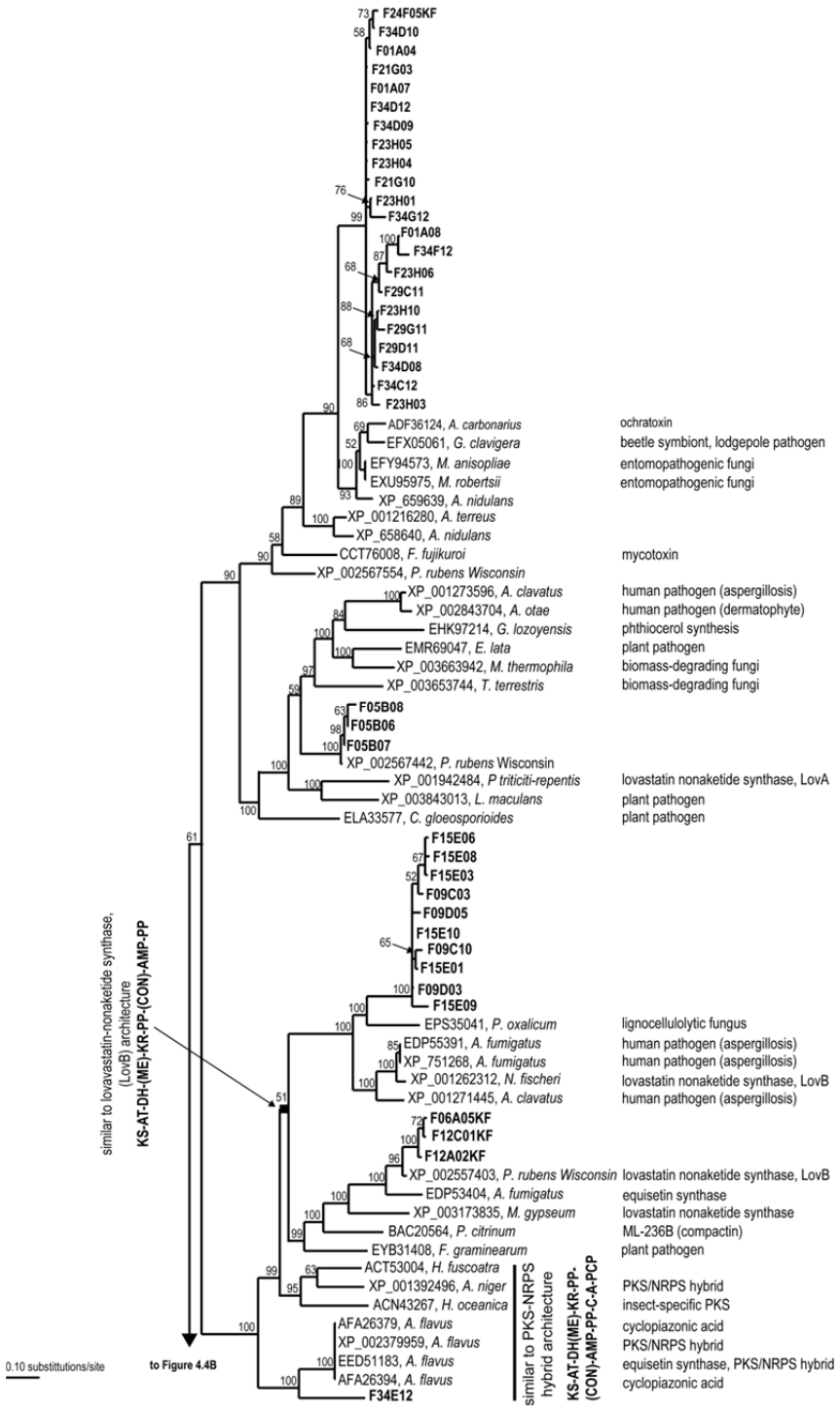
F24F05KF
F34D10
F01A04
F21G03
F01A07
F34D12
F34D09
F23H05
F23H04
F21G10
F23H01
F34G12
F01A08
F34F12
F23H06
F29C11
F23H10
F29G11
F29D11
F34D08
F34C12
F23H03
ADF36124, A. carbonarius
ochratoxin
EFX05061, G. clavigera
beetle symbiont, lodgepole pathogen
EFY94573, M. anisopliae
entomopathogenic fungi
EXU95975, M. robertsii
entomopathogenic fungi
XP_659639, A. nidulans
XP_001216280, A. terreus
XP_658640, A. nidulans
CCT76008, F. fujikuroi
mycotoxin
XP_002567554, P. rubens Wisconsin
XP_001273596, A. clavatus
human pathogen (aspergillosis)
XP_002843704, A. otae
human pathogen (dermatophyte)
EHK97214, G. lozoyensis
phthiocerol synthesis
EMR69047, E. lata
plant pathogen
XP_003663942, M. thermophila
biomass-degrading fungi
XP_003653744, T. terrestris
biomass-degrading fungi
F05B08
F05B06
F05B07
XP_002567442, P. rubens Wisconsin
XP_001942484, P triticiti-repentis
lovastatin nonaketide synthase, LovA
XP_003843013, L. maculans
plant pathogen
ELA33577, C. gloeosporioides
plant pathogen
F15E06
F15E08
F15E03
F09C03
F09D05
F15E10
F09C10
F15E01
F09D03
F15E09
EPS35041, P. oxalicum
lignocellulolytic fungus
EDP55391, A. fumigatus
human pathogen (aspergillosis)
XP_751268, A. fumigatus
human pathogen (aspergillosis)
XP_001262312, N. fischeri
lovastatin nonaketide synthase, LovB
XP_001271445, A. clavatus
human pathogen (aspergillosis)
similar to lovavastatin-nonaketide synthase, (LovB) architecture
KS-AT-DH-(ME)-KR-PP-(CON)-AMP-PP
F06A05KF
F12C01KF
F12A02KF
XP_002557403, P. rubens Wisconsin
lovastatin nonaketide synthase, LovB
EDP53404, A. fumigatus
equisetin synthase
XP_003173835, M. gypseum
lovastatin nonaketide synthase
BAC20564, P. citrinum
ML-236B (compactin)
EYB31408, F. graminearum
plant pathogen
ACT53004, H. fuscoatra
XP_001392496, A. niger
PKS/NRPS hybrid
ACN43267, H. oceanica
insect-specific PKS
AFA26379, A. flavus
cyclopiazonic acid
XP_002379959, A. flavus
PKS/NRPS hybrid
EED51183, A. flavus
equisetin synthase, PKS/NRPS hybrid
AFA26394, A. flavus
cyclopiazonic acid
F34E12
similar to PKS-NRPS hybrid architecture
KS-AT-DH(ME)-KR-PP-(CON)-AMP-PP-C-A-PCP
0.10 substitutions/site
to Figure 4.4B

Figure 4.4A

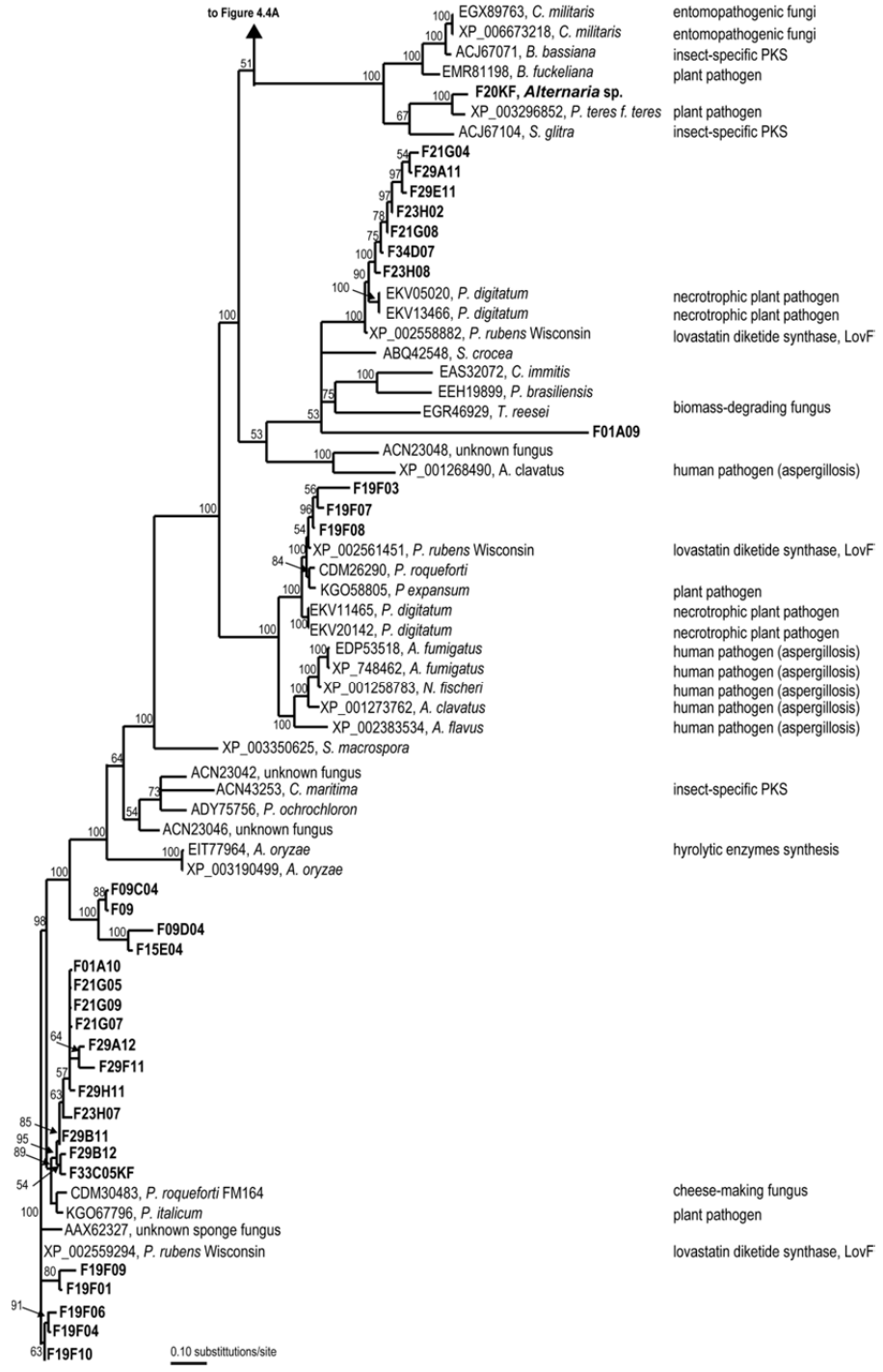
to Figure 4.4A
EGX89763, C. militaris
entomopathogenic fungi
XP_006673218, C. militaris
entomopathogenic fungi
ACJ67071, B. bassiana
insect-specific PKS
EMR81198, B. fuckeliana
plant pathogen
F20KF, Alternaria sp.
XP_003296852, P. teres f. teres
plant pathogen
ACJ67104, S. glitra
insect-specific PKS
F21G04
F29A11
F29E11
F23H02
F21G08
F34D07
F23H08
EKV05020, P. digitatum
necrotrophic plant pathogen
EKV13466, P. digitatum
necrotrophic plant pathogen
XP_002558882, P. rubens Wisconsin
lovastatin diketide synthase, LovF
ABQ42548, S. crocea
EAS32072, C. immitis
EEH19899, P. brasiliensis
EGR46929, T. reesei
biomass-degrading fungus
F01A09
ACN23048, unknown fungus
XP_001268490, A. clavatus
human pathogen (aspergillosis)
F19F03
F19F07
F19F08
XP_002561451, P. rubens Wisconsin
lovastatin diketide synthase, LovF
CDM26290, P. roqueforti
KGO58805, P expansum
plant pathogen
EKV11465, P. digitatum
necrotrophic plant pathogen
EKV20142, P. digitatum
necrotrophic plant pathogen
EDP53518, A. fumigatus
human pathogen (aspergillosis)
XP_748462, A. fumigatus
human pathogen (aspergillosis)
XP_001258783, N. fischeri
human pathogen (aspergillosis)
XP_001273762, A. clavatus
human pathogen (aspergillosis)
XP_002383534, A. flavus
human pathogen (aspergillosis)
XP_003350625, S. macrospora
ACN23042, unknown fungus
ACN43253, C. maritima
insect-specific PKS
ADY75756, P. ochrochloron
ACN23046, unknown fungus
EIT77964, A. oryzae
hyrolytic enzymes synthesis
XP_003190499, A. oryzae
F09C04
F09
F09D04
F15E04
F01A10
F21G05
F21G09
F21G07
F29A12
F29F11
F29H11
F23H07
F29B11
F29B12
F33C05KF
CDM30483, P. roqueforti FM164
cheese-making fungus
KGO67796, P. italicum
plant pathogen
AAX62327, unknown sponge fungus
XP_002559294, P. rubens Wisconsin
lovastatin diketide synthase, LovF
F19F09
F19F01
F19F06
F19F04
F19F10
0.10 substitutions/site

Figure 4.4B

Table 4.4. (See page 109 for caption)

Isolate[a]	Clones	Closely related protein sequences and NCBI accession number[b]	Similarities	Amino acid sizes (bp)	Intron sizes[c] (bp)
	F01A04	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	80%	312	292
	F01A07	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	312	279
F01	F01A08	polyketide synthase [Metarhizium robertsii], EXU95975	81%	278	66
	F01A09	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	43%	112	No
	F01A10	Beta-ketoacyl synthase [Penicillium roqueforti FM164], CDM30483	95%	286	53
	F05B06	Pc21g03930 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002567442	99%	313	261
F05	F05B07	Pc21g03930 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002567442	99%	330	38, 60
	F05B08	Pc21g03930 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002567442	99%	374	49, 60
F06	F0605KF	Pc12g05590 [*Penicillium chrysogenu* m Wisconsin 54-1255], XP_002567442	92%	218	46, 46
	F09	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	83%	140	No
	F09C03	LovB-like polyketide synthase [*Aspergillus fumigatus* Af293], XP_751268	74%	316	No
	F09C04	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	83%	140	51
F09	F09C10	hypothetical protein PDE_10006 [*Penicillium oxalicum* 114-2], EPS35041	72%	327	No
	F09D03	hypothetical protein PDE_10006 [*Penicillium oxalicum* 114-2], EPS35041	73%	342	No
	F09D04	polyketide synthase [*Thielavia terrestris* NRRL 8126], XP_003650812	43%	177	292, 58
	F09D05	hypothetical protein PDE_10006 [*Penicillium oxalicum* 114-2], EPS35041	75%	311	No
F12	F12A02KF	Pc12g05590 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002557403	92%	231	46, 52
	F12C01KF	Pc12g05590 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002557403	92%	222	46, 46
	F15E01	hypothetical protein PDE_10006 [*Penicillium oxalicum* 114-2], EPS35041	72%	327	No
	F15E03	hypothetical protein PDE_10006 [*Penicillium oxalicum* 114-2], EPS35041	70%	341	33
	F15E04	putative beta-ketoacyl synthase [*Colletotrichum sublineola*], KDN61564	53%	231	292
F15	F15E06	polyketide synthase, putative [*Aspergillus clavatus* NRRL 1], XP_001271445	76%	331	No
	F15E08	polyketide synthase, putative [*Aspergillus fumigatus* A1163], EDP55391	70%	348	No
	F15E09	polyketide synthase, putative [*Aspergillus clavatus* NRRL 1], XP_001271445	75%	226	No
	F15E10	polyketide synthase, putative [*Aspergillus fumigatus* A1163], EDP55391	76%	316	No
	F19F01	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	99%	136	55
	F19F03	Pc16g11480 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002561451	97%	194	84, 427, 51, 45
	F19F04	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	99%	274	55
F19	F19F06	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	98%	270	55
	F19F07	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM26290	94%	204	45, 50
	F19F08	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM26290	96%	204	45, 50
	F19F09	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	88%	328	55, 55
	F19F10	Pc13g08690 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002559294	99%	286	55
F20	F20KF	hypothetical protein PTT_07049 [*Pyrenophora teres f. teres* 0-1]	89%	227	No
	F21G03	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	251	No
	F21G04	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO62596	96%	301	56
	F21G05	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM30483	95%	286	53
F21	F21G07	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM30483	94%	286	53
	F21G08	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*],KGO62596	96%	332	56, 94
	F21G09	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164],CDM30483	95%	271	56
	F21G10	polyketide synthase [*Metarhizium robertsii*], EXU95975	81%	278	No
	F23H01	putative polyketide synthase [*Metarhizium anisopliae*], KFG85673	83%	294	170, 87
	F23H02	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO62596	94%	354	56
	F23H03	putative polyketide synthase [*Metarhizium anisopliae*], KFG85673	81%	293	66
	F23H04	polyketide synthase, putative [*Metarhizium anisopliae* ARSEF 23], XP_007826133	80%	306	262
F23	F23H05	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	79%	332	75
	F23H06	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	80%	325	66
	F23H07	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium italicum*], KGO67796	94%	304	53
	F23H08	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO62596	95%	344	56
	F23H10	polyketide synthase [*Metarhizium robertsii*], EXU95975	83%	304	66
F24	F24F05KF	Acyl transferase/acyl hydrolase/lysophospholipase [i], KGO58480	68%	283	No
	F29A11	Pc13g04470 [*Penicillium chrysogenum* Wisconsin 54-1255], XP_002558882	96%	215	56
	F29A12	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM30483	95%	288	53
	F29B11	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium italicum*], KGO67796	95%	307	53
	F29B12	Beta-ketoacyl synthase [Penicillium roqueforti FM164], CDM30483	84%	290	73, 106, 53, 50
F29	F29C11	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	82%	319	66
	F29D11	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	83%	304	66
	F29E11	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO62596	96%	289	56
	F29F11	Beta-ketoacyl synthase [*Penicillium roqueforti* FM164], CDM30483	95%	272	53
	F29G11	polyketide synthase [*Metarhizium robertsii*], EXU95975	79%	309	66
	F29H11	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium italicum*], KGO67796	95%	286	53
F33	F33C05KF	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium italicum*], KGO67796	93%	230	257, 431
	F34C12	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	345	66
	F34D07	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO62596	97%	158	56, 194
	F34D08	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	82%	304	66
	F34D09	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	312	290
F34	F34D10	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	312	298
	F34D12	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	312	220
	F34E12	truncated polyketide syntase A [*Aspergillus flavus*], AFA26394	71%	296	88, 61
	F34F12	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	81%	310	66
	F34G12	Acyl transferase/acyl hydrolase/lysophospholipase [*Penicillium expansum*], KGO58480	79%	352	84

[a] Fungus isolate code and identity are given in Table 1

[b] Closely related PCR products were determined by similarity searches using BLASTP

[c] Putative intron sizes correspond to the PKS gene fragments in the adjacent column. "no" indicates that the gene fragment had no introns. PKS fragment can have more than one introns, *i.e.* F05B07 (38, 60).

Discussion

Sponge-derived fungi are among the most prolific organisms for secondary metabolite production with a wide range of biotechnologically interesting compounds such as trichoharzin, gymnasterons, penicillinide and citrinide (Kobayashi*, et al.*, 1993, Amagata*, et al.*, 1998, Bhadury*, et al.*, 2006, Saleem*, et al.*, 2007). Many of the secondary metabolites have been suggested to act as pheromones (*i.e* sesquiterpenoids), antifeedants (*i.e* anthraquinone) or repellents and regulators in the development of the sponge host (Brauers*, et al.*, 2000, Debbab*, et al.*, 2012, Wang*, et al.*, 2014). Several previous studies have shown that marine sponges indeed harbour a substantial diversity of cultivable fungi within their tissue (Höller*, et al.*, 2000, Pivkin*, et al.*, 2006, Paz*, et al.*, 2010, Wiese*, et al.*, 2011, Zhou*, et al.*, 2011, Yu*, et al.*, 2012). However, it has been frequently demonstrated that cultivable fungal taxa from sponges resemble those from terrestrial habitats (Paz*, et al.*, 2010, Wiese*, et al.*, 2011, Yu*, et al.*, 2012). This is in accordance with our findings where *Penicillium* spp., followed by *Alternaria* sp., represented the largest number of cultivable fungi from the sponges *Aplysina aerophoba*, *Petrosia ficiformis* and *Corticium candelabrum* (Figure 4.2). Although none of our filamentous fungal isolates were affiliated with known fungal isolates obtained from marine sources (Figure 4.2), they should be considered as marine-fungi since they could sporulate in culture media containing ASW (Kohlmeyer & Kohlmeyer, 1979, Mayer*, et al.*, 2007). As of now, the only marine filamentous fungus known to have a unique physical association with crustaceous sponges is an ascomycete from the genus *Koralionastes* (Kohlmeyer & Volkmann-Kohlmeyer, 1990). It is interesting to note that filamentous fungi from this study were closely related to known plant endophytes (*i.e*. *P. solitum*, *P. tardum* and *P. freii*, Figure 4.2). Plant endophytes are well known to produce a wide range of chemicals that play a role in host defence (Arnold*, et al.*, 2003, Vega*, et al.*, 2008), and it is tempting to speculate that this could also be the case for sponge-associated fungi.

In our study, we also found yeasts that are closely related to *Rhodotorula* spp., which are commonly found in the deep sea (Kutty & Philip, 2008). Yeasts are commonly found in marine environments but far less common in sponges, although few studies have recorded the isolation of yeasts such as *Pichia* sp., *Metchnikowia* sp., *Tremellales* sp.,and *Rhodotorula* sp. from sponges (Burgaud*, et al.*, 2010, Liu*, et al.*, 2010, Vaca*, et al.*, 2012).

Examination of tissues of sponges from the genus *Chondrilla* indicated the presence of intra- and extracellular unidentified yeasts living in symbiosis with the sponge host (Maldonado*, et al.*, 2005). Recent evidence from the study of sponges from the Mediterranean and North Sea using next-generation sequencing suggested that yeasts could be a major player in the sponge holobiont (Chapter 3).

Penicillium is a fungal genus widely distributed in different environments and among the taxa most frequently isolated from sponges (Höller*, et al.*, 2000, Wang*, et al.*, 2008). Phylogenetic analysis of rRNA sequences of putative sponge-associated microbes available in public database lead to the conclusion that several bacteria and archaea are symbionts of sponges, and similarly the presence of sponge-specific *Penicillium* clades has been proposed (Simister*, et al.*, 2012). *Penicillium* spp. associated with sponges are known to produce a large variety of bioactive compounds with a wide range of pharmacological activities (Bringmann*, et al.*, 2004, Bringmann*, et al.*, 2005, Qi*, et al.*, 2013), but they are known to be heterogenous with respect to their genomic content and difficult to classify to the strain level based on morphological characteristics and the 18S rRNA gene sequence (Bakri*, et al.*, 2007). The use of a simple repeat primer was shown to be instrumental for discriminating strains of the same *Penicillium* species based on their genomic content (Bakri*, et al.*, 2007, Roslan*, et al.*, 2009), and this method has previously been employed to increase the taxonomic resolution of *Penicillium* spp. isolated from sponges (Zhou*, et al.*, 2011). It was demonstrated that several *Penicillium* spp. strains included in the same clade showed different antimicrobial activity (Zhou*, et al.*, 2011), indicating that is important to analyse each strain. In our study, no clear correlation was found between RAPD banding patterns and types of PKS encoding gene sequences that were retrieved from different fungal isolates. However, it is interesting to note that fungal isolates (*i.e.* F05, F09, F15 and F19) harboured more than one type of putative polyketide synthases (Table 4.4) and formed a distinct cluster.

Despite the evidence presented on bacterial symbionts from sponges as the producers of metabolites originally assumed to be produced by the sponge (Konig*, et al.*, 2006), comparable evidence for fungi is lacking. For example, evidence of bacteria involved in host-defence was derived from the uncultured bacterial community in the marine sponge *Theonella swinhoei* and *Paederus* beetles that produce the anticancer polyketide

onnamide, was speculated to confer an advantage to animal hosts rather than to the producers themselves (Piel, 2002, Piel*, et al.*, 2004). This also holds true for the antitumor agent psymberin from the marine sponge *Psammocinia aff. Bulbosa*, which is produced by its uncultured bacterial symbionts (Fisch*, et al.*, 2009). Polyketide compounds such as onnamide and psymberin have to date not been detected in non-symbiotic bacteria, hence the idea was since it is essential in the host defence, the biosynthetic genes would be preferentially maintained in symbionts (Piel*, et al.*, 2005, Piel, 2006). Detection of polyketide synthase (PKS) genes and corresponding compounds in sponge-associated fungi hints that these fungi play similar role in the chemical defence of the host (Amagata*, et al.*, 2010, Proksch*, et al.*, 2010, Zhou*, et al.*, 2011, Almeida*, et al.*, 2012). However, to conclude on the ecological relevance of these PKS clusters in sponge-derived fungi, it is important to complement these studies with cultivation-independent approaches. In our study the fungi isolated from *Aplysina aerophoba* were not found in the tissue from the same individual by molecular characterisation of the fungal diversity.

It is well documented that in fungal PKS, the KS domain is the most conserved (Bingle*, et al.*, 1999, Kroken*, et al.*, 2003) while the KS-AT interdomain region is usually more variable (Amnuaykanjanasin*, et al.*, 2009). Targeting the KS and KS-AT conserved region makes it possible to identify PKS genes without the need to sequence larger portions of every gene cluster (Kroken*, et al.*, 2003). The KA primer series designed by Amnuaykanjanasin and co-workers (2005) was originally designed for the purpose of detecting the lovastatin-type PKS, which is responsible for the production of a cholesterol lowering agent (Borchardt, 1999, Meenupriya*, et al.*, 2011). Our phylogenetic analysis (Figure 4.4.A-B) suggested that the majority of sponge-associated fungi-derived PKS gene fragments are predicted to encode proteins of the reducing type PKS, which is closely related to lovastatin-type biosynthesis (LovA, LovB, LovF). In addition, we reported putative fungal PKS-NRPS hybrids which are responsible for the large functional diversity of PKS leading to a wide range of compounds such as fusarin c (mycotoxin), equisetin (HIV-1 inhibitor) and cyclopiazonic acid (mycotoxin) (Boettger*, et al.*, 2012, Boettger & Hertweck, 2013, Fisch, 2013). Structural diversity of fungal polyketides results from the number of iteration reactions, the number of reduction reactions, types of subunits used, degree of chemical reduction of the β-keto thioester, extent of

stereochemistry of the α-keto group at each condensation and in the case of aromatic polyketides, subsequent processing of the nascent polyketide chain (*i.e.* cyclization) (Keller*, et al.*, 2005, Boettger & Hertweck, 2013). In our study, *Penicillium* spp. and *Alternaria* spp. screened were positive for polyketide synthase encoding genes. Polyketides known from *Penicillium* spp. and *Alternaria* spp. include griseofulvin, ochratoxins A and B, patulin and citrinin (Kozlovskii*, et al.*, 2013).

Conclusion

In this study, we have identified genetically diverse sponge-associated fungi, which are phylogenetically related to plant fungal endophytes. Filamentous fungi from the genus *Penicllium* could sporulate in media prepared with artificial seawater, indicating that they can be characterized as marine fungi. Diverse PKS genes from sponge-derived fungi were discovered, of which many are similar to sequences encoding lovastatin type PKS. Putative PKS-NRPS hybrid genes which are responsible for the large functional diversity of fungal PKSs were also detected from sponge-derived fungi,. Our finding highlights once more that sponge-derived fungi are a potentially prolific biological resource for novel PKS and varieties of known PKS.

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Conflict of interest

The authors declared that there are no conflicts of interest.

Supplementary Materials

Table S4.1 is available upon request.

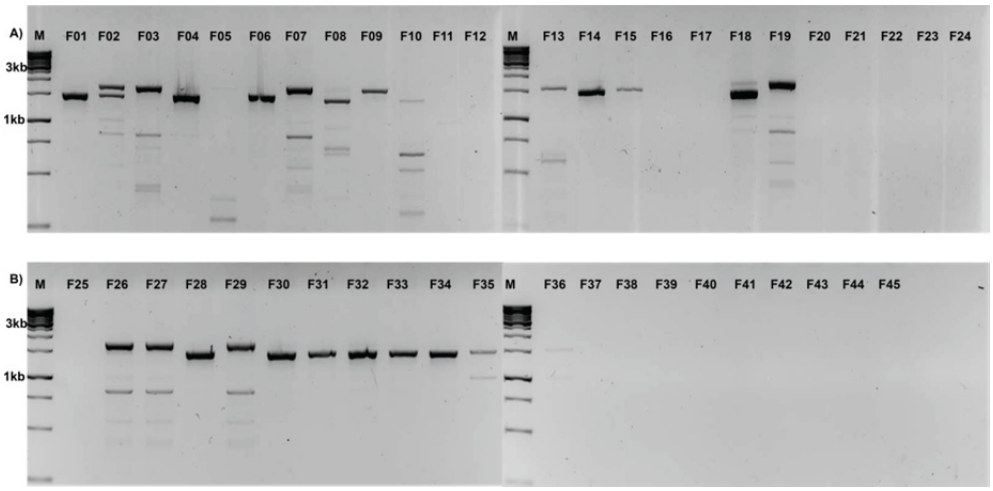

Fig.S4.1. Random amplified polymorphic DNA (RAPD) pattern of fungal isolates from sponges generated with primer M13. A) Fungal isolate F01 to F24 and B) Fungal isolate F25 to F45. M refers to DNA ladder used (Fermentas) with the size 1kb and 3kb denoted.

Chapter 5:

Systematic assessment of cultivable bacteria from the marine sponge *Halichondria panicea*

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Summary

Sponges are known to harbour a substantial bacterial diversity in their tissue. In the present work we focus on cultivation of bacteria from the marine sponge *Halichondria panicea* by utilizing a large variety of different cultivation media and cultivation conditions. This cultivation-dependent approach was complemented with cultivation-independent assessment of bacterial community composition through pyrosequencing of the 16S rRNA gene pool from the same sponge individual. For the sponge microbiota, it was demonstrated that a predominant actinobacterial phylotype represented 29 % of all 16S rRNA gene sequences retrieved from the sponge tissue, while a *Rhodobacter*-like phylotype represented 14.6 % of the reads. A total of 1283 isolates belonging to 75 different genera were obtained from this sponge specimen. Although the dominant sponge-associated bacteria were not recovered, 3.7 % of the bacterial phylotypes present in the sponge were also obtained in culture.

Keywords: sponge-associated bacteria, pyrosequencing, cultivation, *Halichondria panicea*

Introduction

Sponges (phylum *Porifera*) are ancient sessile invertebrates that have been around at least 650 million years (Maloof*, et al.*, 2010). The sponge holobiont is a complex system comprising microbial representatives from the three domains of life, namely *Eukarya*, *Bacteria*, and *Archaea*. To date, 32 different bacterial and archaeal phyla and candidate phyla have been detected in sponges (Hentschel*, et al.*, 2012, Schmitt*, et al.*, 2012). It is well known that sponges are prolific source for bioactive compounds ranging from antibacterial, anticancer, anticoagulant, antifungal, antiviral to other biological activities (Sipkema*, et al.*, 2005a, Sipkema*, et al.*, 2005b, Blunt*, et al.*, 2011, Imhoff*, et al.*, 2011). Often the production of such compounds was attributed to sponge-associated bacteria (Faulkner*, et al.*, 2000, Thomas*, et al.*, 2010). For example, bacterial symbionts in sponges have been shown to be the producers of cytotoxic secondary metabolites such as psamberin and pederin (Piel*, et al.*, 2005, Fisch*, et al.*, 2009). Bacteria can contribute to more than 40% of the total sponge biomass (Vacelet & Donadey, 1977) and can exceed the bacterial concentration in the surrounding seawater by two to three orders of magnitude (Friedrich*, et al.*, 2001). Electron microscopy studies showed that some sponges harbour dense microbial consortia within their mesohyl tissues, while microbial presence in other sponges was much less prominent (Reiswig, 1974, Vacelet & Donadey, 1977, Imbs & Rodkina, 2004). This led to two general classifications based on the abundance of bacteria. Firstly, a distinction was made between “bacteriosponges” for sponges that harbour large numbers of bacteria and “non-symbiont harbouring, normal sponges” for species that seemed void of bacteria (Reiswig, 1981). Secondly, in order to acknowledge the presence of archaea in sponge tissues, the terms “high microbial abundance” (HMA) and “low microbial abundance” (LMA) sponge were coined later (Hentschel*, et al.*, 2003). Bacterial phyla such as *Acidobacteria, Actinobacteria*, candidate phylum *Poribacteria*, *Proteobacteria* and *Chloroflexi,* are more common in HMA sponges that are also generally more microbially diverse in comparison to LMA sponges (Weisz*, et al.*, 2007, Erwin*, et al.*, 2012, Schmitt*, et al.*, 2012, Giles*, et al.*, 2013, Moitinho-Silva*, et al.*, 2014, Naim*, et al.*, 2014, Poppell*, et al.*, 2014). LMA sponges are generally either dominated by either a single proteobacterium (alpha-, beta-, or gamma-) or a cyanobacterium (genus

Synechococcus) (Schmitt*, et al.*, 2012, Giles*, et al.*, 2013, Naim*, et al.*, 2014). Cultivation and isolation of microorganisms is indispensable for studying the physiological and metabolic characteristics of individual microbial strains (Vartoukian*, et al.*, 2010). However, the majority of microorganisms from sponges has to date not been amenable to cultivation (Sipkema*, et al.*, 2011, Schippers*, et al.*, 2012, Montalvo*, et al.*, 2014, Hardoim*, et al.*, 2015).

Most cultivation studies from sponges focus on novel isolates and production of bioactive compounds, but little attention is given to unravelling the host-symbiont relationship. Only recently unconventional approaches, such as mimicking the natural environment inside the sponge body, *i.e.* using oxygen gradients and diffusion growth chambers, have been applied for the cultivation of sponge-associated bacteria (Lavy*, et al.*, 2014, Steinert*, et al.*, 2014). Studies applying a large diversity of cultivation methods have shown that up to 14% of the bacterial richness associated to sponges can be isolated (Sipkema*, et al.*, 2011).

In the present work, we have concentrated our efforts on enhancing the cultivability of bacteria associated with the marine sponge *Halichondria panicea*. *H. panicea* is a typical LMA sponge, which has been found to be dominated by a single α-proteobacterial symbiont (Althoff*, et al.*, 1998). A recent 16S ribosomal RNA (rRNA) gene-targeted pyrosequencing study confirmed the dominance of this alphaproteobacterial symbiont, with more than 80% of the reads belonging to a *Rhodobacter*-like α-proteobacterium (Naim*, et al.*, 2014). In addition to the use of general oligotrophic cultivation media on agar plates, we defined a set of cultivation media and environmental conditions targeted on the isolation of the *Rhodobacter*-like bacterium. Thirdly, we applied liquid growth media to grow this bacterium and other sponge-associated bacteria. We complemented the cultivation experiment by pyrosequencing of the 16S rRNA gene pool from the same *H. panicea* individual.

Materials and Methods

Sample collection

A specimen (HP1) of *Halichondria panicea* (Class *Demospongiae;* Order *Halichondriida;* Family *Halichondridae*) was collected by SCUBA diving at a depth of approximately 8 meters on September 14th, 2009, in the Eastern

Scheldt estuary at the dive site Lokkersnol near Zierikzee, The Netherlands (N51°38'54.84", E3°53'6.68"). Sample preparation of HP1 was done according to the method described by Sipkema *et al.* (Sipkema & Blanch, 2010). Briefly, the sponge was rinsed three times with sterile artificial seawater before grinding the tissue with an autoclaved mortar and pestle. A homogeneous cell suspension was obtained by addition of two tissue volumes of sterile artificial seawater. The cell suspension was divided in aliquots of 1.0 ml. One aliquot of the cell suspension was immediately used for cultivation. The other aliquots were mixed with 0.5 ml 50% glycerol in artificial seawater and stored at -80 ºC. Natural seawater (NSW) was collected from the vicinity of the sponge sampling site, and upon arrival at the laboratory it was filtered with a 0.45 µm polycarbonate filter (GE Osmonics, Minnetonka, MN), autoclaved and stored at 4 °C until further use for preparation of cultivation media.

Cultivation media

Three types of cultivation media were applied. We first applied i) basic cultivation media to isolate as many different bacteria as possible from *H. panicea*, then ii) targeted cultivation media to attempt to isolate the *Rhodobacter*-like bacterium reported to be present in high numbers in *H. panicea* (Althoff*, et al.*, 1998, Naim*, et al.*, 2014) and iii) liquid growth media (Table 5.1).

i) Basic cultivation media

For basic cultivation of *H. panicea*-associated bacteria, nine different agar media were selected from a previous study (Sipkema*, et al.*, 2011). The nine different agar media used were: **1.** Basic agar (1 l sterile Natural seawater [NSW]); **2**. Actinomycete isolation agar (1 l NSW, 1 g peptone from casein, 0.1 g asparagine, 4 g sodium propionate, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 1 mg $FeSO_4.7H_2O$, 1 ml glycerol); **3.** Raffinose-histidine agar **(**1 l NSW, 2 g raffinose, 0.2 g histidine) (modified from (Webster*, et al.*, 2001)); **4.** Glycerol-arginine agar (1 l NSW, 2 ml glycerol, 0.3 g arginine); **5.** Peptone starch agar (1 l NSW, 2 g starch, 0.2 g peptone from casein) (modified from (Mincer*, et al.*, 2002)); **6.** Fluid thioglycollate agar (1 l NSW, 1 g peptone from casein, 0.3 g yeast extract, 0.3 g D-glucose, 0.05 g L-cystine, 0.5 g sodium thioglycollate); **7.** Cellobiose agar (1 l NSW, 2 g Cellobiose); **8.** Delicious agar (1 l NSW, 0.3 g peptone from casein, 0.1 g yeast extract, 0.01 g D-glucose); **9.** Tryptone soy agar (1 l NSW, 15 g

tryptone soy broth). All media, except Tryptone soy agar, were supplemented with 15 g agar to produce solid media (Tryptone soy agar already contains 15 g agar). Furthermore, all media were supplemented with 1 ml trace metal solution, 1 ml phosphate solution (Olson & McCarthy, 2005) and 1 ml vitamin solution (based on BME vitamins, Sigma). The vitamin solution was added after autoclaving the media to prevent degradation. Four agar plates were poured with each medium. The agar surfaces of the plates were inoculated with 50 µl of 10^{-1}, 10^{-2} and 10^{-3} dilutions of the cell suspension (see part sample collection), making use of sterilized glass beads. Additionally, one plate was included as a negative control. The plates were incubated in the dark between 18 ºC to 20 ºC for at least 20 days.

ii) Targeted cultivation media

For the targeted cultivation approach, a number of selective media for cultivation of the *Rhodobacter*-like bacterium were used in combination with different environmental parameters with respect to light and oxygen (Table 5.1). The media applied were: **10**. Glycerol-arginine agar (similar to **4**); **12.** Marine rich agar (3.74 g marine broth 2216, 900 ml NSW, 100 ml dH_2O); **11**. Marine gradient agar (3 different mixtures of glycerol-arginine agar (GAA) and marine rich agar (MRA). Mixtures tested were 25% GAA - 75 % MRA, 50% GAA - 50 % MRA and 75% GAA - 25 % MRA; **13**. *Rhodobacter* agar (1 l NSW, 1 g yeast extract, 1 g disodium succinate, 0.5 g KH_2PO_4, 0.4 g NH4Cl). 5 ml ferric citrate solution , 0.5 ml ethanol, and 1 ml Rhodobacter trace elements solution was added according to Atlas (2010). The composition of the Rhodobacter trace elements is (0.3 g H_3BO_3, 0.2 g $CoCl_2$, 0.1 g $ZnSO_4$, 0.03 g Na_2MoO_4, 0.02 g $NiCl_2$, 0.01 g $CuCl_2$); **14**. R8AH agar (1 l NSW, 2.5 g malic acid, 1 g yeast extract, 0.9 g K_2HPO_4, 0.6 g KH_2PO_4, 0.07 g EDTA, 0.02 g ferric citrate). 7.5 ml vitamin solution (BME vitamins, Sigma) and 1 ml trace elements solution SL-6 was added (Atlas, 2010). The composition of the trace elements solution SL6 is (0.3 g ferric citrate, 0.05 g EDTA, 0.02 g $CaCl_2$, 0.02 g $MnSO_4$, 0.02 g $(NH_4)_6Mo_7O_{24}$, 0.001 g H_3BO_3, 0.001 g $CuSO_4$, 0.001 g $ZnSO_4$); **15**. *Rhodospirillaceae* agar (1 l NSW, 1 g succinic acid, 0.5 g KH_2PO4, 0.4 g NH_4Cl, 0.2 g yeast extract).). 5 ml ferric citrate solution, 1 ml vitamin B_{12} solution and1 ml trace elements solution was added as described by Atlas (2010). Composition of trace elements solution SL-7 is (0.2 g $CoCl_2$, 0.1 g $MnCl_2$,

0.07 g $ZnCl_2$, 0.06 g H_3BO_3, 0.04 g Na_2MoO_4, 0.02 g $CuCl_2$, 0.02 g $NiCl_2$, 1 ml 25% HCl solution).
The pH ranged from 5.8 to 7.2 for the different media (Table 5.1). All media were supplemented with 15 g agar per l to produce solid media and (if applied) the vitamin solution was added to the media after autoclaving. Three agar plates were poured per medium with one additional plate as negative control for the cultivation experiment. The agar surfaces were inoculated with 50 µl of a 10^{-3} dilution of the cryopreserved HP1 cell suspension (see part sample collection), and spread with the use of sterilized glass beads. The plates were incubated at 20 ºC with different environmental parameters (Table 5.1). Media 10-15 were incubated aerobically either in the dark (indicated as 10a, 11a, 12a, 13a, 14a and 15a) or under tungsten light (indicated as 10b, 11b, 12b, 13b, 14b and 15b). Media 10-15 were also used for anaerobic cultivation experiments (indicated as 10c, 11c, 12c 13c, 14c and 15c). For the anaerobic experiments, the media were additionally supplemented with 0.42 g/l $NaNO_3$, 0.96 g/l sodium propionate and 0.001 g resazurin. Anaerobic jars were used for incubation and an anaerobic chamber was used for removing oxygen present at the start of the experiment, manipulation of plates such as checking for growth and transferring to a new plate. All plates were incubated between 18 ºC to 20 ºC for at least 35 days and a maximum of 60 days before analysis.

iii) Liquid media

For enrichment of bacteria from *H. panicea*, liquid cultures were prepared as follows: **16**. Synthetic *Thaumarchaeota* medium (previously described as *Crenarchaeota* medium (Koenneke*, et al.*, 2005) (1 l NSW, 5 g $MgCl_2$, 5 g $MgSO_4$, 1.5 g $CaCl_2$ and 0.1 g KBr. The pH was adjusted to 7.0–7.2 using NaOH. Three 250 ml screw cap bottles plus one negative control were filled with 100 ml media and after autoclaving the following solutions (Widdel & Bak, 1992) were added aseptically: 0.2 ml non-chelated trace element mixture, 0.2 ml vitamin solution, 2 ml KH_2PO_4 solution (4 g l^{-1}), 0.2 ml selenite-tungstate solution, 0.2 ml bicarbonate solution (1 M), and 0.2 ml ammonium chloride (1 M). Medium **17, 18** and **19** were the same as **13, 14** and **15**, but no agar was added. Three 10 ml screw-cap bottles filled with 5 ml of medium were used with one additional bottle as negative control for each of the media 17, 18 and 19; **20.** Pfennig's medium was prepared

according to methods described previously (Balows, *et al.*, 1992). It contained (1 l of distilled water, 1 g KH_2PO_4, 0.05 g $CaCl_2{\cdot}2H_2O$, 0.5 g NH_4Cl, 100 g NaCl, 2 g $MgCl_2{\cdot}6H_2O$, 1 g $MgSO_4{\cdot}7H_2O$, 2 g $NaHCO_3$, 0.75 g $Na_2S{\cdot}9H_2O$, 1 ml vitamin solution V7, and 1 ml trace element solution SL12. The pH was adjusted to pH 7.2–7.4. **21.** Marine anaerobic medium1 (1L NSW, 11.2 g $MgCl_2$, 1.4 g $CaCl_2$, 0.7 g KCl, 0.1 g KBr). 10 ml ammonium/phosphate-solution). 1 ml vitamin-solution, 1 ml tungsten/selenite-solution, 30 ml bicarbonate-solution, 1.5 ml sodium sulfide-solution and 1 ml trace elements-solution was added as described previously (Holliger *et. al*, 1993). **22.** Marine anaerobic medium2 (1L NSW 1 g KH_2PO_4, 3.48 g $Na_2HPO_4{\cdot}2H_2O$, 0.81 g NH_4Cl, 0.033 g $MgCl_2{\cdot}6H_2O$, 0.0090 g $CaCl_2{\cdot}2H_2O$, 0.01 g Fe(NH_4) citrate. Vitamins and trace elements were added in the final concentrations as described by (Holliger, *et al.*, 1993); **23** *Rhodovulum adriaticum* (Hiraishi & Ueda, 1994) medium (previously known as *Rhodobacter adriaticus* (Neutzling, *et al.*, 1984)) (1L NSW, 3 g $NaHCO_3$, 1 g K_2HPO_4, 1 g NH_4Cl, 0.5 g sodium ascorbate). 1 ml vitamin solution (BME vitamins, Sigma) and 1 ml trace elements solution SLA was added according to Atlas (2010). The composition of trace elements SLA is (5 g $CuCl_2$, 1.8 g $FeCl_2$, 0.5 g H_3BO_3, 0.25 g $CoCl_2$, 0.1 g $ZnCl_2$, 0.07 g $MnCl_2$, 0.03 g Na_2MoO_4, 0.01 g Na_2SeO_3, 0.01 g $NiCl_2$); **24.** *Rhodobacter veldkampii* enrichment medium was prepared as described previously (Hansen & Imhoff, 1985). It contained: Solution 1: 2.5 g sodium acetate, 2 g $CaCl_2$ dissolved in 2.5 l NSW. It was distributed as 53 ml aliquots in 100 ml serum bottles and autoclaved; Solution 2: 50 ml of heavy metal solution (Atlas, 2010), 3 ml of vitamin B_{12} solution (Atlas, 2010), 15 ml of vitamin solution (Atlas, 2010), 1 g KH_2PO_4, 1 g KCl, 0.8 g NH_4Cl, 0.8 g $MgCl_2{\cdot}6H_2O$, 32 ml distilled water. Solution 3: 4.5 g $NaHCO_3$, 900 ml distilled water. Solution 3 was flushed with CO_2 for 30 minutes. After CO_2 saturation, solution 3 was immediately added to solution 2. This combined solution 2 and 3 was filter sterilized using CO_2 pressure to push the liquid through (no suction) and this sterile-filtered solution was added to the 100 ml bottles containing 53 ml of solution 1 until the bottles were completely filled. Solution 4: 3 g $Na_2S{\cdot}9H_2O$ dissolved in 200 ml distilled water. It was autoclaved with a magnetic-stirrer rod in the flask and partially neutralized after sterilization by adding drop by drop 2 ml sterile 2M H_2SO_4. Solution 4 was added to the bottles containing solution 1, 2 and 3 by removing 6 ml from the bottles and replacing it by an equal volume of solution 4. The

bottles were allowed to stand overnight to develop a hazy white precipitate before they were inoculated with 10^{-3} dilution of cryopreserved HP1 cells as described earlier. For media 20-24, three anaerobic serum bottles of 100 ml, plus one additional negative control each were sealed with butyl-rubber stoppers and crimp seals, and they were filled with 100 ml medium, leaving no headspace. Bottles were flushed with dinitrogen gas (1.5 atm). . All bottles were inoculated with 1 ml 10^{-3} dilution of the cryopreserved HP1 cell suspension. Enrichment cultures 17,18,-19,20, 23 and 24 were grown in tungsten light. If included in the medium, the vitamin solution was added to the media after autoclaving. After a minimum incubation period of 30 days at 18 °C to -20°C, depending on the growth, 10% of the enrichment cultures were transferred to a new bottle with fresh media. This process was repeated for three times (another 30 days and so on) to reduce the number of bacterial richness as described previously (Bedard*, et al.*, 2006, Sousa*, et al.*, 2007).

Table 5.1. Culture media used, cultivation conditions applied, pH and number of isolates/clones sequenced per medium.

Medium No	Name	Abbrev.	Culture condition	pH	No of isolates/ clones sequenced [b]
	BASIC MEDIA				
1	Basic Agar	BA	dark, 18°C	7.5	17
2	Actinomycetes Isolation Agar	AIA	dark, 18°C	7.5	27
3	Raffinose-Histidine Agar	RHA	dark, 18°C	7.5	36
4	Glycerol-arginine Agar	GAA	dark, 18°C	7.5	41
5	Peptone starch Agar	PSA	dark, 18°C	7.5	61
6	Fluid thioglycollate Agar	FTA	dark, 18°C	7.5	63
7	Cellbiose Agar	CA	dark, 18°C	7.5	no growth
8	Delicious Agar	DA	dark, 18°C	7.5	98
9	Tryptone Soya Agar	TSA	dark, 18°C	7.5	125
	TARGETED MEDIA				
10a	Glycerol-arginine Agar	0%LO	aerobic, light, 18 - 20°C	7.2	62
10b	Glycerol-arginine Agar	0%DO	aerobic, dark, 18 - 20°C	7.2	72
10c	Glycerol-arginine Agar	GD0%SAN	anaerobic, dark, 18 - 20°C	7.2	31
11a	Marine Gradient Agar[b]	GDXLO	aerobic, light, 18 - 20°C	7.2	52
11b	Marine Gradient Agar[b]	GDXDO	aerobic, dark, 18 - 20°C	7.2	58
11c	Marine Gradient Agar[b]	GDXSAN	anaerobic, dark, 18 - 20°C	7.2	36
12a	Marine Rich Agar	GD100%LO	aerobic, light, 18 - 20°C	7.2	9
12b	Marine Rich Agar	GD100%DO	aerobic, dark, 18 - 20°C	7.2	13
12c	Marine Rich Agar	GD100%SAN	anaerobic, dark, 18 - 20°C	7.2	12
13a	Rhodobacter medium	RBLO	aerobic, light, 18 - 20°C	6.9	54
13b	Rhodobacter medium	RBDO	aerobic, dark, 18 -20°C	6.9	78
13c	Rhodobacter medium	RBSAN	anaerobic, dark, 18- 20°C	6.9	62
14a	R8AH medium	RHLO	aerobic, light, 18 - 20°C	6.9	46
14b	R8AH medium	RHDO	aerobic, dark, 18 - 20°C	6.9	18
14c	R8AH medium	RHSAN	anaerobic, dark, 18 - 20°C	6.9	29
15a	Rhodospirillaceae medium	RSLO	aerobic, light, 18 - 20°C	5.8	57
15b	Rhodospirillaceae medium	RSDO	aerobic, dark, 18 - 20°C	5.8	29
15c	Rhodospirillaceae medium	RSSAN	anaerobic, dark, 18 - 20°C	5.8	no growth
	LIQUID MEDIA				
16	Crenarchaeota Medium	ARC	aerobic, dark, 18 - 20°C	7.2	37
17	Rhodobacter medium	RB	aerobic, light, 18 - 20°C	6.9	50
18	R8AH medium	RH	aerobic, light, 18 - 20°C	6.9	no growth
19	Rhodospirillaceae medium	RS	aerobic, light, 18 - 20°C	5.8	52
20	Pfennig's medium	PF	anaerobic, light, 18 - 20°C	6.8	24
21	Marine X medium	MA1	anaerobic, dark, 18 - 20°C	7.3	13
22	Marine Anaerobic medium	MA2	anaerobic, dark, 18 - 20°C	7.3	11
23	*Rhodovulum adriaticum* medium	RA	anaerobic, light, 18 - 20°C	7	no growth
24	*Rhodobacter veldkampii* medium	RV	anaerobic, light, 18 - 20°C	6.7	no growth

[a] Isolates or clones sequenced based on number of high-quality PCR products generated

[b] Marine Gradient Agar is the combination of Marine Rich Agar and Glycerol-arginine Agar with fixed percentages 25%, 50% and 75%. X refers to the percentage used, for instance, GD25%DO means 25% of Marine Rich Agar plus 75% of Glycerol-arginine agar and so on.

DNA extraction from sponge tissue and liquid cultures

DNA was extracted from cryopreserved cells of HP1 and liquid cultures using the FastDNA® Spin Kit for Soil (MP Biomedicals). Starting material for each sample was cells harvested either from 2 ml of cryopreserved sponge cell solution, or from 5 ml liquid cultures by centrifugation at 10,000 g for 5 min. DNA extraction was done according to the manufacturer's instructions. DNA concentration and purity were measured using a NanoDrop spectrophotometer (ThermoFisher Scientific, St. Leon-Rot, Germany) and visualised by 1% (w/v) gel electrophoresis.

16S rRNA gene PCR of the isolates

Colonies growing on the agar plates were numbered and picked using sterile tooth picks and transferred to 96-well, 200 µl microtiter plates filled with 10 µl nuclease free water per well (Promega). The filled microtiter plates were stored at -20 ºC overnight. Then, 1 µl of these samples was used to amplify the 16S rRNA gene in a 50 µl PCR reaction. The PCR mixtures were prepared as follows: 10 µl green GoTaq reacton buffer (5X), 1 µl PCR nucleotide mix (10mM each), 0.5 µl GoTaq DNA polymerase (1.25u), 1 µl (10 pmol) each, of universal bacterial forward primer 27F (Weisburg*, et al.*, 1991) and bacterial reverse primer 1492R (Wilson*, et al.*, 1990), see (Table 5.2). and 34.5 µl nuclease-free water. The PCR program was as follows: initial denaturation for 5 min at 94 ºC, followed by 35 cycles of denaturation for 30 sec at 94 ºC, annealing for 40 sec at 52 ºC and extension for 1.30 min at 72 ºC followed by a final extension for 5 min at 72 ºC. The PCR amplicons were visualized on a 1% (w/v) agarose gel supplemented with SybrSafe (LifeTechnologies GmbH, Darmstadt, Germany) dye. The amplified fragments were purified using the Roche High Pure PCR Micro Cleanup Kit (Roche Diagnostics Nederland BV, Almere) and Sanger sequenced using the 27F primer (Lane, 1992) .

The partial 16S rRNA gene from the liquid growth cultures was PCR amplified, visualised and purified as described above. Subsequently, they were used to construct clone libraries. Clone libraries were prepared using the pGEM®-T Easy Vector Systems (Promega) according to the manufacturer's instructions. The ligation products were transformed into XL1-Blue competent cells (Agilent Technologies GmbH, Böblingen, Germany). The transformed cells were plated on LB agar plates (DSMZ 318) containing ampicillin, X-Gal and IPTG, which allowed for colour

selection of colonies. White clones were selected for sequencing using the T7 primer.
The 16S rRNA gene sequences of isolates and clones were deposited at the NCBI GenBank under accession numbers KP684272 - KP684433 and . KP684434 - KP684480, respectively.

Characterisation of the isolated bacteria

16S rRNA gene sequences were checked for possible chimeric origins using the DECIPHER webserver (http://decipher.cee.wisc.edu/FindChimeras.html)(Wright*, et al.*, 2012). Sequences were classified using the online version of RDP Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Wang*, et al.*, 2007). To identify sequence similarity between isolated bacteria and OTUs from the pyrosequencing (see below), all 16S rRNA gene sequences of isolates were blasted against a database containing the pyrosequencing OTUs using a locally installed version of Blast2-2-24 (Zhang*, et al.*, 2000).

Multivariate analysis

To assess the effect of cultivation media and cultivation conditions on the diversity of cultivable bacteria (genus level taxonomy), redundancy analysis was performed using CANOCO 4.5. (ter Braak, Wageningen University). A matrix of relative abundance data at genus level obtained under the different conditions was square-root transformed. The five media for which we did not obtain growth were left out of the analysis. Cultivation conditions (solid/liquid, light/dark and aerobic/anaerobic) were used as environmental variables. The significance of the first RDA axis and all RDA axes combined was tested using Monte Carlo permutation tests (ter Braak & Smilauer, 2002).

Molecular identification of the sponge host

The partial 18S rRNA gene of *H. panicea* (HP1) was amplified from the extracted DNA using the primer combination EUKF/EUKR (Table 5.2) (Medlin*, et al.*, 1988). PCR amplification was performed in a volume of 50 µl using 1× GoTaq buffer 0.2 mM dNTP mixture, 0.8 U GoTaq DNA polymerase (New England BioLabs, Frankfurt am Main, Germany), 0.5 µM of each primer and 1 µl template DNA. The PCR was done according to the following programme: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s and

elongation at 72°C for 2 min; and a final extension for 72°C for 10 min. The amplified fragment was purified using the Roche High Pure PCR Micro Cleanup Kit (Roche Diagnostics Nederland BV, Almere), ligated into the pGEM®-T Easy Vector and introduced in *E. coli* (Promega) according to the manufacturer's instructions. Cloned PCR products were Sanger sequenced using the T7 primer from the pGEM®-T Easy kit. Sequences were used as query against the NCBI Genbank non-redundant nucleotide database on January 17th, 2015, to identify closest relatives. The 18S rRNA gene sequences were deposited at the NCBI GenBank under accession numbers KC899017-KC899021.

Microbial diversity by tag pyrosequencing analysis

Tag-encoded amplicon pyrosequencing was conducted for *H. panicea* (HP1) DNA. The primers used were universal 16S rRNA gene primers PRK341F and PRK806R (Table 5.1) to amplify an approximately 466 bp fragment of the bacterial and archaeal 16S rRNA genes comprising the V3 and V4 regions (Yu*, et al.*, 2006). PCR amplification was performed in a volume of 40 µl using 1× Phusion HF buffer, 2.5 mM magnesium chloride, 0.2 mM dNTP mixture, 0.8 U Phusion Hot Start DNA polymerase (Finnzymes, Espoo, Finland), 0.5 µM of each primer and 1 µl template DNA (DNA extracted from cryopreserved HP1). PCR was performed using the following conditions: an initial denaturation at 98°C for 30s, followed by 30 cycles of denaturation at 98°C for 5s, annealing at 56°C for 20s, elongation at 72°C for 20s, and a final elongation at 72°C for 5 min. The sample was checked on a 1.25% (w/v) agarose gel and purified using the Millipore DNA Gel Extraction Kit (Millipore, Billerica, MA, USA). A second round of PCR was performed as described above, except that a pyrosequencing adapter (CCT AYG GRB GCA SCA G) and a barcode of 10 nucleotides length was used with the forward primer. Furthermore, the number of cycles of denaturation, annealing and elongation was reduced to 15. The PCR product was visualised on a 1% (w/v) agarose gel and excised from the gel and purified as described above. The amplified fragment with adapter and tag was quantified using a Qubit™ fluorometer (Invitrogen) and mixed in with other samples at approximately equal concentrations (4×10^5 copies μL^{-1}) to ensure equal representation of each sample in the pool. A 454-sequencing run was performed on a GS FLX Standard PicoTiterPlate (70 × 75) using a

GS FLX pyrosequencing system according to the manufacturer's instructions (Roche, Mannheim, Germany) at the Technical University of Copenhagen. Pyrosequencing data were deposited at the European Bioinformatics Institute in the sequence read archive under sample accession number ERS719000 (http://www.ebi.ac.uk/ena/data/view/ERP001839).

Pyrosequencing data was analysed using the QIIME 1.4.0 pipeline (Caporaso*, et al.*, 2010a). Low quality sequences were removed using default parameters (i. reads with fewer than 200 or more than 1000 nucleotides; ii. reads with more than 6 ambiguous nucleotides, iii. homopolymer runs exceeding 6 bases, iv. reads with missing quality scores and reads with a mean quality score lower than 25; v. reads with mismatches in the primer sequence), and operational taxonomic units (OTUs) were identified at the 97% identity level. Representative sequences from the OTUs were aligned using PyNAST (DeSantis*, et al.*, 2006b). The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% (Wang*, et al.*, 2007) against the 12_10 Greengenes core set. Possible chimeric OTUs were identified using QIIME's ChimeraSlayer and removed from the initially generated OTU list, producing a final set of non-chimeric OTUs.

Table 5.1: Primers used in this study

Primer	Sequence (5′-3′)	Target	Annealing temperature (°C)	Use	Reference
27F	aga gtt tga tcc tgg ctg ag	bacterial 16S rRNA gene	52	colony PCR, clone libraries	Weisburg *et al.*, 1991
1392R	acg ggc ggt gtg trc	bacterial 16S rRNA gene	52	colony PCR product sequencing	Lane *et al.*, 1991
1492R	ggt tac ctt gtt acg act t	bacterial 16S rRNA gene	52	colony PCR, clone libraries	Wilson *et al.*, 1990
PRK341F	cct ayg ggr bgc asc ag	Bacterial and archaeal 16S rRNA gene	56	pyrosequencing	Yu *et al.*, 2005
PRK806R	gga cta cnn ggg tat cta at	Bacterial and archaeal 16S rRNA gene	56	pyrosequencing	Yu *et al.*, 2005
EUKF	aac ctg gtt gat cct gcc agt	Eukaryotic 18S rRNA gene	55	clone libraries	Medlin *et al.*, 1988
EUKR	tga tcc ttc tgc agg ttc acc tac	Eukaryotic 18S rRNA gene	55	clone libraries	Medlin *et al.*, 1988

Results

Overview of the *H. panicea* microbiome by cultivation-independent means

The sponge specimen (HP1) used for both the cultivation-independent and cultivation-dependent analysis of associated bacteria and archaea was identified as *Halichondria panicea*. All five partial 18S rRNA gene clone sequences showed the highest similarity (99%) with other previously identified *H. panicea* specimen (data not shown).

The bacteria and archaea associated with *H. panicea* were determined by pyrosequencing of partial 16S rRNA genes obtained from the sponge. After quality filtering and chimera removal 11,199 bacterial and archaeal 16S rRNA gene reads were considered for analysis of microbial diversity. The reads were represented by 1039 operational taxonomic units (OTUs) at the approximate species level of 97% sequence similarity. These OTUs represented 1 archaeal phylum (*Thaumarchaeota*), albeit present at a very low level (0.03%), and 19 bacterial phyla and candidate phyla. *Actinobacteria* were represented by the highest number of reads (45.2%), followed by *Proteobacteria* (38%), *Cyanobacteria* (6.0%), *Verrucomicrobia* (4.7%) *and Bacteroidetes* (1.3%) (Figure 5.1). Other phyla included *Acidobacteria*, *Armatimonadetes*, candidate phylum MSBL-6, candidate phylum TM6, candidate phylum WS-2, candidate phylum *Saccharibacteria* (also known as candidate phylum TM7) *Chlamydiae*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes* and *Spirochaetes*, but individually these phyla each represented less than 0.5% of all reads.

Thirty six OTUs had a relative abundance that was larger than 0.25% in the sponge, and these were defined as predominant OTUs in the *H. panicea* microbiome (Table 5.3). A Blastn query using these 36 OTUs against the NCBI non-redundant nucleotide database revealed that 17 of the predominant OTUs were similar to sequences also found in marine bacterioplankton. This includes an actinobacterial phylotype (OTU1015) that represented 29% of the reads of HP1. Although sequences with 100% identity have also been found in the marine sponges *Hymeniacidon perleve* (GQ144907) and *Hymeniacidon flavia* (HM100929) and the coral *Montastraea annularis* (Klaus*, et al.*, 2011), most related sequence reports are from seawater (Blast search data not shown). The second most abundant OTU with a relative abundance of 14.6% corresponded to the *Rhodobacter*-like bacterium that was previously found to be the dominant bacterium in *H. panicea* (Althoff*, et al.*, 1998, Wichels*, et al.*, 2006, Naim*, et*

al., 2014). Also a number of other predominant proteobacterial and actinobacterial OTUs were previously reported from *H. panicea* and *H. perleve* (Table 5.3).

Table 5.3 Predominant OTUs (>0.25% of the reads) in *H. panicea* and their nearest neighbours as determined by a Blastn query against the NCBI database. The source and percentage of similarity to the first Blast hit is also indicated.

OTU ID	[a]Relative abundance (%)	Phylum	[b]Taxonomic identification	Nearest neighbour and NCBI accession number	Similarity	Source
1015	29.0	*Actinobacteria*	o__*Actinomycetales*	actinobacterium clone Shb-BH1 16S rRNA gene, KJ851374	100%	marine bacterioplankton
37	14.6	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone HP1-2.1 16S rRNA gene, KJ453525	100%	marine sponge (*H. panicea*)
1045	4.7	*Proteobacteria*	f__*Comamonadaceae*	*Variovorax* sp. FSGRA14 16S riRNA gene, KJ200411	100%	plant endophyte (sugarcane)
1159	3.7	*Actinobacteria*	o__*Actinomycetales*	actinobacterium clone Shb-BH1 16S rRNA gene, KJ851374	98%	marine bacterioplankton
688	2.3	*Proteobacteria*	o__*Rickettsiales*	bacterium clone BL_Jun2011_2,5m_H10 16S rRNA, KF817310	100%	marine bacterioplankton
961	2.2	*Actinobacteria*	o__*Actinomycetales*	marine bacterium clone J8-C3 16S rRNA gene, KF185707	98%	marine bacterioplankton
482	1.8	*Cyanobacteria*	f__*Synechococcaceae*	bacterium clone WATa27 16S rRNA gene, KJ007896	100%	marine bacterioplankton
749	1.6	*Cyanobacteria*	f__*Synechococcaceae*	bacterium clone 0818N7_3_93_D08821 16S rRNA gene, KJ811961	100%	marine bacterioplankton
94	1.4	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone BF5_1510 16S r RNA gene, KC307670	100%	jellyfish
17	1.3	*Actinobacteria*	o__*Actinomycetales*	actinobacterium clone b-25 16S rRNA gene, GQ144906	96%	marine sponge (*H. perleve*)
338	1.2	*Cyanobacteria*	f__*Synechococcaceae*	bacterium clone 269_365 16S ribosomal RNA gene, KF596607	99%	marine bacterioplankton
1129	1.0	*Verrucomicrobia*	f__*Verrucomicrobiaceae*	*Verrucomicrobia* clone SHAB753 16S rRNA gene, GQ348811	100%	marine bacterioplankton
1434	1.0	*Verrucomicrobia*	f__*Verrucomicrobiaceae*	marine bacterium clone J8-G4 16S rRNA gene, KF185753	100%	marine bacterioplankton
695	0.9	*Actinobacteria*	o *Actinomycetales*	bacterium clone SS_WC_10 16S ribosomal RNA gene, FJ973580	97%	marine bacterioplankton
429	0.6	*Proteobacteria*	f__*Comamonadaceae*	*V. paradoxus* isolate BD18-E04 16S rRNA gene, HF584859	96%	plant endophyte (grapevine)
452	0.6	*Actinobacteria*	o__*Actinomycetales*	actinobacterium clone B5 16S ribosomal RNA gene, FJ999592	99%	marine sponge (*H. simulans*)
1256	0.5	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone sponge_clone10 16S rRNA gene, AY948358	97%	marine sponge (*H. panicea*)
1212	0.5	*Proteobacteria*	f__*Moraxellaceae*	bacterium clone 4I6-C12 16S rRNA gene, EU149114	99%	beetle gut
326	0.5	*Actinobacteria*	c__*Actinobacteria*	bacterioplankton clone P1-1B_23 16S rRNA gene, KC000230	99%	marine bacterioplankton
676	0.5	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone sponge_clone1 16S rRNA gene, AY948354	99%	marine sponge (*H. panicea*)
1407	0.5	*Proteobacteria*	f__*Rhodobacteraceae*	Uncultured bacterium HNS27# HpaNS1-3 16S rRNA gene, Z88567	97"%	marine sponge (*H. panicea*)
189	0.4	*Verrucomicrobia*	f__*Verrucomicrobiaceae*	bacterium clone BF2010_Aug_21m_D4 16S rRNA gene, JX864470	100%	marine bacterioplankton
643	0.4	*Actinobacteria*	o__*Actinomycetales*	actinobacterium clone HP44 16S ribosomal RNA gene, EU140905	97%	marine sponge (*H. perleve*)
674	0.4	*Actinobacteria*	o__*Actinomycetales*	bacterium clone sponge_clone8 16S rRNA gene, AY948357	97%	marine sponge (*H. panicea*)
1062	0.4	*Proteobacteria*	f__*Rhodobacteraceae*	*Paracoccus* sp. isolate MN4 16S rRNA gene, AB845230	99%	worm gut
71	0.4	*Verrucomicrobia*	f__*Verrucomicrobiaceae*	bacterium clone Past_D19 16S rRNA gene, GU118986	99%	coral (*P. astreoides*)
353	0.4	*Proteobacteria*	o__*Oceanospirillales*	marine bacterium clone J2-H10 16S rRNA gene, KF186005	98%	marine bacterioplankton
408	0.3	*Verrucomicrobia*	f__*Verrucomicrobiaceae*	*Verrucomicrobia* clone PI_4z12f 16S rRNA gene, AY580844	96%	marine bacterioplankton
147	0.3	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone HP1-2.1 16S rRNA gene, KJ453525	97%	marine sponge (*H. perleve*)
1068	0.3	*Cyanobacteria*	f__*Synechococcaceae*	Cyanobacterium clone WS08_021 16S rRNA gene, KJ566267	98%	marine bacterioplankton
154	0.3	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone HP1-2.1 16S rRNA gene, KJ453525	96%	marine sponge (*H. panicea*)
1364	0.3	*Actinobacteria*	o__*Actinomycetales*	bacterium clone G7DUZBG01BAZ4W 16S rRNA gene, JX922747	98%	shrimp gut
307	0.3	*Proteobacteria*	f__*Rhodobacteraceae*	bacterium clone HP1-2.1 16S rRNA gene, KJ453525	98%	marine sponge (*H. panicea*)
1097	0.3	*Proteobacteria*	f__*Comamonadaceae*	*Comamonas* sp. isolate A4-H2 16S rRNA gene, KJ534600	99%	ant gut
797	0.3	*Cyanobacteria*	f__*Synechococcaceae*	bacterium clone OTU356 16S rRNA gene, HM921226	98%	marine bacterioplankton
918	0.3	*Actinobacteria*	o__*Acidimicrobiales*	bacterium clone EE8-001_B04 16S rRNA gene, HE979671	99%	marine bacterioplankton

a - relative abundance of the OTU

b - most detailed taxonomic assignment as determined from the RDP II classifier incorporated in QIIME. Abbreviations: c_, o_ and f_ indicate class, order and family respectively

Cultivable bacteria from *H. panicea*

Using a broad range of media that differed in nutrient composition and concentration and cultivation conditions, a total of 1359 colonies and liquid cultures were obtained. After PCR amplification 1283 isolates and liquid culture-derived clones could be identified (Figure 5.2.A). They all belonged to the phyla *Proteobacteria* (742 isolates), *Firmicutes* (361), *Actinobacteria* (128) and *Bacteroidetes* (50). One isolate belonged to *Chlorobi* and one isolate could not be classified (Figure 5.2). Most *Proteobacteria* isolated were *Alphaproteobacteria* (509) and *Gammaproteobacteria* (182), but also *Deltaproteobacteria* (45), *Betaproteobacteria* (5) and one epsilonproteobacterium were isolated.

The isolates represented 75 genera, and the richness of genera was positively correlated to the number of isolates that was obtained for each phylum (or class for the *Proteobacteria*). Most genera belonged to *Alphaproteobacteria* (21 genera), *Actinobacteria* (17), *Gammaproteobacteria* (13), *Bacteroidetes* (10), *Firmicutes* (8), *Delta-* and *Betaprotebacteria* (2 each) and *Epsilonproteobacteria* and *Chlorobi* (1 each). The most commonly isolated genera were *Bacillus* (319 isolates), *Paracoccus* (305) and *Shewanella* (83) (Figure 5.2.B). One *Rhodobacter* sp. colony was obtained from glycerol-arginine agar (4.GAA).

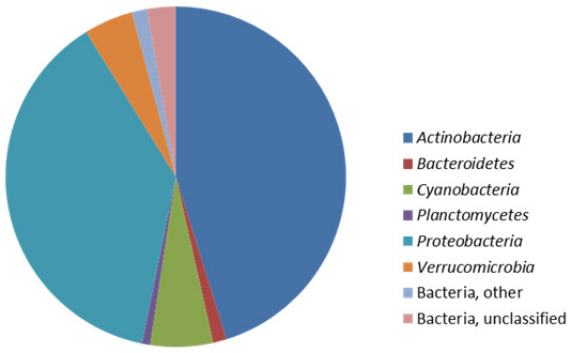


Figure 51: Relative abundance of the predominant microbial phyla that represent more than 0.5% of the microbial community in the marine sponge *Halichondria panicea* (HP1).

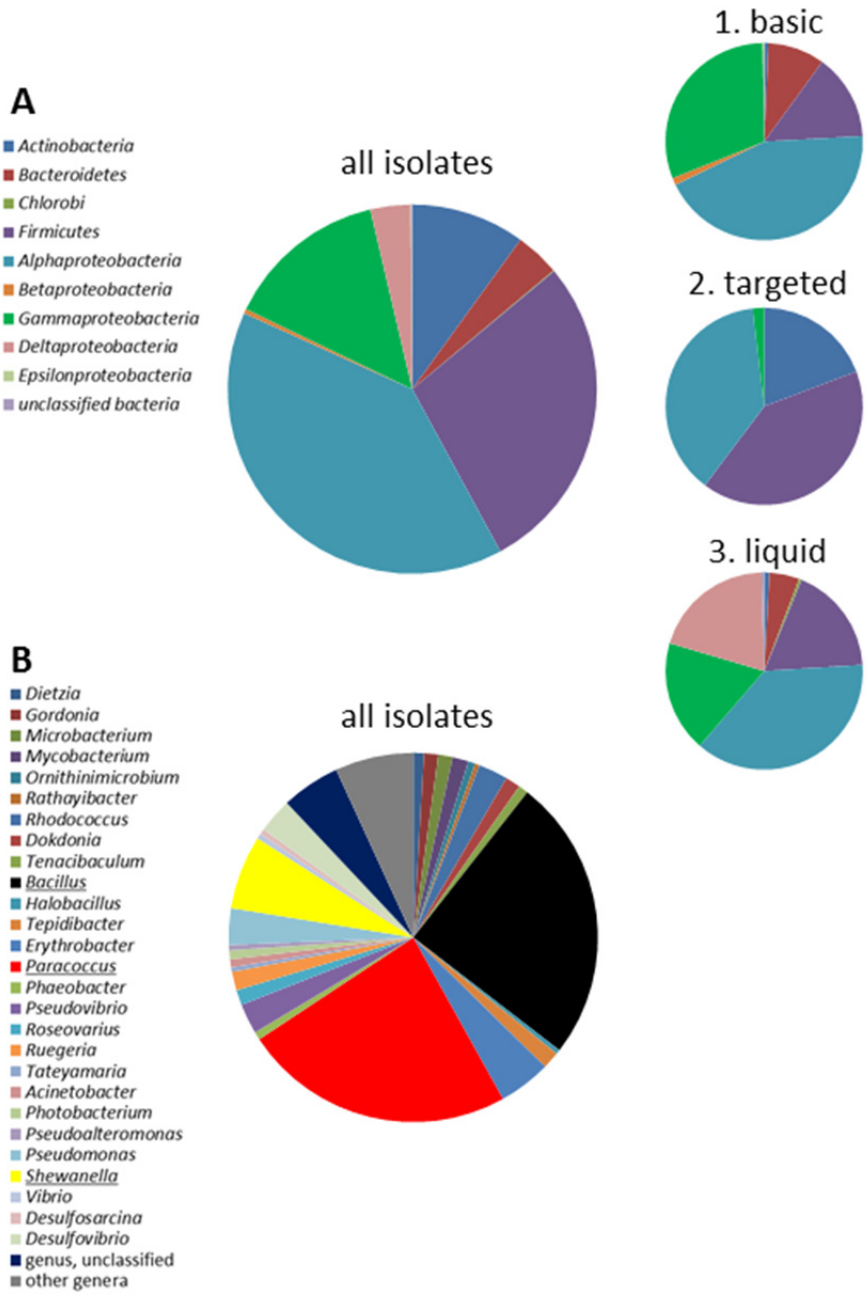
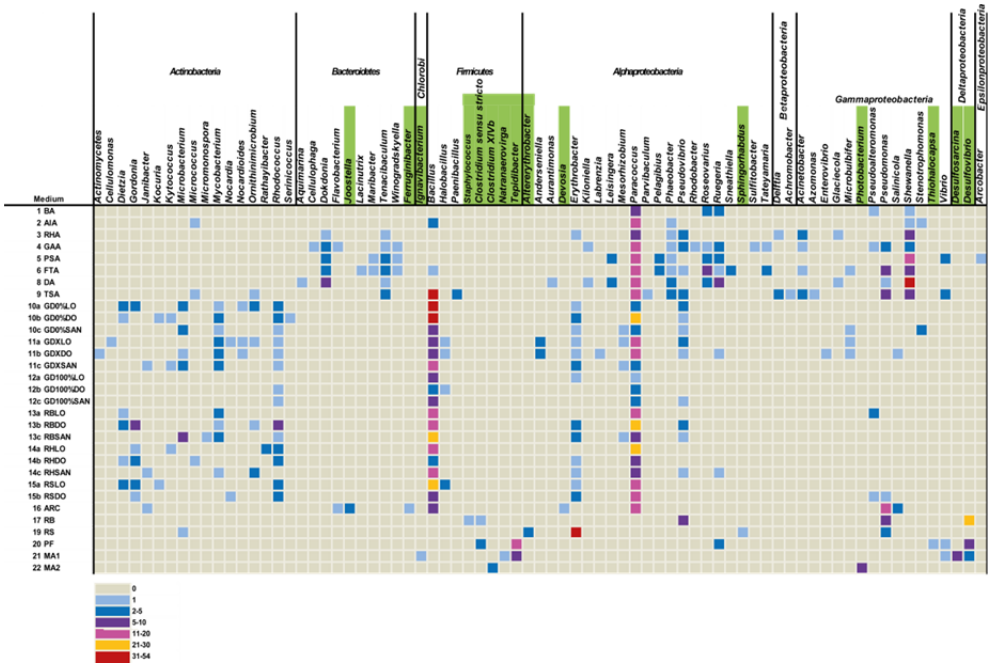


Figure 5.2 A..Left: Phyla (or classes in case of *Proteobacteria*) represented by the isolates obtained from *H. panicea*. Right: Phyla obtained from the three cultivation experiments (1) basic, (2) targeted and (3) liquid. **B.** Genera isolated from *Halichondria panicea*. *Bacillus*, *Paracoccus* and *Shewanella* were the most common isolates and are underlined in the legend. Genera that were represented by less than 5 isolates are grouped under the name “other genera”.

Figure 5.3 Heatmap based on number of isolates belonging to one genus for each medium used. Isolates that could not be classified at the genus-level were omitted. For a detailed overview of the medium abbreviations see Table 5.1. Genera highlighted in green were exclusively identified from liquid culture.



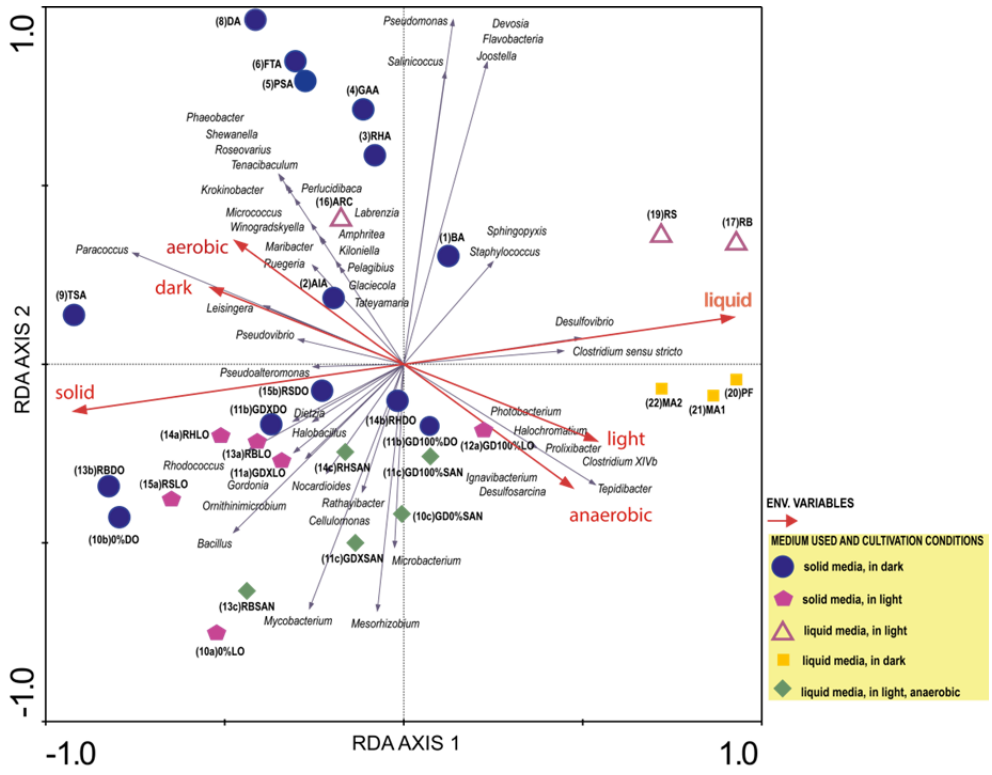


Figure 5.4 Triplot of RDA axes 1 and 2 accounting for 69.5% of the variance of cultivation media used with respect to environmental variables. The eigenvalue for axis 1 (horizontal) is 0.16 and for axis 2 (vertical) is 0.06. Genus abundance data were square root transformed. Environmental variables used in the analysis are shown in red arrows.

Effect of cultivation media and conditions

In comparison to the basic cultivation experiment (1), the targeted cultivation experiment led to isolation of substantially more *Actinobacteria* and *Firmicutes*, while *Gammaproteobacteria* were much less abundant and *Bacteroidetes* were no longer found (Figure 5.2.A). With the liquid growth experiment (3) the most prominent deviation from the basic isolation experiment was the growth of a substantial number of *Deltaproteobacteria* (45 sequences) while none were obtained from agar plates. Sixty genera were obtained from agar plates (basic + targeted cultivation experiment), while 26 genera were obtained with liquid enrichment cultures. Fifteen of the 26 genera obtained with liquid enrichment cultures were not obtained from agar plates.

Glycerol-arginine agar (4.GAA) and 6.FTA were the most prolific media used with respect to genus richness obtained. Both media yielded 18 different genera, which is particularly high when it is taken into account that for 4.GAA only 41 colonies were obtained. The richest medium (9.TSA) yielded the highest number of colonies (107), which represented 16 genera. The lowest diversity (2 genera) was obtained with 22.MA2 (Figure 5.3). Five media, of which three were liquid media did not yield any growth (Table 5.1). *Paracoccus* was obtained from every agar plate growth experiment for which growth was obtained (Figure 5.3).

We subsequently explored the relationship between cultivation media used, environmental variables used (*i.e.* solid medium, liquid medium, light, dark, aerobic, anaerobic) in relation to the genera isolated using redundancy analysis (RDA). The cultivation method (solid/liquid) was the most prominent environmental factor that determined the profile of the bacteria isolated (Figure 5.4). Especially the isolation of *Desulfovibrio* correlated with the use of liquid media. The factors “light” and “anaerobic” seemed correlated, which may be for the simple reason that all cultures that were grown under light were also aerobic. Genera that were solely enriched in light (and anaerobic) conditions were *Photobacterium*, *Clostridium* and *Tepidibacter*.

Comparison of cultivation-based diversity with molecular diversity

Since it was our aim to isolate bacteria that are present in the sponge in the natural environment, the microbial diversity in *Halichondria panicea* based on molecular methods was compared to the microbial diversity obtained through isolation. Since both the data obtained from the molecular diversity study and the cultivation experiment were done with the same specimen a direct comparison between the two methods is possible. All 1039 OTUs obtained from the pyrosequencing analysis were used as a database and all 1283 isolates as query to assess which OTUs found in the sponge tissue could be recovered through isolation. Only two OTUs that were found to represent more than 0.25% of the *H. panicea* microbiota were recovered in the cultivation experiment (OTU94 and OTU1062). In addition to these two predominant OTUs, 49 other OTUs that were found in the sponge were also recovered by cultivation (Table 5.4). These OTUs amounted to a total of 3.7% of the bacteria of *H. panicea* (as defined by the cumulative relative abundance of these OTUs). The dominant actinobacterium (OTU1015) and the *Rhodobacter*-like bacterium (OTU37) that represented the dominant bacteria found in tissue of *H. panicea* were not isolated. The closest relative of OTU1015 that was isolated was GD27DO50%, but it had only 92.9% similarity based on the 16S rRNA gene sequences. The only *Rhodobacter* sp. that was isolated (AIA 5976730) had only 93.5% similarity with OTU37.

Table 5.4 Comparison of 16S rRNA sequences of isolates obtained with OTUs as established for the cultivation-independent analysis. The data are sorted by a descending relative abundance of the OTUs. OTUs that represented more than 0.25 % of the *H. panicea* microbiota are in bold.

pyrosequencing OTU[a]	Rel. abundance[b]	isolate[c]	genus[d]	Phylum/Class	Similarity[e]
94	**1.4376**	**RS70DO**	***Paracoccus***	***Alphaproteobacteria***	**100**
1062	**0.4197**	**TSA 5896781**	***Paracoccus***	***Alphaproteobacteria***	**99.72**
442	0.2232	RS70DO	*Paracoccus*	*Alphaproteobacteria*	97.7
1340	0.2232	RH19SAN	*Ornithinimicrobium*	*Actinobacteria*	98.65
987	0.1429	RS84DO	*Rhodococcus*	*Actinobacteria*	99.17
538	0.1072	RB76DO	*Pseudovibrio*	*Alphaproteobacteria*	98.12
371	0.0893	RS32DO	*Gordonia*	*Actinobacteria*	98.84
413	0.0893	RS79DO	*Pseudomonas*	*Gammaproteobacteria*	97.29
498	0.0804	RS34LO	*Dietzia*	*Actinobacteria*	99.31
666	0.0714	FTA 6169149	*Tenacibaculum*	*Bacteroidetes*	98.36
518	0.0536	RS79DO	*Pseudomonas*	*Gammaproteobacteria*	99.56
1004	0.0536	RS50LO	*Kocuria*	*Actinobacteria*	98.3
905	0.0446	RS83DO	*Erythrobacter*	*Alphaproteobacteria*	97.99
341	0.0357	RH70DO	*Micrococcus*	*Actinobacteria*	99.43
471	0.0357	GA0%49DO	*Kocuria*	*Actinobacteria*	97.31
612	0.0357	TSA 5896794	*Parvibaculum*	*Alphaproteobacteria*	99.65
762	0.0357	RS83DO	*Erythrobacter*	*Alphaproteobacteria*	99.26
1087	0.0357	RS70DO	*Paracoccus*	*Alphaproteobacteria*	99.56
1197	0.0357	GD43DO50%	*Bacillus*	*Firmicutes*	97.93
1416	0.0357	RS32DO	*Gordonia*	*Actinobacteria*	97.69
64	0.0268	RB10SAN	*Phyllobacteriaceae*	*Alphaproteobacteria*	97.18
389	0.0268	RB68SAN	*Mesorhizobium*	*Alphaproteobacteria*	98.02
47	0.0179	FTA 5818270	*Paracoccus*	*Alphaproteobacteria*	97.2
69	0.0179	RHA 6168981	*Acinetobacter*	*Gammaproteobacteria*	98.08
197	0.0179	GA0%11LO	*Bacillus*	*Firmicutes*	98.61
244	0.0179	TSA 5896803	*Tenacibaculum*	*Bacteroidetes*	97.12
305	0.0179	RS30LO	*Bacillus*	*Firmicutes*	99.07
830	0.0179	RH20SAN	*Janibacter*	*Actinobacteria*	99.75
1253	0.0179	GA0%36LO	*Nocardioides*	*Actinobacteria*	98.25
1270	0.0179	FTA 5818270	*Paracoccus*	*Alphaproteobacteria*	98.55
1457	0.0179	RH84LO	*Bacillus*	*Firmicutes*	99.52
31	0.0089	RHA 6168993	*Flavobacteriaceae*	*Bacteroidetes*	97.1
120	0.0089	RB68SAN	*Mesorhizobium*	*Alphaproteobacteria*	97.53
235	0.0089	RS70DO	*Paracoccus*	*Alphaproteobacteria*	98.19
294	0.0089	GD30SAN0%	*Stenotrophomonas*	*Gammaproteobacteria*	99.11
489	0.0089	AIA 5976944	*Paracoccus*	*Alphaproteobacteria*	99.62
582	0.0089	TSA 5896794	*Parvibaculum*	*Alphaproteobacteria*	98.85
611	0.0089	GD30SAN0%	*Stenotrophomonas*	*Gammaproteobacteria*	97.67
633	0.0089	PSA 6169080	*Winogradskyella*	*Bacteroidetes*	97.91
656	0.0089	RHA 6168991	*Rhodobacteraceae*	*Alphaproteobacteria*	97.5
728	0.0089	PSA 6169073	*Ruegeria*	*Alphaproteobacteria*	98.73
742	0.0089	RHA 6168987	*Flavobacteriaceae*	*Bacteroidetes*	99.04
755	0.0089	RB41SAN	*Paracoccus*	*Alphaproteobacteria*	98.56
862	0.0089	GD39LO50%	*Pseudovibrio*	*Alphaproteobacteria*	98.31
1060	0.0089	PSA 6169077	*Roseovarius*	*Alphaproteobacteria*	99.72
1083	0.0089	RB34LO	*Paracoccus*	*Alphaproteobacteria*	97.14
1127	0.0089	PSA 6169081	*Ruegeria*	*Alphaproteobacteria*	99.03
1263	0.0089	DA 5818232	*Ruegeria*	*Alphaproteobacteria*	97.13
1312	0.0089	GD77LO100%	*Bacillus*	*Firmicutes*	97.22
1456	0.0089	RS91DO	*Erythrobacter*	*Alphaproteobacteria*	97.83
1463	0.0089	DA 6169036	*Roseovarius*	*Alphaproteobacteria*	99.46

a-representative 16S rRNA sequence of pyrosequencing analysis clustering at 97% similarity
b-relative abundance in the sponge microbiome (%)
c-16S rRNA sequence of isolate with best hit based on similarity with a minimum alignment length of 200 bp
d- genus of isolate (family if genus-level classification was not achieved with 80% confidence)
e-percentage of similary determined by Blast2 comparison (Zhang *et al* ., 2000)

Discussion

H. panicea is a shallow water sponge with a wide geographical distribution (Vethaak*, et al.*, 1982). A large number of bioactive compounds has been detected in *H. panicea*, such as brominated compounds (Kotterman*, et al.*, 2003), a water-soluble compound to deter barnacle settling (Toth & Lindeborg, 2008), neuroactive compounds (Perovic*, et al.*, 1998), demospongic acids (Ando*, et al.*, 1998) and the sesquiterpene isothiocyanate (Nakamura*, et al.*, 1992). This makes the sponge host and its associated microorganisms an interesting model for biotechnological exploitation.

Dominant bacteria of *H. panicea*

Evidence from pyrosequencing analysis in this study showed that *Actinobacteria* is the most abundant phylum (45.2 % of the reads) followed by *Alphaproteobacteria* (39 %) *Cyanobacteria* (6.0 %), *Verrucomicrobia* (4.7 %) and *Bacteroidetes* (1.3 %). This is in stark contrast to the findings from previous investigations (Nakamura*, et al.*, 1992, Althoff*, et al.*, 1998, Naim*, et al.*, 2014) where *Rhodobacter*-like phylotypes were found to represent the large majority of the bacteria in *H. panicea*. This *Rhodobacter*-like bacterium was also found in our study (OTU37) but represented 'only' 14.6 % of the microbiota of specimen HP1. Other studies of *H. panicea*-associated microbiota have also found large fractions of *Actinobacteria* comprising 3 to 20 % of the bacterial community in the sponge (Imhoff & Stöhr, 2003, Hentschel*, et al.*, 2006). This could be explained either by intraspecific variation which may be more apparent in LMA sponges as the relative abundance of their microbiota is more easily affected because of the lower absolute numbers of microorganisms present in their tissues. In addition, seasonal changes of associated bacteria have been observed for *H. panicea* (Wichels*, et al.*, 2006). We sampled HP1 in September 2009 while for a previous study other *H. panicea* individuals were collected in December 2008 (Naim*, et al.*, 2014). In the latter study nearly 2 % of the *H. panicea* community was found to be represented by novel lineages if *Chlamydiae*. Although we did detect *Chlamydiae* in HP1, they were present at a much lower relative abundance (0.24%). The *Actinobacteria*-affiliated most abundant OTU in *H. panicea* has been found in other sponges (*Hymeniacidon perleve* (GQ144907), *Hymeniacidon flavia* (HM100929) and the coral *Montastraea annularis* (Klaus*, et al.*, 2011)), but

is mainly found in seawater. A bacterium that is 99% identical to this OTU (OTU1015) has been isolated from seawater using high-throughput dilution-to-extinction culturing (Stingl*, et al.*, 2007).

Diversity of cultivable bacteria from *H. panicea*

Preceding efforts to cultivate sponge-associated bacteria have shown that it is notoriously challenging to isolate these bacteria. Cultivation success ranged between 0.1% to 11% of the sponge-associated bacteria (Santavy*, et al.*, 1990, Friedrich*, et al.*, 1999, Webster & Hill, 2001, Olson & McCarthy, 2005). Diversification of cultivation media with different compositions and nutrient concentrations and cultivation conditions has shown to lead to an improved cultivation recovery rate (Sipkema*, et al.*, 2011). Therefore, we attempted to maximize the use of different isolation media varying in nutrient compositions and nutrient concentrations, and with environmental variables. In addition, in a targeted approach we used growth media and conditions aimed to isolate the *Rhodobacter*-like species from *H. panicea*. Since the *Rhodobacter*-like bacterium and also the other dominant OTU were not isolated, the coverage of *H. panicea* bacteria recovered by cultivation was limited to 3.7 %. This 3.7 % was the cumulative relative abundance of 51 OTUs that were recovered by cultivation. It is noteworthy that 20 additional OTUs that were labelled as chimera were also recovered as isolates (data not shown). As the relative abundance of chimera-labelled reads of HP1 was low, this will not have a major impact on the cultivation recovery rate. However, it does indicate that identification of chimera may be too progressive for samples, such as sponges in which still many novel bacteria are being discovered.

Outlook

A large and diverse cultivation effort that was setup for this study did not lead to the isolation of the most dominant *H. panicea*-associated bacteria (OTU37 and OTU1015). Instead, bacteria that were present at a much lower relative abundance were enriched. A similar result has been described for bacterial cultivation exercises with other sponges (Sipkema*, et al.*, 2011, Ozturk*, et al.*, 2013, Montalvo*, et al.*, 2014, Hardoim*, et al.*, 2015). Furthermore, similar outcomes are not restricted to marine sponges, but are also obtained with other environmental samples, such as soil where factors as cell viability, long incubation time and unknown substrate often are cited as the reasons for the uncultivability (Felske*, et al.*, 1999). Current

developments in single cell genomics and the reconstruction of genomes from metagenomes for uncultivated bacteria may provide novel insights in the near future that will give clues on how to isolate these bacteria. Single cell genomics has yielded insight in the metabolism of *Poribacteria* and *Tectomicrobia* for which no cultured species exist (Siegl*, et al.*, 2011, Wilson*, et al.*, 2014). In addition, a metatranscriptomics approach has been use to design a mucin-based medium to isolate a *Rikinella*-like bacterium, which is the dominant symbiont of the medicinal leech *Hirudo verbana* (Bomar*, et al.*, 2011).

Conclusion

A comprehensive cultivation efforts to isolate bacteria from the marine sponge *Halichondria panicea* resulted in the isolation of 75 genera. Variation of cultivation media and conditions had a large impact on the bacterial diversity obtained. Although the majority of the isolates were found to be *Proteobacteria* and *Actinobacteria* they were different than the *Proteobacteria* and *Actinobacteria* detected in the sponge by molecular means. Although the dominant sponge-associated bacteria were not recovered, 3.7 % of the bacteria present in the sponge were obtained in culture.

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Chapter 6:

Genotypic and phenotypic diversity of *Pseudovibrio* spp. isolated from marine sponges

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Abstract

Marine sponges have been around for at least 650 million years. They harbour a large number of microorganisms, including archaea, bacteria and fungi. Comparing the outcome of culture-dependent and molecular surveys of bacterial diversity in sponges, a number of species are regularly isolated, but do not represent the majority of bacteria in the sponge microbiome. Among these, members of the alphaproteobacterial genus *Pseudovibrio* have been frequently isolated and have been shown to produce bioactive compounds with diverse properties. In this study, a total of 76 *Pseudovibrio* spp. isolates from six Mediterranean sponges (*Aplysina aerophoba, Petrosia ficiformis, Corticium candelabrum, Ircina* sp., *A. acuta* and) were characterized based on their 16S ribosomal RNA gene sequence, as well as phenotypic attribute based on antibiotic resistance) and additional genotypic (BOX PCR, halogenase-encoding gene-targeted PCR) features. 75 isolates were most closely related to *P. ascidiaceicola* and one isolate was found to be closely related to *P. axinellae*. Isolates from *P. ficiformis* showed different phenotypic profiles in that they were sensitive to ampicillin and penicillin in comparison to other isolates that were resistant to these antibiotics. Results from the phenotypic and genotypic characteristics could be used for selecting *Pseudovibrio* spp. with different genetic potential for further characterization.

Keywords: genotypic, phenotypic, *Pseudovibrio* spp., comparative genomics, sponge

Introduction

Sponges host different microorganisms such as *Archaea*, *Bacteria,* eukaryotes and viruses (Webster & Taylor, 2012). Investigations from sponges employing culture-dependent and culture independent approaches have revealed the presence of at least 32 bacterial and archaeal phyla in sponges (Hentschel*, et al.*, 2012, Schmitt*, et al.*, 2012). Comparing the outcome of culture-dependent and molecular surveys revealed that the majority of bacteria isolated from sponges are in general not present at high relative abundance in the sponge microbiome (Schippers*, et al.*, 2012). One prime example is the alphaproteobacterial genus *Pseudovibrio* , members of which have been consistently isolated from many sponge species regardless of the biogeography (Thiel & Imhoff, 2003, Enticknap*, et al.*, 2006, Muscholl-Silberhorn*, et al.*, 2008, Sipkema*, et al.*, 2009, Abdelmohsen*, et al.*, 2010, Sipkema*, et al.*, 2011) but not detected in surrounding seawater (Enticknap*, et al.*, 2006). In addition to sponges, *Pseudovibrio* spp. have also been isolated from coastal seawater (Hosoya & Yokota, 2007), tunicates (Fukunaga*, et al.*, 2006, Sertan-de Guzman*, et al.*, 2007) and corals (Chen*, et al.*, 2012). Members of the *Pseudovibrio* genus are prolific producers of secondary metabolites, and the analysis of the large genomes of several representatives indicated that they are metabolically versatile and well-equipped for symbiosis (Sertan-de Guzman*, et al.*, 2007, O' Halloran*, et al.*, 2011, Penesyan*, et al.*, 2011, Bondarev*, et al.*, 2013). Secondary metabolites produced by *Pseudovibrio* spp. include compounds such as phenazine (Schneemann*, et al.*, 2011) and tropodithietic acid (TDA) (Penesyan*, et al.*, 2011), which are potentially attractive for applications in biotechnology and pharmaceutical industry. *Pseudovibrio* spp. have particularly been described as producers of substances with antimicrobial properties (Santos*, et al.*, 2010, O' Halloran*, et al.*, 2011).

The aim of the current study was to identify genotypic and phenotypic diversity of *Pseudovibrio* spp. isolated from marine sponges as determined by genomic DNA fingerprints and their phenotypic properties. Their widespread occurrence and high abundance as culturable symbionts with interesting secondary metabolites makes the *Pseudovibrio* clade an attractive target for comparative genomics at a more detailed level. To this end, the results of this study provide the necessary basis for the selection of *Pseudovibrio* spp. isolates to be sent for genome sequencing.

Materials and Methods

Culture condition

A total of 76 strains comprised of; 72 strains of different *Pseudovibrio* spp. from different sponges from Mediterranean sea (*Aplysina aerophoba, Petrosia ficiformis, Corticium candelabrum*) isolated previously from different cultivation media (Versluis*, et al.*, 2015), and one strain each from marine sponges (*Acanthella acuta*, *Ircinia* sp. and *Chondrilla nucula*) and the bryozoan (*Cellephora. pumicosa*) were regrown from -80°C glycerol stocks on either Difco Marine Agar 2216 or in Difco Marine Broth 2216 (BD Biosciences, Franklin Lakes, USA). Modifications to the protocol were made, briefly: i) the media were diluted three times (9.98 g medium, 0.8 L distilled water), ii) per 0.8 L medium, 0.8 ml trace metal elements (0.3 g H_3BO_3, 0.2 g $CoCl_2$, 0.1 g $ZnSO_4$, 0.03 g Na_2MoO_4, 0.02 g $NiCl_2$, 0.01 g $CuCl_2$) was added and iii) per 0.8 L medium, 0.8 ml of BME vitamins was added (Sigma, Zwijndrecht, The Netherlands). The isolates were grown at 20 °C in the dark for 3 to 5 days. For some isolates, longer incubation times up to 10 days were needed to increase the biomass. Colony morphology of isolates and their appearance on Difco Marine Agar 2216 were observed and noted.

Phenotypic screening of *Pseudovibrio* spp.

For discrimination of *Pseudovibrio* spp. isolated from marine sponge *A. aerophoba*, *C. candelabrum* and *P. ficiformis*, all 72 *Pseudovibrio* spp. isolated from those sponges are tested for antibiotic resistance. Selected isolates were regrown on Mueller-Hinton agar (BD Biosciences, Franklin Lakes, USA) prepared using artificial seawater (33 g Reef Crystal dissolved in 1L of distilled water). Mueller-Hinton agar with artificial seawater was first autoclaved and then poured onto plastic petri dishes. Subsequently, different antibiotics were added namely polymyxin (specific for Gram-negative bacteria), daptomycin, lincomycin, linezolid, penicillin vancomycin (specific for gram-positive bacteria) and antibiotics that target both Gram-positive and Gram-negative bacteria (see Table 6.2). Antibiotics were used at the following concentrations: ampicillin, 50 mg/L; cefotaxime, 100 mg/L; chloramphenicol, 50 mg/L; ciprofloxacin, 15 mg/L; daptomcyin, 5 mg/L; erythromycin, 15 mg/L; kanamycin, 100 mg/L; lincomycin, 15 mg/L ; linezolid, 15 mg/L; penicillin, 50 mg/L; polymyxin B, 15 mg/L; rifampicin, 7.5 mg/L; sulfamethoxazole, 95 mg/L; tetracyline, 50 mg/L; trimethoprim, 5 mg/L; and vancomycin, 50 mg/L. The plates were incubated at room

temperature for 3 to 5 days. Plates with no growth indicated that the isolates are sensitive to the antibiotics while plates with growth indicate antibiotic resistance strains.

Genomic fingerprinting by BOXA1R colony PCR

Genomic fingerprints of *Pseudovibrio* spp. isolates were generated using a BOXA1R single primer (Table 6.1). Firstly, bacterial colonies growing on the agar plate were picked using micropipette tips and then transferred to microtiter plate wells containing 20 µl of nuclease free water (Promega, Madison, USA). Bacterial cell lysis was induced by heating the cells for 15 minutes at 95 °C prior to the PCR reactions. Genomic fingerprints were generated by repetitive PCR (rep-PCR) targeting repetitive prokaryotic BOX elements (Versalovic, 1994). Each PCR reaction was carried out in a total volume of 25 µl. Each reaction consisted of 1 µl of lysed bacterial cells, 0.625 U of GoTaq® HotStart DNA Polymerase (Promega, Madison, USA), 0.5 µL of 10 µM BOXA1R single primer (Biolegio BV, Nijmegen, The Netherlands), 0.5 µL of 10 mM of an equimolar mixture of the four deoxyribonucleotide triphosphates (Roche Diagnostics GmbH, Mannheim, Germany), 5 µl of 5x PCR buffer and 17.875 µl of Milli Q (DNA free water). Genomic DNA of isolate 1D03 from *Aplysina aerophoba* was used as a positive control and 1 µl water instead of template as negative control. PCR amplification was performed in a Bioké thermal cycler (ThermoScientific, Waltham, USA) using the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 1 min, extension at 68°C for 1 min, and a final extension step at 68°C for 10 min.

Subsequently, 15 µl of each of the PCR mixtures was loaded into a 1.5% (w/v) agarose gel and electrophoresed and stained with SYBR® Safe DNA Gel Stain (Life Technologies GmbH, Darmsdtadt, Germany) for 45 minutes at 100V. The banding patterns were analysed using Bionumerics 3.5 (Applied Maths, Sint-Martens-Latum, Belgium) to obtain densitomeric curves and subsequently similarity indices (SI's). Images were normalized using the markers prior to analysis. Markers used were GeneRuler™ 1 kb DNA ladder and GeneRuler™ 1kb plus DNA ladder (ThermoScientific, Waltham, USA). Levels of similarity between fingerprints were calculated according to the Pearson's correlation (Fromin*, et al.*, 2002) by applying the unweighted pair group method with mathematical averages (UPGMA) using the similarity matrix generated.

PCR-based screening of flavin-dependent halogenase gene

Detection of flavin-dependent halogenase-encoding genes was carried out using the primer combination Ha002/Ha005 (Table 6.1). These degenerate primers were originally designed to detect conserved regions of tryptophan 5-halogenase (Zehner*, et al.*, 2005). Firstly, bacterial colonies growing on the agar plate were picked using micropipette tips and then transferred to a microtiter plate of which each well contained 20 µl of nuclease free water (Promega, Madison, USA). Bacterial cell lysis was induced by heating the cells for 15 minutes at 95 °C prior to the PCR reactions. One µl of cell-lysis-water was transferred into the 49-µl PCR mixture which contained 27.5 µl of nuclease-free water,3 µl of each primer (10 µm), 5 µl of 10X PCR buffer II (Applied Biosciences, Foster City, CA), 3 µl of 2.5 mM $MgCl_2$ (Applied Biosciences, Foster City, CA), 0.5 µl of AmpliTaq Gold DNA polymerase (5U/ µl) (Applied Biosciences, Foster City, CA), 2 µl of deoxynucleoside triphosphate (dNTP) mixture (10mM), and 5 µl of 7% dimethyl sulfoxide (DMSO). 1 µl of plasmid DNA (10 ng/µl) of *E. coli* clones from previous study of flavin-dependent halogenase compound, pyrrolnitrin (Hammer*, et al.*, 1997) was used as a positive control. The PCR protocol consisted of 15 min denaturation at 95°C; 1 cycle of 1 min at 95°C, annealing of 1 min at 65°C, and a 1 min extension at 72°C followed by 34 cycles of 1 min at 95°C, 1 min at 60°C, and a 1-min extension at 72°C. A final extension step was done for 10 min at 72°C. The PCR products were checked for the expected amplicon size (~700 bp) by loading 25 µl PCR product onto 1.5% (w/v) agarose gel supplemented with SyBr Safe DNA Stain (Life Technologies) and left running for 45 min at 100V.

Table 6.1. Primers used in this study

Primer	Sequence (5′-3′)[a]	Target	Annealing temperature (°C)	Use	References
BOXA1R	tac ggc aag gcg acg ctg acg	Bacteria BOXA1R gene	52	Colony PCR	(Versalovic, 1994)
27F	aga gtt tga tcm tgg ctc ag	Bacteria 16S rRNA gene	52	Colony PCR	(Lane, 1992)
1492R	acc ttg tta cga ctt	Bacteria 16S rRNA gene	52	Colony PCR	(Lane, 1992)
907R	ccg tca att cmt ttr agt tt	Bacteria 16S rRNA gene	52	Sanger sequencing	(Lane, 1992)
Ha002	tcg gyg tsg gcg arg cga ccr tcc c	Halogenase gene	60	Colony PCR	Zehner *et al.*, 2005
Ha005	ccg gag cag tcg ayg aas agg tc	Halogenase gene	60	Colony PCR	Zehner *et al.*, 2005

[a]: M = A or C; R = A or G.

16S rRNA gene amplification

Amplification of the 16S rRNA gene was done by colony PCR using the same bacterial cells that were lysed and used for the BOXA1R PCR to confirm the identity of *Pseudovibrio* spp. The primers used for amplification of the 16S rRNA gene were universal bacterial primers 27F and 1492R ((Lane, 1992); Table 6.1). Each PCR reaction was carried out in a total volume of 50 µl and consisted of 1 µl of lysed bacterial cells of each isolate, 0.25 µl of 5U GoTaq® HotStart DNA Polymerase (Promega, Madison, USA), 1 µl of 10 µM of both primers (Biolegio BV, Nijmegen, The Netherlands), 1 µl of 10 mM deoxyribonucleotide triphosphate (Roche

Diagnostics GmbH, Mannheim, Germany), 10 µl 5x PCR buffer and 35.75 µl Milli Q (DNA-free water). PCR amplification was performed using a Bioké thermal cycler (ThermoScientific, Waltham, USA) based on the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. Amplicon size (~1465 bp) was checked by loading 5 µl of each PCR reaction mixture into a 1% (w/v) agarose gel supplemented with SybrSafe DNA Stain (Life Technologies) and run for 45 minutes at 100V. Amplicons with the expected size were sequenced using primer 907R ((Lane, 1992); Table 6.1) at GATC-Biotech (Konstanz, Germany).

Sequence data

The sequence data was edited with Vector NTI Advance® 10 (Invitrogen, Carlsbad, USA), briefly; i) called secondary peaks from the chromatogram, ii) unambigous regions at the start of 5'-end and 3'-end were trimmed and iii) the chromatogram was manually inspected for any incosistencies. 16S rRNA genes were checked for possible chimeric origins using DECIPHER online web tools (Wright*, et al.*, 2012). Nearest neighbours were searched from the non-redundant nucleotide database of the National Centre for Biotechnology Information (NCBI) using the query function of Basic Local Alignment Search Tool (BLAST) (January 20, 2014). *Pseudovibrio* spp. 16S rRNA gene sequences from previously published genomes were also included (Bondarev*, et al.*, 2013). The 16S rRNA gene sequences were deposited at NCBI GenBank with accession numbers KP319273-KP319347 except for *Pseudovibrio* sp. isolated from marine sponge *Ircinia* sp of which the 16S rRNA sequence had already been deposited (HM460699).

Phylogenetic analysis

16S rRNA gene sequences of *Pseudovibrio* spp. and their nearest neighbours were imported in the ARB software package (Ludwig*, et al.*, 2004). DNA sequences were aligned using the FastAlign function of the alignment editor implemented in the ARB program and refined manually. Ambiguous regions of the alignment were systematically removed using the program Gblocks v0.91b (Castresana, 2000). The default program parameters were used, except allowing a minimum block length of 5 and gaps in half of the positions. Phylogenetic trees were created by Bayesian analysis, using MrBayes v3.2 (Ronquist*, et al.*, 2012). All parameters were

treated as unknown variables with uniform prior-probability densities at the beginning of each run, and their values were estimated from the data during the analysis. Random starting trees were initiated and run for 10^7 generations. The number of chains was set to four, and Markov chains were sampled every 1000 iterations. Points prior to convergence were determined graphically and discarded. Calculated trees were imported into ARB, visually checked for the tree topologies and the consensus tree was exported as Encapsulated PostScript file (EPS).

Results

Phylogenetic affiliation of *Pseudovibrio* spp.isolated from sponges

Colonies of *Pseudovibrio* spp. isolated from sponges in this study usually had a flattened appearance, sticky and highly mucoid colony. The colony colour ranged from brown to dark brown, similar to previously reported *Pseudovibrio* spp. from sponges (Webster & Hill, 2001, Sertan-de Guzman*, et al.*, 2007, O' Halloran*, et al.*, 2011, Penesyan*, et al.*, 2011, O'Halloran*, et al.*, 2013)

A total of 76 *Pseudovibrio* isolates could be retrieved from different sponges, of which 34 isolates were from *Aplysina aerophoba*, 4 isolates were from *Corticium candelabrum*, another 34 isolates were from *Petrosia ficiformis*, one from *Ircinia* sp., one from *Chondrilla nucula* , one from *Acanthella acuta* and one isolate from the bryozoan *Cellephora pumicosa* (KP319347). Bayesian analysis placed 75 *Pseudovibrio* spp. isolates as closely related to *Pseudovibrio ascidiaceicola* (Figure 6.1.A), which is commonly found in marine sponges and other marine animals such as ascidians (Enticknap*, et al.*, 2006, Fukunaga*, et al.*, 2006, Muscholl-Silberhorn*, et al.*, 2008). One isolate (8H04) which originated from *P. ficiformis* was found to belong to a distinct *Pseudovibrio* cluster containing only sequences from sponges. This suggests the existence of a sponge-specific *Pseudovibrio* clade related to the type strain *P. axinellae* (O'Halloran*, et al.*, 2013), which was isolated from the marine sponge *Axinella dissimilis* (Figure 6.1.B). No isolates from this study were closely related to the other two known *Pseudovibrio* species, namely *Pseudovibrio denitrificans* (Shieh*, et al.*, 2004) and *Pseudovibrio japonicus* (Hosoya & Yokota, 2007).

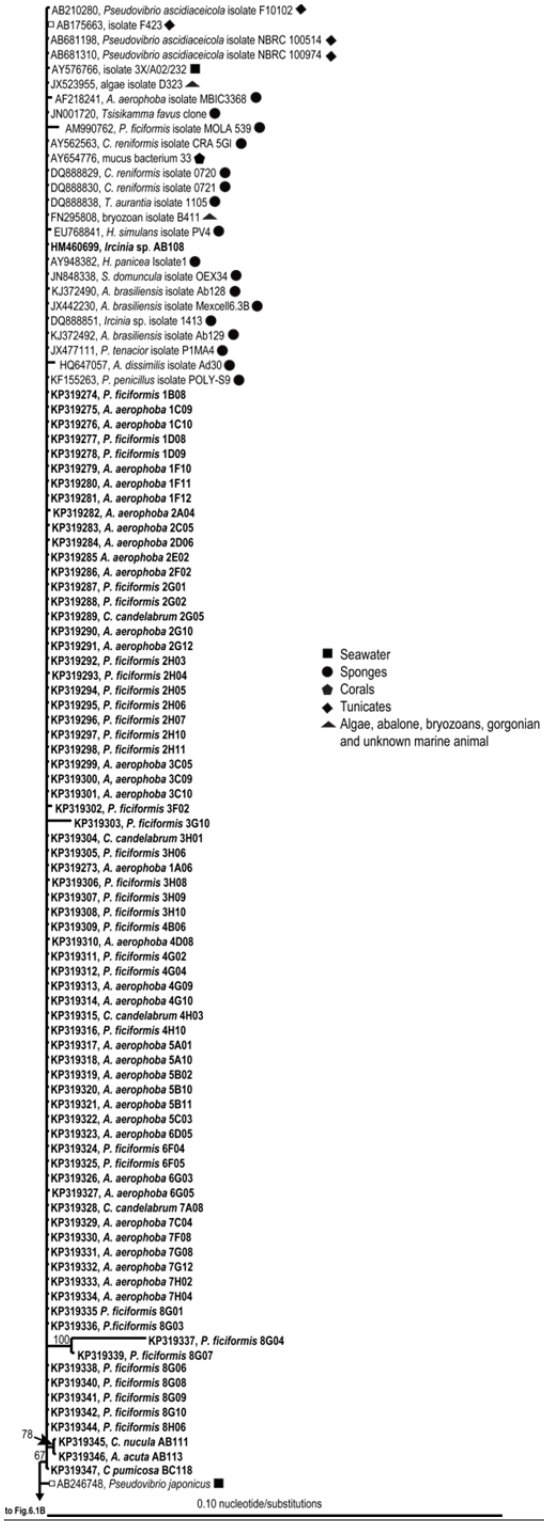

Figure 6.1.A. Bayesian phylogram based on 16S rRNA gene sequences of *Pseudovibrio* isolates from this study, its nearest neighbours from NCBI and reference sequences from the study described previously by Bondarev *et al*., 2013. For each reference sequence, the source of isolation is indicated, which is either from seawater, sponge, coral, tunicate or other organisms (see legend). Isolates from this study are indicated in bold. Branch nodes with a white box (□) represent type strains from different *Pseudovibrio* species, namely *P. ascidiaceicola* (Fukunaga *et al*., 2006), *P. japonicus* (Hosoya & Yokota, 2007), *P. axinellae* (O'Halloran *et al.*, 2011) and *P. denitrificans* (Shieh *et al*., 2004). The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values < 50 are not indicated.

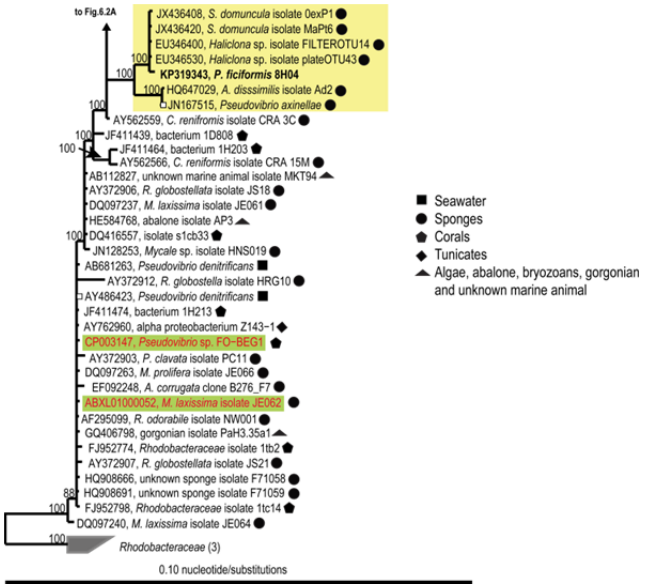

Figure 6.1.B. Bayesian phylogram based on 16S rRNA gene sequences of Pseudovibrio isolates as described in Figure 6.3.A. Isolates highlighted in green are Pseudovibrio isolates of which genomes have been sequenced (Bondarev et al., 2013). The yellow box indicates a Pseudovibrio clade containing Pseudovibrio spp. that have only been isolated from sponges.

Genotypic diversity of Pseudovibrio spp.

BOXA1R colony PCR was employed to analyse genomic diversity among different isolates based on genomic banding patterns of the repetitive BOX elements in prokaryotes. Two to nine bands were obtained per isolate (Figure 6.2). Strains from the same sponge host tended to cluster together. For instance, at 70% similarity, a cluster which included strains of 6G05, 7G08, 1C10 and 1C09 from *A. aerophoba* is distinct from the next cluster of 2G12, 7H02 and 7G12 which is also from *A. aerophoba*. This pattern also applies to strains from *P. ficiformis* and was repeated throughout the phylogram, although there are outliers (*e.g.* 1D09, 1F12 and 4G09). All strains from *C. candelabrum* separated from each other based on their genomic banding paterns. Isolates from other marine sponges (*A. acuta*, *Ircinia* sp. and *C. nucula*) and the bryozoan (*C. pumicosa*) all had their specific banding pattern (Figure 6.2). Only two isolates, one from *A. aerophoba* and one from *P. ficiformis*, were positive in a PCR-based

aerophoba and one from *P. ficiformis*, were positive in a PCR-based halogenase screening. However, these did not cluster together. In addition, there was no apparent correlation between antimicrobial resistance profiles and genomic banding pattern.

Exploring antibiotic resistance phenotypes of *Pseudovibrio* spp.

For a subset of 72 *Pseudovibrio* spp. isolates antibiotic resistance profiles were determined to identify traits that show strain-to-strain variation. A wide range of antibiotics were used which are specific for gram-negative bacteria such as polymyxin, specific for gram-positive bacteria (*e.g.* daptomycin, linezolid, vancomycin) and wide spectrum antibiotics that target both gram-positive and gram-negative bacteria (Figure 6.3.). All *Pseudovibrio* strains could grow on media supplemented with polymyxin, daptomycin, vancomycin, lincomycin and tetracycline, with the exception of strain 3H01 that was found sensitive to tetracycline. Almost all strains could grow on media supplemented with penicillin and ampicillin except strains 2G02, 2H04, 2H06 and 4G04, which did not show any growth. In addition, strain 2H11 was sensitive to penicillin but not to ampicillin while strains 8G08, 8G09 and 8G10 were sensitive to ampicillin but not to penicillin. Only strain 7C04 could grow on a medium supplemented with linezolid.

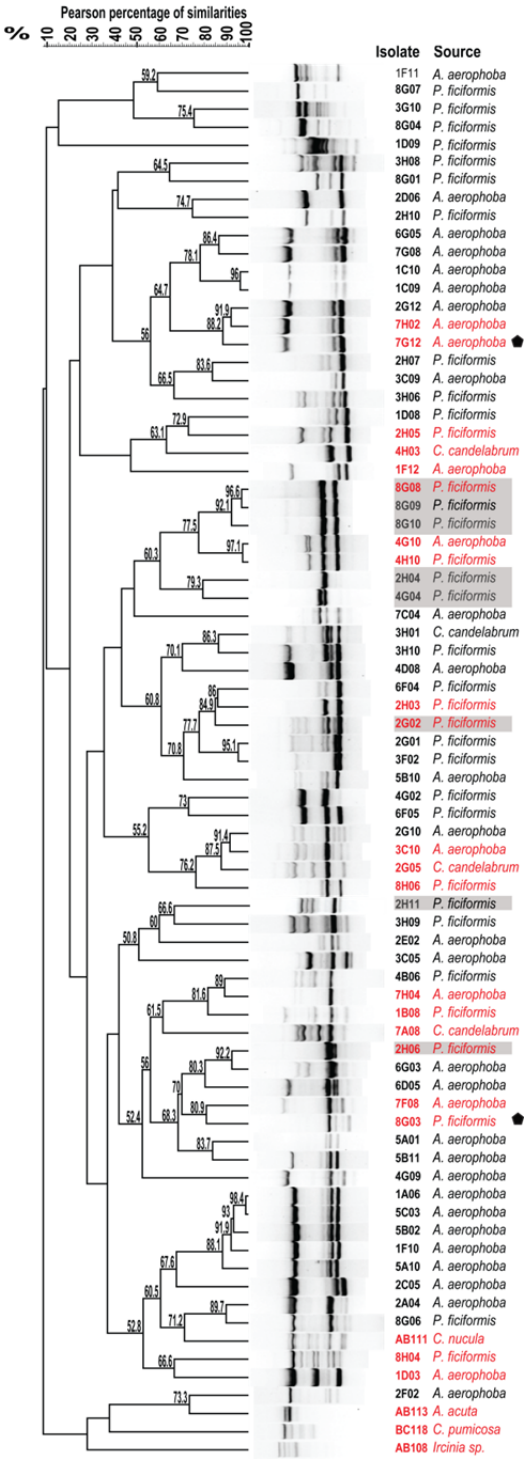

Figure 6.2 Phylogram of BOXA1R colony rep-PCR of *Pseudovibrio* spp. isolates fingerprints. The phylogram was generated using UPGMA clustering with Pearson correlation. The isolate ID is next to the banding pattern (*e.g.* 1F11). Next to the isolate ID are the sponge species from where the isolates originated (*e.g.* 1F11 is isolated from *A. aerophoba*). All *Pseudovibrio* isolates are from marine sponges except for isolate BC118, which was isolated from the bryozoan *Cellepora pumicosa*. The different ABR phenotypes are all indicated with grey boxes, indicating isolates which are sensitive to ampicillin, sensitive to penicillin or sensitive to both ampicillin and penicillin (see also Figure 6.3). Isolates represented by (⬟) indicate the presence of a halogenase gene. Isolates with red font were selected for genome sequencing.

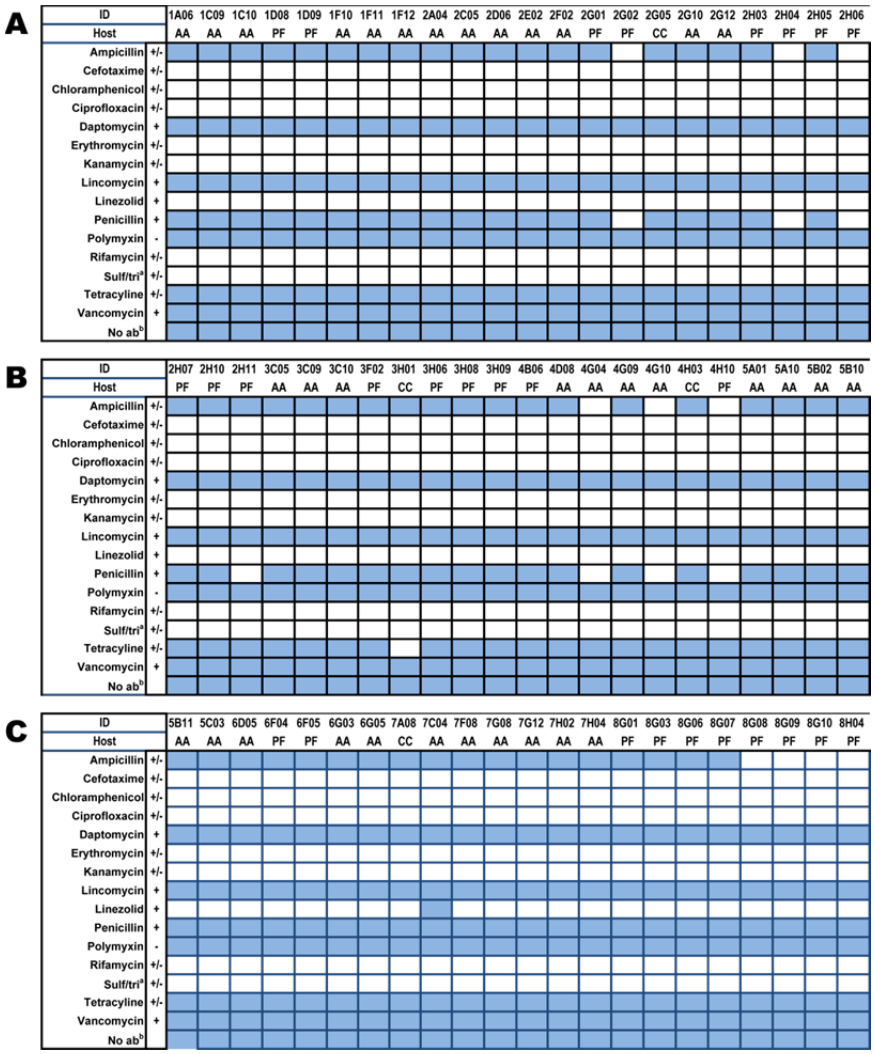

Figure 6.2.A-C. Antibiotic resistance profiles of 72 *Pseudovibrio* spp. isolates based on their ability to grow on Mueller-Hinton medium supplemented with different antibiotics. The minus and plus symbols shown below the antibiotics tested indicate their expected target bacteria, gram negative and gram positive, respectively. Positive growth (antibiotic-resistant) is indicated in blue while white indicated there is no growth (antibiotic-sensitive). Six isolates tested (1B08, 3G10, 3H10, 4G02, 8G04 and 8H06) shown inconclusive results and were omitted from the antibiogram. Sulfi/Tri[a] refers to combination of Sulfamethoxazole/Trimethoprim and No ab[b] indicates that no antibiotics added to the medium plate

Selection of isolates for genome sequencing

Based on the phenotypic and genotypic data, the following criteria were considered for selection of *Pseudovibrio* isolates for genome sequencing. i) isolate from different sponge species: these include isolate AB108 (*Ircinia* sp.), AB111 (*C. nucula*) and AB113 (*A. acuta*). Three isolates from *C. candelabrum* and the isolates from the corresponding sponge host in the same cluster: *e.g.* 2H05 (*P. ficiformis*), 4H03 (*C. candelabrum*), 1F12 (*A. aerophoba*) and 1D03 (*A. aerophoba*), (see Figure 6.4). Isolate BC118 from the bryozoan *C. pumicosa* was also selected. ii) Isolates with different antibiotic resistance profiles: some isolates from *P. ficiformis* showed a different antibiotic resistance pattern compared to isolates from the other sponges (Figure 6.3). Isolates 2H02, 2H06 which were sensitive to ampicillin and penicillin, and 8G08 which was only sensitive to penicillin were selected. For comparison, isolates with different resistance profiles (the other isolates were resistant to ampicillin and penicillin) from clusters with similar genomic fingerprints were chosen. These include isolates 2H03, , 4G10 and 4H10. iii) Halogenase-positive isolates and halogenase-negative isolates from the same cluster: 7H02, 7G12, 7F08 and 8G03. In total, 25 isolates were selected for genome sequencing.

Discussion

In this study, diversity of *Pseudovibrio* isolates from different sponge hosts was analysed using both phenotypic and genotypic approaches. Considerable diversity was observed for strains isolated from *P. ficiformis* based on their resistance to penicillin and ampicillin (Figure 6.3), which may be a result of the high adaptation capacity of the strains to the hostile environment inside the sponge host. A high adaptation capacity of bacterial species to diverse natural environments has been demonstrated previously for *Lactobacillus plantarum* (Bringel*, et al.*, 2001, Siezen*, et al.*, 2010).

The phenotypic study was complemented by a genotypic characterization of *Pseudovibrio* spp. using BOX elements which are found in the bacterial genome and a PCR-based screening of flavin-dependent halogenase genes (Figure 6.2). Although 16S rRNA gene sequences give valuable information for phylogenetic characterisation (Figure 6.1.A-B), these data do not reveal genomic variation among isolates of the same species due to the highly conserved nature of 16S rRNA gene sequences (Rodríguez-Valera, 2002). Genomic DNA fingerprints of the *Pseudovibrio* spp. clearly

show substantial genotypic diversity in comparison to the 16S rRNA sequences of the isolates (Figure 6.2). This is consistent with previous findings where high microdiversity of isolates from sponges has been recorded using repetitive PCR methods such as randomly amplified polymorphic DNA (RAPD) (O' Halloran*, et al.*, 2011) and BOXA1R primer (Esteves*, et al.*, 2013). Interestingly, according to Esteves *et al.*, 2013, BOX-PCR fingerprinting results suggest a specimen-dependent occurrence of prevailing bacterial genomes across sponge individuals. Such comparison could not be drawn from this study since different sponge specimens are also different species, but it was demosntrated that they are host-specific clustering of isolates particularly from *A. aerophoba* and *P. ficiformis* (Figure 6.2). More isolates from similar sponge species should be investigated to check whether this indication for species-dependent occurrence is maintainable.

In sponges, the true producers of many bioactive compounds were found to be sponge-associated bacteria (Piel, 2004). *Pseudovibrio* spp. from sponges in particular are known producers of diverse secondary metabolites (Sertan-de Guzman*, et al.*, 2007, O' Halloran*, et al.*, 2011, Penesyan*, et al.*, 2011, Chen*, et al.*, 2012, Bondarev*, et al.*, 2013). In the marine environment, bromine is available in abundance and the majority of marine halogenated metabolites contain bromine (Herrera-Rodriguez*, et al.*, 2011). $FADH_2$-dependent halogenases have been shown to play a significant role in the biosynthesis of many halogenated secondary metabolites (Gribble, 2003, van Pée & Patallo, 2006). Two isolates were found to harbour a putative $FADH_2$-dependent halogenase gene and may be producers of halogenated secondary metabolites. Genome sequencing will provide more clues on the presence of secondary metabolite gene clusters and the position of the halogenase gene in the two *Pseudovibrio* spp.

Currently there are two publicly available *Pseudovibrio* genomes, one strain was isolated from marine sponge *Mycale laxissima* (*Pseudovibrio* sp. JE062) and another was isolated from a coral (*Pseudovibrio* sp. FO-BEG1) (Bondarev*, et al.*, 2013), which is closely related to *Pseudovibrio denitrificans*. Genomic analysis of *Pseudovibrio* sp JE062 and *Pseudovibrio* sp. FO-BEG1 revealed unique features of genes associated with symbiosis, for instance discovery of gene related to excretion system type VI (T6SSs) and type III secretion system (T3SS), including effector molecules

(Bondarev*, et al.*, 2013). Type VI (T6SSs) excretion system have been described to play a role in biofilm formation (Aschtgen*, et al.*, 2008), quorum sensing (Weber*, et al.*, 2009), interbacterial interactions (Hood et al., 2010) and antipathogenesis (Chow & Mazmanian, 2010, Jani & Cotter, 2010). Comparative genomics of *Pseudovibrio* sp JE062 and *Pseudovibrio* sp. FO-BEG1 also discovered to possess hybrid polyketide synthase-nonribosomal peptide synthase (NRPS-PKS) gene cluster assumed to be involved in the production of secondary metabolites colibactin with yet unknown *in vivo* functions. It was hypothesized that colibactin produced by *Pseudovibrio* is similar in function to colibactin-producing *Enterobacteriaceae* where it was used to prolong the attachment to intestinal epithelium cells in order to successfully colonize the intestine of their host (Nougayrède*, et al.*, 2006).

In this study, 75 isolates were found to be closely related with *Pseudovibrio ascidiaceicola* and one isolate was most closely related to *P axinellae*. Selection of 25 isolates from this study for genome sequencing coupled with different genotypic and phenotypic data found in this study will be the basis for comparison of sponge-associated *Pseudovibrio* spp. and provide more insight into the size and complexity of *Pseudovibrio* genomes.

Conclusion

Pseudovibrio isolates that have been tested in this study showed differences in phenotypic and genotypic properties Several strains from the sponge host *P. ficiformis*, were found to exhibit sensitivity to ampicillin and penicillin while other strains from the same sponge and all isolates from other tested sponges were resistant to these antibiotics. Two isolates were found halogenase-positive based on PCR-based screening.

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Chapter 7:

General Discussion

Cultivation gives context to microbial ecology

One of the inevitabilities in any field of science is *change*, for instance the focus of research changes with time. The changes in the field of sponge microbial ecology in particular over the last decade have been remarkable. The so called metagenomic-, metatranscriptomic- and metaproteomic-based approaches have impacted the whole concept of microbial ecology with accumulation of huge and overwhelming datasets. These culture-independent technologies have become extensively used to capture genes, gene transcripts, and proteins in complex microbial communities. “Meta” approaches have been successfully used to describe the lifestyle of *Poribacteria*, which are thought to be symbiotically associated with marine sponges (Siegl*, et al.*, 2011), predicting the function of an uncultured deltaproteobacterium in the marine sponge *Cymbastella concentrica* (Liu*, et al.*, 2011) and identifying common functions in six sponge microbiomes (Fan*, et al.*, 2012). Based on these trends, there are questions to-be-asked especially in the field of sponge microbiology: what is the significance and importance of isolating yet-to-be cultured organisms in the omics era of analysing massive datasets produced using “meta” approaches? If single cell genomics taken together with metagenomics have allowed the sequencing of the complete genome of an uncultured organism, is the isolation of that organism still needed? In turn, if the “meta” approaches could decipher the entangled metabolism in complex communities, could we take advantage of such information to pave new ways of isolating target organisms from sponges?

For answering those questions, one could start thinking about the percentage of bacteria isolated from sponges. To date, a maximum of about 14% of the bacterial species could be isolated from the sponge microbiome by using multiple approaches to enhance the cultivability (Sipkema*, et al.*, 2011). Our effort as described in Chapter 5 has led to the isolation of to 3.7% of the bacteria associated with *Halichondria panicea* in comparison to the uncultured majority of the sponge microbiome. There are good reasons why culturing is still relevant in the ~omics age. Currently, a large number of predicted physiological functions is based on homologues from the cultured fraction of isolates in the laboratory (Biller*, et al.*, 2014). Without properly characterized isolates, one could not easily give answers to questions like who they are, what they are doing, and what they could

potentially do, even though culture-independent techniques such as stable isotope probing (SIP), fluorescence in situ hybridization (FISH), and metagenomics could provide a clue (Redmond*, et al.*, 2010, Liu*, et al.*, 2011, Behnam*, et al.*, 2012, Fiore*, et al.*, 2013). Prediction of physiological properties based on 16S rRNA or 18S rRNA sequence similarity of environmental sequences to cultured organisms has to be done with more caution to avoid far-fetched conclusions. For instance, fungi from the order *Malasseziales* detected from marine sponges as described in Chapter 3 and corals (Amend*, et al.*, 2012), are responsible for dandruff formation and seborrhoeic dermatitis in human. However, none of the enviromental *Malasseziales* have been cultured so far, posing a valid question as to what actually is their role in the sponge and coral holobiont. . A more rewarding approach would be to combine both approaches, where the omics-based approach and cultivation go hand-to-hand. For example, the results of a metatranscriptomics study have been used to design a mucin-based medium to isolate a *Rikinella*-like bacterium, which is the dominant symbiont of the medicinal leech *Hirudo verbana* (Bomar*, et al.*, 2011). Another prime example on how cultivation coupled with molecular analysis has contributed to the isolation of a subgroup of the phylum *Chloroflexi*, members of which are found in almost all environments including sponges (Yamada & Sekiguchi, 2009, Schmitt*, et al.*, 2011). Despite their cosmopolitan nature, only a small number of cultured microorganisms were known. However, using enrichment cultures and spine-like structures of sludge granules coupled with molecular analyses has enabled isolation and characterization of six genera within subclass 1 of the *Chloroflexi* (Yamada & Sekiguchi, 2009).

The next frontier: Culture-to-isolate and isolate-to-discover

It was revealed that more than half of the known natural products with antimicrobial, antitumor or antiviral activity are of bacterial origin (Berdy, 2005). The majority of these compounds was isolated from cultivated representatives of only five bacterial groups: filamentous actinomycetes, *Myxobacteria*, *Cyanobacteria*, and members of the genera *Pseudomonas* and *Bacillus* (Wilson*, et al.*, 2014). Approximately 70% of all known prokaryotic phyla is without cultured representatives and represents a particularly promising source for new, chemically prolific taxa (Achtman & Wagner, 2008). In this respect, sponges are known to harbour a gigantic

consortium of uncultivated bacteria comprising hundreds of distinct phylotypes (Hentschel*, et al.*, 2012). However, the true metabolic potential of these uncultured microbes remains unexplored except for individual biosynthetic pathways reported from environmental sources (Brady*, et al.*, 2009, Piel, 2011). For example, the pathways involved in the production of onnamide- and theopederin-type polyketides (Piel*, et al.*, 2004) and ribosomal peptides of the polytheonamide group (Freeman*, et al.*, 2012), which were previously discovered in the marine sponge *Theonella swinhoei* were proven to be of microbial origin. The challenge is to access the uncultivated fraction of microbes from sponges to exploit their genetic potential by understanding the biosynthetic pathways and mass-produce the bioactive compounds for the benefit of mankind. In addition, there are also symbionts without apparent function or yet-to-be-characterized such as the dominant alphaproteobacterial symbiont (Althoff*, et al.*, 1998) from the marine sponge *Halichondria panicea* (Chapter 2). Brominated compounds such as bromophenols have been characterized from *H. panicea* (Kotterman*, et al.*, 2003), and their origin could be attributed to its sponge symbiont based on the fact that organobromine compounds are often synthesized by bacteria, via a flavin-dependent halogenase (van Pée, 2001). The recent discovery of a marine brominase with previously unknown decarboxylative-halogenation enzymology confirmed the involvement of marine bacteria to produce such compound (Agarwal*, et al.*, 2014) Although a culture-dependent approach was used to analyse the cultivable assemblages as described in Chapter 5, isolation of this specific alphaproteobacterial symbiont remains elusive. The study confirmed the existence of cultivable bacterial assemblages that do not represent the majority of bacteria from the sponge microbiome, but are commonly isolated from different sponges (Schippers*, et al.*, 2012). One of those commonly isolated bacterial taxa is the genus *Pseudovibrio*. Comparative genome analysis of *Pseudovibrio* spp. isolates from sponge and coral has shown previously that this genus is not only metabolically versatile but fully-equipped for interaction with the sponge host (Bondarev*, et al.*, 2013). This is exemplified by the discovery of two types of secretion systems, namely type VI secretion systems (T6SSs) and type III secretion system (T3SS), that could be involved in symbiont–host interactions. Analysis of the 16S rRNA gene (Bondarev*, et al.*, 2013) made it possible to distinguish at least four different species of *Pseudovibrio* namely *Pseudovibrio japonicus*

(Hosoya & Yokota, 2007), *Pseudovibrio denitrificans* (Shieh*, et al.*, 2004), *Pseudovibrio asciadeceicola* (Fukunaga*, et al.*, 2006) and *Pseudovibrio axinellae* (O'Halloran*, et al.*, 2013). However, this method lacks the resolution to distinguish genetically different individual species and strains of *Pseudovibrio* due to the fact that the sequence of the 16S rRNA gene is highly conserved (99.7–100.0% homology). To this end, we characterized the microdiversity of *Pseudovibrio* spp. as described in Chapter 6, and a total of 25 representative strains is currently prepared for genome sequencing. Availability of these genomes will provide more clues on the lifestyle of the genus *Pseudovibrio* in sponges.

Origin of sponge-derived fungal isolates

When isolating fungi from sponges, there are two possibilities that have to be taken into account. It could be that the fungi have been growing in the sponge, *e.g.* as a symbiont, or that they were present as spores filtered from the surrounding seawater by the sponge host. Although at this point, the distinction cannot be made exclusively for each strain obtained, some trends could be highlighted. For instance, if the associated fungi or symbionts are predominantly present or enriched in the sponge host and capable to grow under the applied conditions *in vitro*, they should represent the 'real' fungal community in the sponge.This trend was shown in a culture-dependent study of dominant fungi from Australian coral reefs where members of the fungal genera *Aspergillus*, *Cladosporium* and *Penicillium* were found in similar proportions in around 70 sponge samples (Morrison-Gardiner, 2002). Such dominance has also been observed for *Penicillium* spp isolated from the marine sponges *A. aerophoba*, *C. candelabrum* and *P. ficiformis* as described in Chapter 4. However, we could also show an apparent lack of congruence with respect to predominant fungal taxa retrieved by cultivation as compared with the molecular data. As such, the possibility that the isolated fungi originated from spores filtered from the water by the sponges cannot be ruled out. Moreover, the presence of fungal propagules in seawater at different sites is well established (Roth Jr*, et al.*, 1964, Schaumann, 1974, Miller & Whitney, 1981), hence there is high likelihood that they were filtered and accumulated by the sponges and persevered inside sponges until they were able to germinate and grow under laboratory conditions. Members of the genera *Aspergillus, Cladosporium, Doratomyces, Fusarium, Penicillium*

and *Verticillium* were reported to be among the most common isolates from sediment, water and air samples from the North Sea and North-East-Atlantic, which is similar to the situation with well-known and widespread terrestrial fungi (Schaumann, 1974). Another explanation would be that fungi are really present in sponges as symbionts and involved in the defence of the host (Proksch*, et al.*, 2010). Detection of fungi such as *Malasseziales, Saccharomycetales, Capnodiales* and *Heliotales* that are closely related to pathogens in animals and plants as described in Chapter 3 could mean that they are originally enriched or filtered in sponges and later stayed as resident. To date, vertical transmission of an unidentified yeast has been only demonstrated in the marine sponge *Chondrilla* sp., which points towards a symbiotic relationship between the yeast and the sponge host (Maldonado*, et al.*, 2005). In addition, secondary metabolites produced by sponge-derived fungi are chemically and structurally different from those produced by other members of the same genera commonly isolated from terrestrial environments, such as *Aspergillus*, *Penicillium* and *Trichoderma* (Bugni & Ireland, 2004, Hiort*, et al.*, 2004). The genetic potential of sponge-derived fungi to produce such compounds, possibly derived from polyketide synthases (PKS) and polyketide synthase-nonribosomal peptidase (PKS-NRPS) that could be involved in the host-defence mechanism has been described in Chapter 4.

Host specificity in marine sponges

Host specificity could be defined by the way in which organisms associate with only a few (specialist) or many (generalist) host species (Taylor*, et al.*, 2004). Understanding host specificity is crucial in biodiversity because it influences a remarkably wide range of biological phenomena. This is illustrated, for example, by host switching in the epidemiology of diseases where animal parasites are transmitted to humans (Despommier*, et al.*, 1994) and ecological co-existence of different flowering plants because of species-specific pollinators (Wright, 2002). In fact, the major theme in the eukaryotic ecological literature is about species of eukaryotes living on or in other organisms (*e.g.* insects on plants, parasitoids on insects, fungi on plant or animal hosts) and the degree of specificity of their interaction, *i.e.* whether they are specific to one or a few hosts or, generalized across many (Futuyma & Moreno, 1988, Thompson, 1994, Little & Currie, 2007). As for fungi, additional evidence showed that frequency of host-specific fungal

species on tropical trees is higher than anticipated in comparison to early estimates of fungal diversity, which are probably orders of magnitude too low (Frohlich and Hyde, 1999). Although such host-specificity was not observed from molecular analysis of sponge-associated fungi (**Chapter 3**), more sponge hosts need to be sampled and analysed before any robust conclusion on host-specificity could be derived. Interestingly, however, host-specificity was observed for the sponge-bacteria association based on the study of the North Sea sponges (*H. panicea*, *H. oculata* and *H. xena*) as described in **Chapter 2** in which each of the sympatric sponges had its own dominant associated bacterium. A deviation from this sponge species-specific pattern was the presence of more than 20 different and novel *Chlamydiae* present in the three sponge species, but not in the seawater. The general host-specific pattern has been documented for many sponge species. For example, the marine sponges *Cymbastela concentrica*, *Callyspongia* sp. and *Stylinos* sp. from Australia host unique and specialized communities of microbes (Taylor*, et al.*, 2004). This host-specificity of Low Microbial Abundance (LMA) sponges is characterised by domination of the sponge-associated microbiota by a host-specific cyanobacterium from the genus *Synechococcus* or a host-specific proteobacterium (Giles*, et al.*, 2013, Poppell*, et al.*, 2014). This characteristic was confirmed by our study, showing that microbial communities associated with *H. panicea*, *H. oculata* and *H. xena* were dominated by a single host-specific α-, β- and γ-proteobacterium, respectively. A recent study of sponge-associated bacteria from seven closely related *Hexadella* spp. confirmed the presence of sponge-specific and species-specific host-bacteria associations, even among extremely low abundant taxa (Reveillaud*, et al.*, 2014). Altogether, these results (**Chapter 2** and the studies mentioned) demonstrate highly diverse, remarkably specific and stable sponge-bacteria associations that extend to members of the rare biosphere at a very fine phylogenetic and geographical scale.

Plasticity of sponge symbionts

The most widely used definition of symbiosis was provided by de Bary (1879) as the ‘‘living together of two differently named organisms’’. Although arguments for alternative definitions of symbiosis persevered in the twentieth century (Lewin, 1982), symbiosis today is usually defined as intimate, enduring associations between individuals of different species

(Goff, 1982). For example, reseachers studying lichen symbiosis consider these associations to be "controlled parasitism" in which the fungal mycobiont is an obligate, biotrophic parasite of the algal phycobiont (Ahmadjian & Jacobs, 1981) and later it was argued that despite its parasitic nature, the fungal mycobiont is more of an optimal harvester (Hyvärinen*, et al.*, 2002). This description recognizes the dynamic interactions of both partners in time and any shifts influencing the outcomes for one or both partners (Bronstein, 1994a, Bronstein, 1994b). To this end, the characterisation of parasitism, commensalism and mutualism are not easy to make for sponge host-microbes associations. Taken into account the various forms of symbioses in marine sponges that existed throughout evolutionary events, mutualism and parasitism may in fact be considered as two sides of the same coin. The symbiotic associations cannot be simply considered as closed-circuits isolated from the external circumstances. In fact, it has been shown how easily symbiotic associations can switch between mutualism and parasitism in response to even the slightest environmental change (Leung & Poulin, 2008). This point has been illustrated in sponge symbiosis where extrinsic factors such as temperature and diseases can influence the symbiotic relationship (Webster*, et al.*, 2008, Webster*, et al.*, 2008), determining that the *a priori* beneficial outcome becomes detrimental if the circumstances change or *vice versa*. From an evolutionary perspective, symbiosis plays a role as key catalyst of evolution by promoting speciation, diversification and evolutionary novelties such as the development of cell types, tissues and organs to harbour mutualists as well as barriers against pathogens, including the development of the immune system (Pérez-Brocal*, et al.*, 2013). The endosymbiotic theory is a classic example on the evolutionary role of symbioses (Margulis, 1981). The theory postulates an initial invasion of *Alphaproteobacteria* with a large capacity to consume oxygen into the ancestor of the eukaryotic cell, from which the mitochondria would eventually arise. This event was followed by a second colonization of prokaryotes with chlorophyll, believed to be similar to cyanobacteria that gave rise to chloroplasts, resulting in photosynthetic cells such as plants, which have both mitochondria and chloroplasts. Related to that aspect, it could be that members of the phylum *Chlamydiae,* which were found in high relative abundance in sponges from the North Sea as described in Chapter 2 could be originally present as pathogen in the sponge hosts, but

throughout sands of time became permanent residents of the sponge hosts. It was proposed that ancient symbionts from the phylum *Chlamydiae* facilitated the establishment of primary plastids in a tripartite symbiosis with cyanobacteria and early eukaryotes (Horn*, et al.*, 2004, Subtil*, et al.*, 2013). By extrapolating the pathogen-symbiont relationship to the sponge host, it could be speculated that members of the phyla *Mesomycetozoa* and *Cercozoa*, which are commonly associated with parasitic interactions could also be originated as pathogens of sponges. Both phyla were found in high relative abundance using a molecular approach as described in Chapter 3.

Sponge holobiont: so near, yet so far

To date, at least 32 bacterial phyla and candidate phyla have been found in sponges, either via cultivation or molecular characterization (Taylor*, et al.*, 2007, Schmitt*, et al.*, 2012, Webster & Taylor). However, bacteria constitute only one of the three domains of life in the sponge holobiont. It was demonstrated that there are other putative symbionts from eukarya such as fungi, *Mesomycetozoea*, and *Cercozoa* that are part of the sponge holobiont as described in Chapter 2. Their ecology, diversity and function need to be further characterized in order to advance our understanding of the sponge holobiont in relation to disease, symbiont recruitment and maintenance and functioning. Research on archaea and viruses (not covered in this thesis) is lagging behind, and these are the areas that need further attention as discussed in the review by Webster and co-workers (2012).

Throughout our study, we used *H. panicea* as a model for the characterization of the sponge holobiont. *H. panicea* is a Low Microbial Abundance sponge commonly found in the North Sea and the North Atlantic Ocean. It is also called breadcrumb sponge due to its “breadcrumb” like appearance with 'volcano' chimneys. Its appearance is often with yellow to orange colour when viewed underwater, commonly found attached to substrates like rocks and sometimes colonized with red algae or green algae (Kravtsova*, et al.*, 2013). *H. panicea* has a distinct smell like exploded gunpowder possibly due to the synthesis of bioactive compounds to protect itself from predators such as water soluble compounds and bromophenols (Kotterman*, et al.*, 2003, Toth & Lindeborg, 2008).

Different culture-dependent and culture-independent methods have been employed for the investigation of bacteria from *H. panicea* in this thesis

(see Chapter 2 and Chapter 5). Members of the bacterial genera *Bacillus*, *Paracoccus* and *Shewanella* were found to dominate the cultured bacteria, but they only represent a small percentage of the sponge microbiome as described in Chapter 5. The dominant bacterial symbionts as found by molecular diversity studies of *H. panicea,* are either *Rhodobacter*-like alphaproteobacteria (Nakamura*, et al.*, 1992, Althoff*, et al.*, 1998, Naim*, et al.*, 2014), *Roseobacter* sp. (Wichels*, et al.*, 2006) or *Actinobacteria* (Imhoff & Stöhr, 2003, Hentschel*, et al.*, 2006), Chapter 5). However, these differences may have partly do to with the ambiguous taxonomic descriptions of the host. The Census of Marine Life has identified 56 aliases for the Breadcrumb sponge *Halichondria panicea* (http://blogs.nature.com/news/2008/06/ocean_census_reveals_the_beast.html). Hence, for future studies involving marine sponges in general, it is highly recommended that every study should be accompanied by 18S rRNA gene sequence analysis of the sponge host for comparative analysis of sponge microbiota. In addition, members of the *Chlamydiae* were also detected in *H. panicea* as described in Chapter 2, with three OTUs occurring at relative abundances between 0.1 to 1%, in all *H. panicea* specimens, suggesting that this phylum could be important to the sponge host. Last but not least, fungal assemblages have also been detected from *H. panicea* (22 OTUs), including OTU 514 which is closely related to *Malasseziales.*

Overview and Future Outlook

In this thesis, a combination of cultivation-dependent and cultivation-independent approaches was employed for unravelling biodiversity within sponge hosts. Although this shed new light on the cultivability (and current uncultivability) of sponge-associated bacteria and fungi and about the diverse associations that can occur in a single sponge species (bacteria, archaea, eukaryotes), an extended study on the following points would help to resolve more on the insights of sponge-microbe symbiosis:

The associated microorganisms in *H. panicea* were dominated by one (yet uncultivable) alphaproteobacterium, which represented more than 90% of all microorganisms present in the sponge, while it was nearly absent in seawater. This dominance in the sponge begs for the question what it is doing in the sponge. A study combining metagenomic, metatranscriptomic, metaproteomic and metabolomic properties (e.g. according to (Muller*, et*

al., 2013) could indicate the function of this bacterium as the dominant symbiont of *H. panicea*. Species-specific bacterial communities of North Sea marine sponges have been demonstrated in Chapter 2 with the dominance of alpha-, beta- and gammaproteobacteria in *H. panicea*, *H. oculata* and *H. xena*, respectively. In contrast, such species-specific dependency was not observed for fungal communities in sponges examined from two different regions as described in Chapter 3. Further investigation needs to be carried out to understand the localization of fungi (whether it is in the mesohyl or other parts of sponge) and whether they are only present as spores or also as functional organisms. This *in situ* examination should also be applied to other eukaryotic putative symbionts of sponges such as *Mesomycetozoea* and *Cercozoa* and also bacterial symbionts, such as *Chlamydiae.* This could be done with stable isotope probing (SIP), Fluorescence *in situ* hybridization (FISH) and electron microscopy like SEM and TEM. The availability of the genome of *Malassezia* associated with skin disease in human (Xu*, et al.*, 2007, Gioti*, et al.*, 2013) and intracellular bacterial symbionts such as *Chlamydiae* (Bertelli*, et al.*, 2010, Collingro*, et al.*, 2011, Bachmann*, et al.*, 2014) provide exciting avenues for future comparative genomics studies including the potential sponge symbionts. This would have come a long way in recognizing it's importance and understanding their functions in the sponge host.

Isolation of symbionts, including the ones that are present as minor constituents in the sponge microbiome such as members of the genera *Paracoccus*, *Bacillus*, *Shewanella* and *Pseudovibrio* coupled with comparative genomics analysis will provide valuable insights in understanding host-symbiont interactions of marine sponges. In fact, bacterial genomes of *Paracoccus* spp., *Bacillus* spp. and *Shewanella* spp. are available (Alcaraz*, et al.*, 2010, Rodrigues*, et al.*, 2011, Dziewit*, et al.*, 2014), and could provide a starting point for future genomic analysis. Such an effort is epitomized by the selection of 25 *Pseudovibrio* spp. isolates for genome sequencing in Chapter 6. Comparative genomic analysis of this particular genus is ongoing and will enhance our current understanding on cultivable, minor constituents of sponge host.

This project envisioned the complete picture of the sponge-associated microbial diversity by piecing together the puzzle pieces of the sponge holobiont. This goal was carried out by molecular surveys of bacterial and

fungal symbionts using next-generation sequencing to understand their composition and host specificity. Furthermore, the culture-dependent approach enabled isolation of fungi that could be tested in the laboratory and highlighted the genetic potential of sponge-derived fungi based on the presence of polyketide synthase (PKS) genes and hybrid polyketide synthase-nonribosomal peptides genes. The combination of molecular analysis and cultivation of bacteria and fungi demonstrated the complementarity between the two approaches, and yielded an outlook on where to go with integrated application of both strategies.

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Appendices:

Nederlandse samenvatting

De vondst van sponsachtige Metazoa van 650 miljoen jaar oud toont aan dat sponzen al op aarde zijn sinds het Precrambrium. De standvastigheid van sponzen tijdens klimaat- en ecologische veranderingen wordt onder andere toegeschreven aan symbiotische relaties met Archaea, Bacteria en Eukarya die in deze globaal verspreide dieren resideren. Het is tot nog toe onbekend wat de criteria zijn voor het behouden en verkrijgen van symbionts, maar het is wel duidelijk dat overdracht van symbionts tussen spons individuen zowel horizontaal als verticaal kan verlopen.

De sponzen *H. panicea, H. oculata* and *H. xena* uit de Noordzee bleken een host-specifieke microbiota te bevatten die gedomineerd werd door respectievelijk *α- β-* en *γ-Proteobacteria*. Naar de rol van deze symbionts moet nog verder onderzoek worden gedaan. *Chlamydiae* die ver verwant zijn met bekende *Chlamydiae* werden aangetroffen in hoge relatieve abundantie in deze sponzen en dit riep vragen op over hun functie in de spons holobiont.

Er is weinig bekend over de schimmels die aanwezig zijn in zeesponzen. Om hier meer inzicht in te krijgen is er hier met behulp van moleculaire technieken en gebruikmakend van het 18S rRNA gen amplicon onderzoek naar gedaan. In het verleden was dit een lastige opgave doordat 'contaminerend' spons DNA in hoge mate aanwezig was wat ook opgepakt kan worden door schimmel-specifieke PCR primers. Tegenwoordig kan de applicatie van next generation sequencing uitkomst bieden aangezien hiermee enorme hoeveelheden reads gegenereerd kunnen worden. Dit experiment leidde tot de ontdekking van nieuwe geslachten van gist binnen de phyla *Ascomycota* en *Basidiomycota* in sponzen uit de Noordzee en het Middellandse Zeegebied, wat aangeeft dat er in deze sponzen zich een grote verscheidenheid aan gisten bevindt die nog niet bestudeert zijn. Zo zijn er bijvoorbeeld gisten gevonden uit de orde van de *Malasseziales* waarvan bekend is dat deze veelvoorkomende pathogenen zijn in zeedieren. Alhoewel deze gisten de schimmel populatie domineerden uitte zich dat in deze sponzen niet tot (schijnbare) ziekte. Een complementair cultivatie-experiment was uitgevoerd voor het verkrijgen van schimmels in pure cultuur. Voor drie sponzen uit Middellandse Zee, namelijk *A. aerophoba*, *P. ficiformis* and *C. candelabrum*, behoorden de gecultiveerde schimmel cultivars grotendeels tot het *Penicillium* genus. Verder werden

schimmels uit de orde van de *Alternaria* en schimmels geaffilieerd met order van de *Rhodotorula* meerdere keren geïsoleerd. Er is geen overlap gevonden tussen de schimmelsoorten die gevonden werden door middel van moleculaire technieken en die gevonden door cultivatie, wat aangeeft dat “the great plate anomaly” ook opgaat voor schimmels. Veel van de geïsoleerde stammen uit de ordes *Penicillium* en *Alternaria* bleken genetisch gezien het vermogen te hebben om polyketide synthases (PKS) of PKS-non-ribosomal peptide synthase (PKS-NRPS) hybrides te produceren. Dit zijn enzyme complexen die regulier betrokken zijn bij de productie van secundaire metabolieten met een hoge biologische activiteit.
Een cultivatie experiment was uitgevoerd met monsters van *H.panicea* gebruikmakend van een grote verscheidenheid aan media en groei condities. Daarnaast werd ook de bacteriële diversiteit in deze monsters bepaald door middel van moleculaire technieken. De cultiveerbare genera, onder andere *Bacillus*, *Paracoccus* en *Shewanella*, bleken maar een klein onderdeel te zijn van de spons microbiota. *Pseudovibrio* is een ander genus waarvan vaak stammen worden geïsoleerd uit sponzen, maar dat in lage relatieve abundantie in de spons aanwezig is. Op basis van een PCR voor BOX elementen, een PCR voor een halogenase-coderend gen en resistentieprofielen voor antibiotica, wordt hier onderscheid gemaakt tussen *Pseudovibrio* stammen tussen welk niet gedistingeerd kon worden op basis van het 16S rRNA gen.
Tot slot, dit proefschrift levert een bijdrage aan het overbruggen en afgrenzen van de kloof tussen studies die afhankelijk en studies die onafhankelijk zijn van cultivatie in het onderzoek naar bacteriën en schimmels in sponzen. De kennis besloten in dit proefschrift kan dienen als basis en als inspiratie voor toekomstige studies naar microbiële diversiteit (in sponzen). Tevens kan het leidraden bieden voor de studie en exploitatie van symbionts van sponzen.

About the author

Mohd Azrul Naim Mohamad was born on June 23rd, 1983 in Kuala Terengganu, Terengganu, Malaysia. During high school, he studied at Malay College Kuala Kangsar and graduated in 2000. Then, he started his BSc studies in Biotechnology at the International Islamic University Malaysia, with specialization in Environmental Biotechnology and graduated in 2006. In 2008, he continued with the MSc Biotechnology at Wageningen University with specialization in Marine Biotechnology and obtained his MSc degree in 2010. His MSc thesis project was entitled "Biodiversity of sponge-associated microbial communities of the Dutch coast" under the supervision of Dr J. A.. M. Perez. In 2010, he started his PhD at the Laboratory of Microbiology, Wageningen University under the supervision of Prof. Hauke Smidt and Dr. Ir. Detmer Sipkema. The PhD thesis is entitled 'Exploring microbial diversity of marine sponges by culture-dependent and molecular approaches' will be defended on April 30, 2015. Since February 2015 he started as a Lecturer at Kulliyyah of Science, International Islamic University Malaysia.

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List of publications

Naim MA, Smidt H and Sipkema D. Molecular analysis of sponge-associated fungi. *Submitted.*

Naim MA, Silva CF, Tunc G, Mcpherson K, Versluis D, Smidt H and Sipkema D. Diversity of polyketide synthase-encoding genes from marine sponge-derived fungi. *In preparation.*

Naim MA, Spaans S, Smidt H and Sipkema D. Systematic assessment of cultivable bacteria from the marine sponge *Halichondria panicea. In preparation.*

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Mohd Azrul Naim Mohamad
April 2015, Wageningen

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- Techniques for writing and presenting scientific papers, Wageningen University (2011)
- Scientific publishing, Wageningen University (2011)
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- *Diversity of cultivable bacterial assemblages from marine sponge Halichondria panicea*. FEMS 4th Congress of European Microbiologists, 26-30 June 2011, Geneva, Switzerland

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