Diversity in the globally intertwined giant barrel sponge species complex

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1

General introduction

Coral reefs are among the most productive and diverse ecosystems on Earth, rivaling the biodiversity of rainforests (Knowlton et al. 2010). They provide numerous ecosystem services, such as in fisheries, shoreline protection, tourism and yielding compounds for the development of new medicine (Woodhead et al. 2019). Over 500 million people depend on coral reefs for their subsistence (Moberg and Folke 1999; Hughes et al. 2017). Over half of all coral reefs worldwide are threatened by climate change and other stressors, potentially affecting the livelihoods of millions of people (Burke et al. 2011). The large array of human-induced and natural pressures related to coral reef decline has been well studied (e.g. Wilkinson 1999; Hoegh-Guldberg et al. 2007; Perry et al. 2013). Among the major anthropogenic stressors are increased terrestrial runoff, coastal development, dredging, unsustainable fisheries and plastic waste (Bannister et al. 2012; Stender et al. 2014; Hughes et al. 2017; Lamb et al. 2018).

Corals are vulnerable to environmental changes, and other groups of organisms may profit from their demise (Mumby et al. 2016; Cruz et al. 2017). Amongst others, algae, cyanobacteria, and sponges have been reported to increase when corals decline (Hughes et al. 2007; Nörstrom et al. 2009; de Bakker et al. 2017). Of these groups, sponges (Porifera) may challenge both hard and soft corals in terms of species richness, abundance, and biomass on coral reefs (Diaz and Rützler 2001). They play a central role in regulating the carbonate budget of the reefs through bio-erosion, and their erosion rates can equal the calcification rates of reef-building corals (Perry et al. 2014; Webb et al. 2017). An equal balance between erosion and accretion is essential for the sustainability of coral reef ecosystems (Bell et al. 2008). Furthermore, through their pumping and feeding behaviour, sponges play central roles in the cycling of various nutrient elements including the nitrogen, sulphur, silicon, and phosphorus cycles (Southwell et al. 2008; Mohamed et al. 2010; Maldonado et al. 2012; Fiore et al. 2013a; Zhang et al. 2015) and contribute to the so-called benthic-pelagic coupling on coral reefs (Pile et al. 1997; Bak et al. 1998).

Benthic-pelagic coupling is the exchange of energy, mass, or nutrients between the seabed (i.e. the benthic environment) and the water column (i.e. the pelagic environment) (Griffiths et al. 2017). Sponges have relatively simple body plans and their cells are not organized in tissues or organs (van Soest et al. 2012). Instead, their bodies are designed to pump water through a system of channels and pores from which they filter food particles and dissolved organic matter (Pile et al. 1997; de Goeij et al. 2008; Koopmans et al. 2010). Water is drawn into the sponge through small surface openings called ostia and is then led through a narrowing system of incurrent canals to choanocyte chambers. The choanocyte chambers are covered with flagellates (choanocytes), which generate water flows by synchronized movements. From the choanocyte chambers, the water passes through microvilli where nutrients are filtered from the water and food particles are phagocytized by the sponge cells. The water then streams through a series of exhalant channels to the spongocoel, a large central cavity, and is then discharged through an osculum, a large opening on the sponge surface (Reiswig

1975; Larsen and Riisgard 1994). Sponge assemblages in natural densities can overturn the entire water column in a period ranging from 56 days to less than one day (Reiswig 1974; Pile et al. 1996; Patterson et al. 1997). The pumping system of sponges is efficient and it is estimated that less than 4% of the total metabolic expenditure in sponges is required for this activity (Riisgard and Larsen 1995).

The food extracted from the water mainly consists of organic matter. Particulate organic matter (POM), for example detritus or live picoplankton, forms an important part of their nutrition (Hadas et al. 2009). All POM in the seawater that is pumped through the sponge, passes through the choanocyte chambers and the observed variation in the uptake and release of different groups of picoplankton suggests that sponges are selectively feeding on the picoplankton (Frost 1980; Ribes et al. 1999; Yahel et al. 2006; Hanson et al. 2009; Maldonado et al. 2010; Riisgard and Larsen 2010). Due to the efficiency with which the sponges filter picoplankton from the sea, the number of bacteria in discharged seawater can be reduced by more than 99% (Wehrl 2007). However, in other sponge species dissolved organic matter (DOM) is the main source of organic carbon in their diets (Yahel et al. 2003; de Goeij et al. 2008; Mueller et al. 2014). The 'sponge loop' describes how sponges make DOM available to higher trophic levels by rapidly expelling filter cells as detritus (de Goeij et al. 2013). The sponge loop has been suggested to be the reason that coral reef ecosystems can exist in oligotrophic waters or 'marine deserts' (de Goeij et al. 2013).

SPONGE MICROBIAL COMMUNITY

Symbiotic microorganisms are believed to play key roles in the physiology of sponges, including many of the abovementioned processes (Osinga et al. 2001; Pita et al. 2018). Certain microorganisms can also harvest energy from light by photosynthesis making some sponges net primary producers (Southwell et al. 2008; Thacker and Freeman 2012; Fiore et al. 2013a). Others can produce bioactive compounds, some of which act as a chemical defense used to deter predators, pathogens and other harmful organisms (Pawlik 1993; Hentschel et al. 2012). The antimicrobial activities of sponge-associated microorganisms show great pharmaceutical potential and sponges are considered the most promising marine source for new therapeutic compounds (Fuerst 2014; Indraningrat et al. 2016; Mori et al. 2018). Of the 15.000 discovered marine natural products, around 30% have been derived from sponges (Leal et al. 2012; Mehbub et al. 2014; Blunt et al. 2018). The pharmaceutical potential of marine natural products in sponges, however, is still largely untapped (Romano et al. 2017). A better understanding of the nature of the symbiotic relationship between sponges and their associated microbiomes and the drivers of changes in this relationship is essential to accelerate these efforts (Taylor et al. 2007a; Webster and Taylor 2012; Valliappan et al. 2014; Marino et al. 2017). Due to their intricate relationship, sponges and their associated microorganisms are together often referred to as the 'sponge holobiont' (Taylor et al. 2007a; 2007b; Webster and Thomas 2016; Pita et al. 2018). New molecular techniques have accelerated the number of studies on the sponge holobiont, and have provided new

insights in the richness, functional roles and evolutionary history of the sponge-associated microbial community.

A dichotomy seems to exist between sponges harbouring dense communities of symbiotic microorganisms and sponges with much lower concentrations (Reiswig 1974). For these two groups, the names 'high microbial abundance' (HMA) and 'low microbial abundance' (LMA) are generally used (Hentschel et al. 2003). The differences in microbial abundance can be clearly observed with Transmission Electron Microscopy (Fig. 1.1). HMA sponges harbour 10^8 – 10^{10} microbial cells per gram of sponge wet weight, while LMA sponges contain only 10^5-10^6 prokaryotic cells per gram of sponge wet weight, which is similar to the concentration of microorganisms in seawater (Hentschel et al. 2006). In HMA sponges, the microbial biomass can account for more than 35% of the total sponge biomass (Vacelet 1975). HMA microbiomes are rich and diverse, whereas LMA microbiomes are mostly made up of Cyanobacteria and Proteobacteria (Hentschel et al. 2006; Weisz et al. 2007; Gloeckner et al. 2014; Moitinho-Silva et al. 2017a). Various physiological and metabolic differences between the groups have been found, and this is receiving more attention in recent studies (Weisz et al. 2008; Ribes et al. 2012; Moitinho-Silva et al. 2017b).

HMA sponges are capable of maintaining a unique microbial community, despite the constant influx of seawater (Glasl et al. 2017a). Sponge host species often have distinctive microbial fingerprints (Thomas et al. 2016) and the differences among hosts can originate at an early reproductive phase (Schmitt et al. 2008). Microorganisms can be assimilated in gametes by the host sponge ensuring the transmission of essential microorganisms to their offspring (Maldonado et al. 2005; Funkhouser and Bordestein 2013). However, the majority

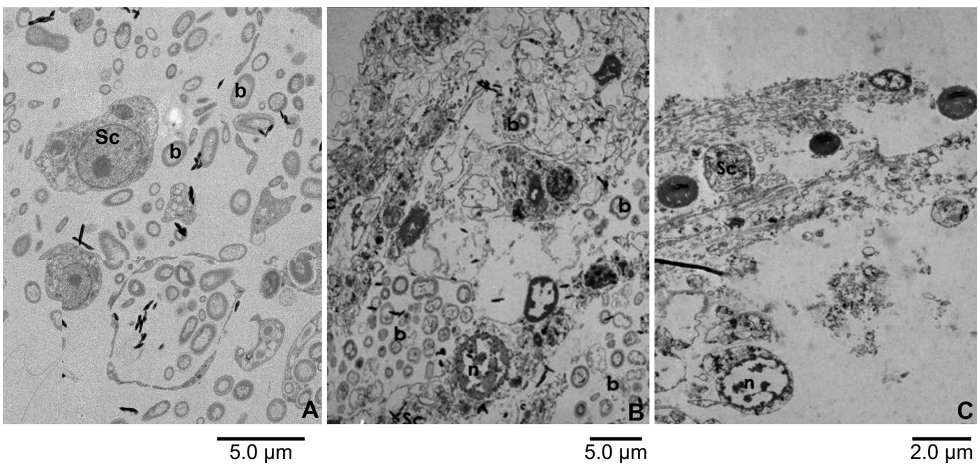

Figure 1.1. Transmission electron microscopy of three sponge species belonging to the order Haplosclerida. Two species are classified as HMA sponges (A, B) and one species is classified as a LMA sponge (C). A = *Xestospongia testudinaria*, B = *Xestospongia vansoesti*, C = *Haliclona fascigera*. Sc = sponge cell; b = bacteria; n = nucleus.

of the Operational Taxonomic Units (OTUs) are likely to be harvested from the surrounding environment, in which they tend to have much lower densities than in the host sponge (Reveillaud et al. 2014; Lynch and Neufeld 2015). As many as 41 different prokaryotic phyla have thus far been identified in sponges, many of which are shared among sponge species (Thomas et al. 2016). The sponge microbiome is thought to be stable across time and space, especially the core community that consists of OTUs that are present in most or all individuals of a certain host species (Erwin et al. 2012; Pita et al. 2013a; 2013b; 2018; Cárdenas et al. 2014; Thomas et al. 2016; Glasl et al. 2018). However, temporal and spatial variation did exist in the microbial communities of some sponge species (Wichels et al. 2006; White et al. 2012; Luter et al. 2015; Weigel and Erwin 2016; Pita et al. 2018).

CLASSIFICATION OF SPONGES

Sponges have evolved over 600 million years ago, placing them among the oldest animal lineages on Earth (Love et al. 2009; Simion et al. 2017). They are generally considered a sister group to all other multicellular animals (Fueda et al. 2017; Pett et al. 2019) and they are subdivided into four distinct classes, the Calcarea, Demospongiae, Hexactinellida and Homoscleromorpha (Gazave et al. 2011; van Soest et al. 2012; van Soest et al. 2012; 2015). The class Demospongiae is the largest and most diverse class, occurs in marine and freshwater environments, and sponges in this class have skeletons composed of spongin fibres and/or siliceous spicules. Approximately 81% of the 7,000 described species belong to this class, and more than 50 new species are described every year (Hooper and van Soest 2002; van Soest et al. 2019).

Demosponges exist in a wide variety of shapes and sizes, from small encrusting layers to large cups, barrels or branching forms (van Soest et al. 2012). Besides high interspecific morphological variation, sponges of the same species may adapt to local environmental conditions such as hydrodynamics, light, and turbidity, resulting in numerous morphologies (Palumbi 1984; Bell et al. 2002). Due to this high morphological variation, the classification of sponges at higher taxonomic levels has long been in debate. The Systema Porifera was a historic publication in 2002 and provided a large revision and comprehensive overview of the taxonomy of sponges (Hooper and van Soest 2002). The classification used in the Systema Porifera is largely based on sponge morphology, especially of the spicules. Although morphology-based classifications provided an excellent baseline, the use of molecular techniques revealed several weaknesses and inconsistencies in this morphology-based classification (Wörheide et al. 2012; Renard et al. 2018). Especially at the lower taxonomic levels in the class Demospongiae, molecular results did not support the existing classification (Redmond et al. 2013). Many scientists and other end users depend on the correct identification of their studied organisms to properly set up experiments and interpret results. Therefore, multiple attempts have been made to further improve the classification of the Demospongiae using molecular techniques, and this remains an

ongoing effort of the scientific community (Redmond et al. 2013; Morrow and Cárdenas 2015; Erpenbeck et al. 2016).

Despite their ecological importance, sponges have been underrepresented in coral reef research (de Voogd et al. 2006; Bell 2008). This is illustrated by the low attendance of international sponge conferences (909 individual participants between 1968 and 2013) compared to international coral reef symposia (over 1000 participants per edition) (Schönberg 2017). Furthermore, coral reef communities are generally assessed on the basis of benthic cover, while sponges are more abundant if the three-dimensional structure of reefs is taken into account, as they are often present in cryptic spaces (Zea 1993; Southwell et al. 2008). If we want to understand coral reefs better, we should look at sponges more.

GIANT BARREL SPONGES

A critical group within the demosponges consists of the giant barrel sponges (belonging to the genus *Xestospongia,* order *Haplosclerida)*. They have a large impact on coral reefs around the globe due to their abundance, size, and ecological relevance. They are among the most conspicuous reef members and occur in the tropical seas of the Atlantic Ocean, Indian Ocean, and western Pacific Ocean. They can reach sizes of over a meter in height and width and due to their slow growth rates of ±1.85 cm per year, large specimens in the Caribbean are thought to be over 1000 years old. One photographed specimen in Curaçao was estimated to be ±2,300 years old (Nagelkerken et al. 2000; McMurray et al. 2008). Due to their size and longevity, giant barrel sponges have been nicknamed 'Redwoods of the Reef' (McMurray et al. 2008). On Indo-Pacific reefs, however, giant barrel sponges grow at least twice as fast and are less long-lived, suggesting that they are more comparable to 'Pines in the Indo-Pacific' (McGrath et al. 2018).

In the Caribbean, giant barrel sponge populations may cover more than 9% of the available reef surface area and have a biomass and filtering capacity greater than any other benthic invertebrate (Zea 1993; McMurray et al. 2008). They are capable of pumping vast quantities of water per day and retain picoplankton at high efficiencies (McMurray et al. 2014; 2016). Giant barrel sponges alone can overturn a water column of 30 m deep every 2.8 to 6.0 days in the Florida Keys, and between 2.3 and 18 days in the Bahamas (McMurray et al. 2014). Their diet consists mostly of dissolved organic carbon (±60-70% of the total organic carbon) and detritus (±20-35%) (McMurray et al. 2017; Wooster et al. 2019). They can also offer shelter to corals (Hammerman and García-Hernández 2016) and harbour other organisms such as sea cucumbers, brittle stars, and lobsters (Hammond and Wilkinson 1985; Baba 1994).

Giant barrel sponges reproduce by broadcast spawning and do not reproduce by fragmentation (McMurray and Pawlik 2009). They release negatively buoyant egg cells and positively buoyant sperm cells during mass spawning events (Ritson-Williams et al. 2005).

Recordings of spawning events are rare but were made during multiple seasons in both the Indo-Pacific and the Caribbean (Ritson-Williams et al. 2005; McMurray et al. 2008; Swierts et al. 2013). They produce a large number of gametes (Fromont and Bergquist 1994) that are unpalatable to fish predators (Lindquist and Hay 1996). Not much is known about the larval behavior, but the dispersal of larvae is believed to be influenced by ocean currents (López-Legentil and Pawlik 2008). Recent increases in the abundance of giant barrel sponges are observed on multiple reefs throughout the Caribbean, with some reefs showing an increase in abundance of more than 300% in 12 years (McMurray et al. 2010; 2015).

Nowadays, three different species are described and generally accepted: *Xestospongia muta* occurs in the Caribbean region, *Xestospongia testudinaria* is spread across large parts of the Indo-Pacific and *Xestospongia bergquistia* is endemic to reefs in Australia (Schmidt 1870; Lamarck 1885; Fromont 1991). The species names of *X. muta* and *X. testudinaria* have been subject to many changes. According to the World Porifera Database, *Xestospongia muta* was formerly known as *Schmidtia muta* and *Petrosia muta*, and *Xestospongia testudinaria* was previously named *Alcyonium testudinarium*, *Reniera crateriformis, Reniera testudinaria* and *Petrosia testudinaria* (van Soest et al. 2015).

Xestospongia testudinaria and *X. bergquistia* are sympatric in Australia and the morphological differences between the species are subtle, with the most profound difference being the amount of spongin fibres present between the spicules (Fromont 1991). It is not possible to distinguish between the species based on visual characteristics in the field, although the strength and elasticity of the sponge is an indication of species identity when they are being pierced or cut (Fromont 1991). No unique characters distinguishing all three species have been found so far. The spicule size ranges of the three species overlap and *X. muta* and *X. testudinaria* have similar morphologies and skeletal structures, and the main distinction between the two is the ocean they live in (Setiawan et al. 2016a).

The giant barrel sponge species have a large intraspecific variability of spicule types and sizes (Subagio et al. 2017) and individuals within each species are also highly variable in size, shape, and external surface morphology. They can have a smooth external surface, or be covered with digitate or lamellate structures (Kerr and Kelly-Borges 1994). Previously, this external morphological variation was believed to represent multiple species (Wilson 1925), and the name *Xestospongia testudinaria* var. *fistulophora* was given to a variety of giant barrel sponge from the Philippines of which the outer surface had fistular structures instead of vertical ridges. Congruent patterns between external morphology, mitochondrial DNA markers and nuclear DNA markers on reefs around Lembeh Island in Indonesia support the hypothesis that multiple species co-exist in the Indo-Pacific (Swierts et al. 2013). Furthermore, in the Caribbean, distinct chemotypes of *X. muta* exist (Fromont et al. 1994) which are not correlated with sampling location, depth, or morphotype (Kerr and Kelly-

Borges 1994). These chemotypes were also suggested to represent different biological species. Furthermore, the three giant barrel sponge species cannot be differentiated using the I3-M11 partition of the CO1 mitochondrial gene (Setiawan 2016a; 2016b). This gene was suggested as a candidate marker for species-level phylogenies in sponges other than the standard barcoding marker using the Folmer primers (Erpenbeck et al. 2006a). Microsatellite data further suggests that the current taxonomy may not represent the true diversity in giant barrel sponges, potentially hindering our understanding of their evolutionary history and the correct interpretation of experimental results (Bell et al. 2014; Richards et al. 2016). Unfortunately, no DNA could be extracted from the dried syntypes of *X. muta* and the holotype of *X. testudinaria* has been lost, and it is, therefore, impossible to examine the genetics of these specimens.

Giant barrel sponges are HMA sponges, and their microbiome consists primarily of Chloroflexi, Proteobacteria, Acidobacteria and Actinobacteria (Montalvo et al. 2014; Montalvo and Hill 2011; Polonia et al. 2017; Cleary et al. 2015a; De Voogd et al. 2015). The bacterial communities of *X. muta* and *X. testudinaria* are very similar, but species-specific differences also exist (Montalvo and Hill 2011). Since the sponges live in different oceans, it is not clear whether the differences are a result of the different local environments or because the sponges are two different species. In the Caribbean local environmental differences significantly affected the microbial communities of giant barrel sponges (Lesser et al. 2016; Morrow et al. 2016; Villegas-Plazas et al. 2018). However, these differences may also represent the different giant barrel sponge species that have been suggested to exist in the Caribbean (Fromont 1994 et al. 1994). These examples illustrate the importance of proper identification of giant barrel sponge species and their evolutionary history.

THESIS OUTLINE

The state of coral reef ecosystems is precarious and it remains difficult to predict how they are affected by current and future environmental changes. The importance of sponges in coral reef ecosystems has long been neglected but is becoming more and more acknowledged. Nevertheless, some fundamental aspects of sponges and their microbiomes remain poorly understood. Inconsistencies between taxonomical classifications and phylogenies are unresolved, the interactions between individual drivers of the prokaryotic community of sponges are unexamined and the role of the sponge holobiont in the wider coral reef ecosystem is unclear. These gaps in our knowledge often even exist in sponges that are used as model groups, such as giant barrel sponges.

This thesis addressed these challenges and aims to answer the following questions: 1. How many giant barrel sponge species exist around the globe? 2. What is the evolutionary history of the giant barrel sponge species? 3. What are the drivers of variation in the prokaryotic community composition of giant barrel sponges? 4. How does the richness, diversity, and

evenness of the (giant barrel) sponge prokaryotic community relate to those of other coral reef organisms? This is studied through a series of in-situ observational studies and by multiple laboratory analyses on giant barrel sponge samples collected from the tropical regions of the Atlantic, Indian and Pacific Ocean.

The section *Genetics* of this thesis unravels the complex phylogeny and evolutionary history of giant barrel sponges. **Chapter 2** shows that at least three giant barrel sponge species exist in the Caribbean and at least three species exist in the Indo-Pacific. The species in each of the ocean basins are sympatric, difficult to distinguish morphologically in the field and do not form monophyletic lineages. In other words, a sponge from Curaçao can genetically be more closely related to a giant barrel sponge in Indonesia, than to another giant barrel sponge on the same reef. This suggests that multiple giant barrel sponge species already occurred before the Atlantic and Indo-Pacific realms were geographically separated by the closure of the Tethys Sea. Although the species diversity in giant barrel sponges seems cryptic, **Chapter 3** contains a photo with a short description of an observation in Tanzania, demonstrating two color morphologies of giant barrel sponges that correspond with different species identities.

The section *Prokaryotes* discusses the diversity of host associated prokaryotes in the giant barrel sponge species complex. **Chapter 4** shows that the prokaryotic community of giant barrel sponges is more strongly influenced by geography than host phylogeny in the Indo-Pacific. This is also true for the Caribbean, as is shown in **Chapter 5**, but this chapter also confirms the role of host identity as a driver of the prokaryotic community, particularly at smaller spatial scales - including giant barrel sponges - relates to other reef organisms such as corals, algae, holothurians, nudibranchs, sea urchins and sponge denizens. Prokaryotic microorganisms are often shared among multiple coral reef host organisms, and the sponge prokaryote community does not appear to be as sponge-specific as previously thought. The section *Reef interactions examines* two ways in which giant barrel sponges interact with other reef organisms. **Chapter 6** explains how the prokaryotic community of multiple sponge species. **Chapter 7** contains several photos with a short description illustrating how giant barrel sponges in Taiwan can facilitate the recovery of coral fragments after a tropical storm.

Chapter 8 synthesizes the main findings from the previous chapters and provides directions for future research on giant barrel sponges and their role in coral reef ecosystems.

METHODS USED IN THIS THESIS

Mitochondrial genes have proven useful for population genetic and phylogeographic studies as they are maternally inherited, have short coalescence times and are expected to undergo lineage sorting relatively fast (Avise et al. 1987; Palumbi et al. 2001). The results

of population genetic studies in sponges, however, showed low levels of genetic variation between populations with the standard mitochondrial DNA barcoding primers (Folmer et al. 1994), even when populations were more than 7,000 km apart (Duran et al. 2004; Wörheide 2006). Erpenbeck et al. (2006a) found that the I3-M11 partition of the CO1 mitochondrial gene in sponges presented more variability at the species level, making it a more suitable marker for taxonomic studies and DNA barcoding. However, this marker alone did not give enough resolution to fully understand the evolutionary history of giant barrel sponges on a global scale nor on regional scales (Setiawan et al. 2016a). Sponges from the Caribbean and the Indo-Pacific were sharing haplotypes, and the haplotypes from both oceans did not form separate monophyletic lineages (Setiawan et al. 2016a). In the Caribbean, the most pronounced morphologies were represented by different haplotypes of this mitochondrial gene (López-Legentil and Pawlik 2009). Since both haplotypes were also found in specimens presenting the typical vase-shaped morphology with an irregular, rough surface, it was not clear whether these different haplotypes represented different species. The ATP6 mitochondrial gene was found to also be useful in sponges and can be added to other mitochondrial markers to increase the genetic variation (Rua et al. 2011).

Nuclear genetic markers independently evolve from mitochondrial markers and congruency between the two suggests the existence of distinct biological species (Padial et al. 2010). For sponges, including giant barrel sponges, the nuclear gene ATPs provides considerable genetic variation (Bentlage and Wörheide 2007; Setiawan et al. 2016b). Combined sequencing of the I3-M11 partition of the CO1 mitochondrial gene, the ATP6 mitochondrial gene, and the ATPs nuclear gene greatly enhances the opportunities to reconstruct the evolutionary history of giant barrel sponges, especially in combination with morphological and ecological variation. These molecular markers are, together, used in Chapter 2.

Next generation sequencing techniques have allowed for the profiling of microbial communities in all environments, including sponges (Claesson et al. 2010; Buermans and Den Dunnen 2014). Illumina sequencing using the 16S rRNA gene V3V4 hypervariable region has been a preferred method in sponge microbial studies in recent years due to the high output, relatively low price and suitability to assess microbial community structures (Reveillaud et al. 2014; Gaikwad et al. 2016; Thomas et al. 2016). This method has been used in Chapters 4, 5 and 6.

Section one: Genetics