AN INVESTIGATION INTO THE BACTERIAL COMMUNITIES ASSOCIATED WITH PYRROLOIMINOQUINONE PRODUCING SOUTH AFRICAN LATRUNCULID SPONGES.

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List of Abbreviations

BLAST	Basic Local Alignment Sequence Tool
bp	Base pairs
COX 1	Cytochrome C Oxidase subunit I gene
DNA	Deoxyribonucleic acid
gDNA	genomic DNA
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
ROV	Remote Operated Vehicle
rRNA	ribosomal RNA
SCUBA	Self-contained underwater breathing apparatus

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Abstract

Marine sponges belonging to the family Latrunculiidae are known for their production of cytotoxic pyrroloiminoquinone alkaloids and the South African coast provides a unique environment for the exploitation of these potent bioactive compounds. The isolation of structurally similar pyrroloiminoquinone compounds from unrelated, non poriferan sources has led to the suggestion that South African latrunculid pyrroloiminoquinones may be secondary metabolites produced by sponge associated microbial symbionts. Previous studies investigating the bacterial communities of South African latrunculid sponges have shown the conservation of distinct microbial populations with unusual bacterial taxa dominated by a novel betaproteobacterial and spirochete species. This study describes the further investigation into these associated bacterial communities, their conservation and sponge microbiome comparisons across spatial, temporal and environmental scales. The bacterial communities associated with seven latrunculid species representing three genera (*Tsitsikamma, Cyclacanthia* and *Latrunculia*) were characterized as well as a *Mycale* and *Tethya rubra* species. Latrunculid sponge microbiomes were significantly different from those associated with sympatric outlier sponge species and the surrounding environment. The bacterial communities associated with latrunculid sponges appear host specific with the conservation of two dominant bacterial symbionts which mirror the phylogeny of their host species.

Chapter One

Literature review

1.1 Introduction

The marine environment covers two thirds of the earth's surface, which presents extensive, largely unexplored biologically diverse ecosystems encompassing vast biodiversity and varied environmental conditions (Costello and Chaudhary, 2017). The oceans are home to a multitude of macro- and microorganisms each with different physiology and capacity to adapt to their environment (Abdelmohsen *et al.,* 2014). Some marine organisms not only survive, but have developed the ability to produce unique secondary metabolites making the marine environment an exceptional storehouse of novel bioactive natural compounds (Malve, 2016). With diminishing terrestrial resources and the increasing prevalence of human diseases, the ocean is a promising source for new drug development and human therapeutic applications by harnessing bioactive natural compounds whose structurally and chemically unique features are generally not found in terrestrially derived natural products (Malve, 2016). The discovery of marine natural products has accelerated over the last two decades and among the marine organisms investigated, sessile filter feeding animals, in particular, are the most promising organisms from which bioactive compounds can be sourced due to their exposure to a host of specific physiological, predatory and ecological challenges (Müller, 2004).

Sponges (phylum Porifera) are recognized as prolific sources of bioactive small molecules contributing nearly 30 % of all marine natural products since the first formally reported bioactive nucleoside derivatives isolated from the sponge, *Tethya crypta*, by Bergmann and Feeney in the 1950s (Bergmann and Feeney, 1951). Sponges are considered to be among the most primitive animal lineages with evolutionary roots dating back to over 700 million years ago (Brain et al., 2012; Maloof et al., 2010). Of these earliest metazoans, modern day sponges are sessile, aquatic organisms which, despite a simple body plan lacking in differentiation and specialized organs, are remarkably efficient at filtering nutrients from the surrounding seawater (Van Soest et al., 2012).

According to the World Porifera Database (Van Soest et al., 2016), more than 8500 species have been reported, with the majority classified within the class Demospongiae (demosponges), and the remaining species belonging to the classes Hexactinellida (glass sponges), Calcarea (calcareous sponges) and Homoscleromorpha (previously within Demospongia) according to their body plan and skeletal composition. Sponges are found globally inhabiting a wide variety of marine and freshwater systems throughout tropical, temperate and polar regions (Hooper and van Soest, 2002), and it is the adaptability of their body plan to changes in environmental characteristics and competing biota which is believed to be the link in the continued survival of these exclusively aquatic animals (Müller, 2003).

1.2 Sponge morphology and function

The sponge body plan is regarded as morphologically ultra-conservative as it consists of a basic level of cellular organization that has not changed significantly over the centuries (Hooper & van Soest, 2002). The sponge body is hollow with its organization centred around a system of pores, ostia, canals and chambers in order to pump high volumes of water through its tissues for the supply of all essential exchanges (Van Soest et al., 2012). Sponges lack specialized circulatory, nervous, muscular, digestive, endocrine, reproductive, respiratory and excretory systems as seen in higher organisms, however instead consist of a collection of different cell types and unifying features that are characteristic of the phylum *Porifera* (Hooper & van Soest, 2002; Funayama, 2010). Beyond the basic body plan sponge morphology is highly diverse due to the presence of species related siliceous or calcareous spicules (Erpenbeck et al., 2006) which make up the skeletal component and aid in sponge taxonomy, in combination with collagenous tissues such as spongin which aid in structural support (Taylor et al*.,* 2007).

The sponge basic body plan consists of layers of several different cell types shared among all species (Van Soest et al., 2012). The outermost surface or unicellular exopinacoderm layer, the ectosome, is composed of pinacocytes, which serve as an epithelial layer covering the sponge exterior. Some of these epithelial cells form small ostia, or external pores, through which water passes into the sponge, and others form larger oscules through which water is expelled (Riisgard and Larsen, 2010). Inside the sponge, the choanosome or inner canal system is lined with single-layered, loosely associated porocytes forming the endopinacoderm which serves as a physical barrier separating the inner sponge matrix from the surrounding seawater (Van Soest et al., 2012). Along these canals and inner body wall, 'water pumping stations' lined by specialised, flagellated choanocytes are found which drive the unidirectional pumping of water throughout the organism (Leys et al., 2011). This process generates a current that traps food particles, allows the extraction of nutrients and oxygen from incoming water and expels waste products into the excurrent canals (Riisgard and Larsen, 2010; Leys et al., 2011). The inner, gelatinous matrix bound on all sides by the endopinacoderm is referred to as the mesohyl and is considered the connective tissue of the sponge.

The mesohyl is an acellular matrix of collagen protein with an organic skeleton composed of flexible spongin fibres and/or an inorganic skeleton composed of mineral spicules that provides the structural integrity of sponges and allows for their unique differentiation (Van Soest et al., 2012). Within the mesohyl motile totipotent cells are also found including archaeocytes which digest trapped food particles (including bacteria, microalgae and fungi) via phagocytosis as well other specialised cells such as collencytes, which produce fibres and sclerocytes which produce characteristic spicules (Funayama, 2010).

The phylum Porifera is a paraphyletic grouping subdivided into four classes: Calcarea, Hexactinellida, Homoscleromorpha and Demospongiae, the largest sponge class including 81% of all living sponges worldwide. According to Hooper & Van Soest (2002) the Class Demospongiae was reported to contain three subclasses namely Tetractinomorpha, Ceractinomorpha and Homoscleromorpha. However in later years, Homoscleromorpha was recognized to be distinct from Demospongiae forming the fourth major sponge lineage (Gazave et al., 2010). Furthermore, phylogenetic analyses based on nuclear and mitochondrial markers resulted in the subdivision of Homoscleromorpha into two clades including the

Plankinidae and Oscarellidae (Gazave et al., 2010). Demospongiae is considered the most numerous and diverse class of sponges producing a mineral skeleton generally made up of siliceous spicules and secrete spongin (Erpenbeck and Wörheide, 2007). The Class Calcarea contains two subclasses namely Calcinea and Calcaronea which have calcareous spicules and comprise exclusively marine species (Sethmann and Wörheide, 2008). The class Hexactinellida, often referred to as glass sponges, contains two subclasses, Amphidiscophora and Hexasterophora, and like the Demospongiae, are composed of siliceous spicules. However, the Hexactinellida differ in the number of symmetry axes of their megascleres and comprise mostly-deep sea sponges (Uriz et al., 2003). Regardless of the Class to which sponges belong, secretion of mineral or proteinaceous structures gives these organisms their variety of three-dimensional shapes (Uriz et al., 2003). The variety of mineral skeletons consisting of calcite, aragonite and/ or silica secreted by sponges confers strength, protection from physical perturbation and maximizes the number of cells in contact with the surrounding seawater, minimizing the metabolic cost of water exchange (Uriz, 2006; Riisgard and Larsen, 2010). The size and shape of present day sponge spicules are relatively constant within species and although the evolution of all spicule features is not fully understood (Erpenbeck et al., 2006), the identification of same spicule morphologies across geological times indicates the beneficial adaptation of spicules to the various environmental conditions encountered by sponges.

1.3 Sponge identification and taxonomy

Spicule size, type, shape, combination and skeletal arrangement are unique to sponge species and form the foundation of sponge systematics (Erpenbeck et al., 2006). The unambiguous identification of sponge morphological characters for comparative morphology is known to be scarce and difficult even for taxonomic experts (Wörheide et al., 2008). Homoplasies, ecological plasticity and the highly variable nature of sponge morphology make many species notoriously difficult to identify and present great difficulties for systematics and the classification of sponges (Muricy et al., 1996; Wörheide et al., 2008). While the use of fixed differences in skeletal or diagnostic characters typical of conventional morphological taxonomy have been practical and served reasonably well to catalogue diversity, it is questionable that such a typological system reflects the real biological diversity (Wörheide et al., 2008). Sponge shape, colour, texture or surface often depend on microhabitat conditions or season or are present *in situ* only (Jones, 1984), and although the mineral elements have been studied extensively (Jones, 1997; Hooper and van Soest, 2002; Uriz et al. 2003) these characteristics still appear inefficient in addressing broader phylogenetic questions. Challenges especially arise in the grouping of morphologically similar sponges and the inability to correctly distinguish closely related but evolutionary distinct sponge lineages. As an opportunity to overcome the shortcomings in conventional morphology based taxonomy, DNA has provided a considerable amount of additional phylogenetic information with a growing body of literature in Porifera DNA generated data.

The utilization of additional characters such as DNA barcodes has been established as an aid to complement conventional taxonomy and increase the speed and accuracy of sponge identification included in the Sponge Barcoding Project. This was the first worldwide barcoding effort to provide a comprehensive repository spanning the taxonomic range of Porifera (Erpenbeck et al., 2007 and Wörheide et al., 2008). In animals, the mitochondrial cytochrome oxidase subunit 1(*CO1*) gene is the most commonly used region for molecular identification as it is among the most conserved protein-coding genes within the mitochondrial genome (Deagle et al., 2014). Primers derived from conserved regions are used for polymerase chain reaction (PCR) amplification of the approximately 700-bp *CO1* fragment generating informative sequences for phylogenetic analyses (Folmer et al., 1994). The *CO1* gene is one of the most popular markers for population genetic and phylogeographic studies, however, has been found to have limited phylogenetic resolution at the species level, with the potential that individual mitochondrial genes might provide misleading evolutionary inferences (Carella et al., 2016). A number of other genes have also been proposed for barcoding purposes and include the cytoplasmic 28S and 18s ribosomal ribonucleic acid (rRNA) gene fragments and the nuclear protein elongation factor 1-α (EF1-α) (Deagle et al., 2014). From bacteria to vertebrates, the conserved nature of ribosomal RNA's due their role in protein synthesis, has made these molecules the favourable choice for the reconstruction of molecular phylogenies (Deagle et al., 2014).

Early sequence comparisons of 5S and 5.8S rRNA molecules used in the first phylogenetic studies of sponges and other metazoans appeared to uphold the metazoan nature of Porifera (Dams et al., 1982; Komiya et al., 1983). However, the highly conserved 5S and 5.8S rRNA sequences were recognized to be too short and the level of saturation too high to provide a signal suitable enough to unambiguously resolve phylogenetic relationships (Halanych, 1991). In later years, the 18S rRNA and 28S rRNA genes with longer sequences, were compared to re-evaluate the phylogenetic relationships between sponge genera, families, orders and classes (Kelly-Borges et al., 1991; Christen et al., 1991). Among the partial 18S rRNA sequences used in an attempt to resolve phylogenetic relationships within the Hadromerida and Lithistid sponges belonging to Demospongiae, many were found to be too short (~200-500 bases) with few overlapping regions and the low variability of the partial sequences were not enough to obtain conclusive results limiting their value in sponge phylogeny (Kelly-Borges et al., 1991 and Kelly-Borges & Pomponi, 2004). The data provided by the partial 18S rRNA sequencing showed support for Porifera being true metazoans, but did not provide enough information to resolve relationships between genera, families or orders. Alternatively, the 5' terminal domain of the 28S rRNA molecule was more informative, providing greater variability with the resolution of the 28S rRNA phylogenetic trees higher than that obtained with the 18S rRNA sequences (Christen et al., 1991 and Lafay et al., 1992). At the 5' extremity of the 28S rRNA the divergent D2 domain was found to be better suited for resolving phylogenetic relationships between genera and families and the more conservative C1, D1 and C2 domains better at the order or class level (Chombard et al., 1998). Since the review of 75 28S rRNA partial sequences by Borchiellini et al. in 2000 over 2400 28S rRNA sequences assigned to Porifera are present in Genbank (Thacker et al., 2013). However, despite this fast progress public data sets still remain, to a large extent, incomplete.

1.4 Diversity of sponge-associated microbial consortia

Most marine sponges have established persistent associations with a wide variety of microorganisms, predominantly bacteria but also including archaea and fungi providing a preferable ecological niche over the nutrient-poor seawater (Schmitt et al., 2012; Webster and Taylor, 2012; Moitinho-Silva et al., 2017). In addition to transient microorganisms acquired from the surrounding water as a food source, sponges harbour large quantities of bacteria which can constitute up to 40% of the host sponge biomass at densities in excess of 10^9 microbial cells per cubic centimetre of sponge tissue (Taylor et al., 2007; Vacelet and Donadey, 1997; Webster and Taylor, 2012). This is two to four orders of magnitude greater than the density typically found in the surrounding seawater and while sponges can host enormous densities of microbial symbionts, not all species harbour such high microbial abundance (Weisz et al., 2008). Based on the density of the microbial communities they host, sponges can be divided into high microbial abundance HMA and low microbial abundance LMA species each with different implications for sponge biology, physiology and ecology (Weisz et al., 2008; Taylor, 2007).

Bacterial load within sponges is considered to be proportionally correlated with the irrigation status of the sponge which is affected by parameters such as complexity and size of the canal system, ostia size and length, as well as surface:volume ratio (Wilkinson, 1978). Poorly irrigated sponges, such as HMA species, create internal environments that foster microbial diversity and high microbial abundance due to their dense mesohyl body plan and complex aquaferous system of longer, narrower canals, which necessitates slower seawater filtration rates, increasing contact time with particulate organic matter (POM) (Boury-Esnault et al., 1990; Vacelet and Donadey, 1997; Weisz et al., 2008). In contrast, the well-irrigated LMA sponge body plan moves large quantities of water through their porous tissues rapidly acquiring POM independent of associated microorganisms (Boury-Esnault et al., 1990; Hentschel et al., 2003; Weisz et al., 2008). The two evolutionary patterns of morphological and physiological differences, have led to two different life strategies involving or independent of close associations with microbial symbionts illustrating how sponge bacterial symbionts can potentially have substantial effects on host evolution (Wilkinson, 1983; Hentschel et al., 2003; Weisz et al., 2008).

Collectively, marine sponges and their associated microbial consortia are described as ‘holobionts’ and the complexity of these relationships can range from one single dominant symbiont to associations with hundreds of symbiont species (Webster and Taylor, 2012). Sponge-associated microbial populations generally occupy the sponge extracellular matrix or mesohyl, however, some symbionts exist intracellularly inhabiting cell types within the body of the sponge (Vacelet and Donadey, 1977; Wilkinson, 1978; Hentschel et al., 2002 and Taylor et al., 2007; Garate et al., 2017). Bacterial distribution follows a general pattern within every sponge, photosynthetically active cyanobacteria and eukaryotic algae are usually located in the light exposed or outer tissue layers of the sponge, whereas heterotrophic, and possibly autotrophic, microorganisms colonize the inner core of the sponge (Hentschel et al., 2003; Keren et al., 2017). These microbial symbionts make important contributions to many aspects of the sponge's physiology and ecology, including nutrient acquisition (Reiswig, 1975; Vacelet et al., 1995; Wehrl et al., 2007; Thomas et al., 2010), processing of metabolic waste (Bergquist, 1978; Beer and Ilan, 1998; Hentschel, 2002), skeletal support (Hentschel, 2002), and secondary metabolite production in the form of chemical defence (Bewley et al., 1996; Schmidt et al., 2000; Unson et al., 1994; Taylor, 2007; Macintyre et al., 2014; Mohan et al., 2016). Sponge nutrition and health rely on these symbiotic microorganisms, which in turn, receive a nutrient rich habitat with oxygen and organic compounds as well as protection provided by the sponge host (Wilkinson, 1978; Olson and McCarthy, 2005).

The mesohyl is both a site for digestion of microorganisms and a home to extensive symbiotic communities. The presence of these symbionts within this niche is believed to indicate either protection of symbiont microbes against digestion, or the recognition of certain microbial types by the sponge cells (Vacelet and Donadey, 1977; Wilkinson, 1978; Wehrl et al., 2007). Thickened bacterial cell walls and slime capsules revealed by electron microscopy studies were thought to serve as barriers in preventing phagocytosis by sponge archaeocytes (Friedrich et al., 1999). However, a study investigating the uptake rates of bacterial isolates in the sponge *Aplysina aerophoba,* revealed the ability to distinguish between food bacteria and bacterial symbionts by the sponge host itself (Friedrich et al., 1999; Wilkinson et al., 1984; Wehrl et al., 2007). Symbiont proteins containing eukaryotic domains have been recognized as potential molecular mechanisms in the mediation of sponge-symbiont interactions including the establishment of an intracellular lifestyle (Nguyen et al., 2013). These eukaryotic-like protein (ELP) domains are considered to mediate the protein-protein interactions in many biological processes, and metagenomic analyses have shown sponge microbial symbionts to contain an abundance of genes encoding ELPs. Ankyrin repeat proteins (ARPs), tetratricopeptide repeat proteins (TPRs), and leucine-rich repeat proteins (LRPs) are amongst the abundant ELP encoding genes contained by sponge microbial symbionts,

Cultivation of bacteria provides access to genetic and biochemical characteristics of sponge symbionts, however most symbionts are challenging to culture independently from their sponge host rendering investigations regarding the nature of these symbiotic relationships and their exact functional roles difficult to fully determine (Handelsman, 2004). Physiologically and phylogenetically diverse sponge-associated microbes have been identified using cultivation-dependent methods however, these methods have largely been limited in the contribution to microbial diversity due to cultivation processes and the concern of bias selection under artificial culture conditions (Webster and Hill, 2001; Hentschel et al., 2001).

Since the majority of microorganisms seem unable to grow under artificial conditions, the use of molecular tools such as 16S rRNA has provided a culture-independent means to assess diversity. Widely recognised as the ‘gold standard’ for bacterial identification, the 16S rRNA genes comprise of highly conserved and hypervariable regions whose substitution rate and genetic differences serve as reliable phylogenetic markers for the assessment of relatedness between sponge-associated microbes (Rzhetsky, 1995; Bansal and Meyer, 2002). Cloning and sequencing of 16S rRNA genes provides data used to describe complete microbial community compositions making it possible to obtain detailed phylogenetic information on the complex microbial consortia associated with sponges (Hentschel et al., 2002). Based on both cultivation- independent and dependent techniques, 28 bacterial phyla including formally described and candidate phyla have been recorded to be associated with sponges (Simister et al., 2012; Hentschel et al., 2002).

Studies that compared microbial communities from closely related sponges found at different locations, and phylogenetically distant sponges found in the same location, confirmed a ‘core microbial community’ consistently present under various environmental and time conditions (Hentschel et al., 2002; Webster et al., 2010; Schmitt et al., 2012; Jeong et al., 2013; Montalvo et al., 2014; Matcher et al., 2017). This core microbial community includes Proteobacteria (Alpha, Beta, Gamma and

Delta), Planctomycetes, Actinobacteria, Acidobacteria, Bacteriodetes, Chloroflexi, Cyanobacteria, Nitospira and firmicutes and the degree to which these microorganisms seem host specific varies greatly among phyla (Vacelet and Donadey, 1977; Webster et al*.,* 2001; Simister et al., 2012; Hentschel et al., 2012; Walmsley, 2012).

The term 'sponge-specific' was described by Hentschel et al., (2002) who showed conservation among sponge-associated bacterial communities regardless of their origin host sponge and/or sampling location. A particular study by Althoff et al., (1998) involving specimens of the marine sponge *Halichondria panacea* found to be common to the North Sea, Adriatic Sea and the Baltic Sea, collected at these different locations, found housed within the mesohyl the same genera of bacteria dominated by the genus *Rhodobacter* suggesting symbiotic relations between the bacteria and host . Both cultivation-based and molecular methods have further reported the phylogenetic signature of the sponge-associated microbial consortium is distinctly different from that of open seawater, with many known sponge-associated microbes only occurring within sponges (Wilkinson, 1978; Santavy and Colwell, 1990; Hentschel et al., 2002; Olson and McCarthy, 2005; Hill et al., 2006; Taylor et al., 2007; Waterworth et al., 2017).

Spatially and temporally stable microbial communities in multiple sponge species across geographic distances, seasonal times and depth appear to indicate that sponge-associated microbial communities are indeed unique and at least partially sponge specific (Hentschel et al., 2002; Lafi et al., 2009; Montalvo et al., 2005; Webster et al., 2010). Microbial communities differ in both quantitative and qualitative composition, and although the numbers of 16S rRNA gene sequences have increased since the sponge-derived clusters defined by Hentschel et al., (2002), this number only reflects a minor proportion of the expected microbial diversity. Sponge derived 16S rRNA sequences are usually deposited in public databases making the quality of the 16S rRNA comparative sequence analyses, and the resulting phylogenetic affiliation, highly dependent and in many cases limited by the dataset available. Gene sequences encoding rRNAs have however, provided a basis for estimating microbial phylogenetic diversity in turn generating taxonomic inventories of marine microbial populations.

The development of next generation sequencing technologies such as 454 pyrosequencing has provided greater insight into the immensely diverse sponge-associated bacterial communities with its ability to generate extremely large amounts of sequence information. A study by Sogin et al., (2006) indicated that true microbial diversity has been underestimated by previous molecular methods, and that dominant populations have often masked the detection and diversity of low-abundance Operational taxonomic units (OTUs). More recently, analysis of deep sequence datasets by Taylor et al., (2013) reported ‘sponge-specific’ clusters present in marine seawater and sediments, although at significantly lower abundances. In addition to providing evidence for the presence of ‘so-called sponge-specific’ bacteria in other marine environments Taylor et al., (2013) further confirmed more than half of the previously defined sponge-specific clusters were indeed putatively sponge-specific. Even with the assistance of molecular techniques it can be extremely difficult to distinguish between seawater-derived contaminants and true ‘sponge-associated bacteria’, and the detection of an organisms DNA in a sample does not necessarily equate with *in situ* activity but may in theory reflect inactive dispersal stages for these bacteria (Webster 2010; Taylor et al., 2013).

1.5 Sponge bacterial symbionts

So how do sponges acquire the majority of their symbionts? Symbioses through host generations are maintained by symbiont transmission, which has a pivotal role in their evolution. Sponges can reproduce both sexually and asexually and many of these sponge symbiotic bacteria are believed to be obtained both from mother sponge to their offspring via vertical transmission, as well as from the surrounding environment via horizontal transmission (Webster and Thomas, 2016). Documented studies (Sciscioli et al., 1994; Schmidtt et al., 2007; Sharp et al., 2007; Steger et al., 2008; Waterworth et al., 2017) highlighted vertical transmission as a mechanism of maintaining microbial assemblages. Fragmentation, budding or the production of gemmules can effectively transfer sponge symbionts of the asexual parent sponge to their progeny in contrast to the release of gametes or fully developed larvae in respective viviparous and oviparous sponges who reproduce sexually (Hentschel et al., 2002; Sciscioli et al., 1994; Ereskovsky et al., 2005; Schmidtt et al., 2007).

Vertical transmission provides the host with symbiont assurance necessary for fitness however this may result in the reduction of the symbiont genome and potential metabolic activity, which may affect progeny whose larval distribution settles in a habitat which is suboptimal for the symbiont (Moran, 2002). In addition to, or as an alternative, sponge hosts may acquire their symbionts from their surrounding environment via horizontal transmission (Taylor et al., 2007; Sipkema et al., 2015). Sponges seem to be particularly fit for environmental acquisition of their associated microorganisms, although this method poses risks including availability of symbionts within the surrounding environment and exploitation by infectious or pathogenic strains (Taylor et al., 2007; Schmitt et al., 2012). As previously mentioned, shared microbiomes have been found in distantly related sponges across isolated geographical regions, and while combined vertical and horizontal symbiont transmission may aid in explaining this occurrence it is unclear as to what degree each method acts upon the hologenome, or the additional contribution by the symbionts themselves in establishing and maintaining these relationships (Webster and Thomas, 2016).

1.6 Sponges as an important source of biologically active marine natural products

Natural products have played crucial roles in healthcare and therapeutics throughout history and have been the most successful source of potential drug leads due to their unique structural diversity. The majority of current natural product-derived therapeutics from aspirin to morphine have terrestrial origins however, extensive exploration of the terrestrial environment has emphasized the need for novel sources of biologically active natural products (Newman and Cragg, 2012; Malve, 2016). The unique adaptations harnessed by marine life in addition to the genetically and chemically diverse ecological resources of the sea, provides a promising source for new drug development (Malve, 2016). In sharp contrast to the terrestrial environment where production of bioactive natural products by plants far exceeds that of animals, natural products from the marine environment, under clinical evaluation, are predominantly produced by invertebrates such as sponges, bryozoans, tunicates and molluscs, and not by algae (Newman and Cragg, 2012). The wealth of bioactive metabolites isolated from soft-bodied, sessile or slow-moving marine invertebrates who lack physical defence structures is no co-incidence, but rather a reflection of the ecological importance potent chemical defence mechanisms play in their survival (Taylor et al., 2007; Montaser and Luesch, 2011; Dias et al., 2012).

Pioneer work by the researchers Bergmann and Feeney in 1951 initiated marine natural product chemistry with the search for biologically active marine natural products, and by the early 1960s, chemical studies were the focus of marine natural product research with few compounds tested for any relevant biological activity (Bergmann and Feeney, 1951; Weinheimer and Spraggins, 1969; Carté, 1996). A decade later, the biomedical potential of marine natural products became apparent and in the 1980s the first marine metabolites entered preclinical studies and clinical trials after effective collaborations between marine chemists and pharmacologists (Tachibana et al., 1981; Faulkner, 1984; Carté, 1996; Munro et al., 1999).

The antitumor, antimicrobial, antiviral, anti-inflammatory and cytotoxic properties of marine natural products highlights their importance as lead compounds for the pharmaceutical industry with two lead drugs developed in the nineteenth century still in use today for the treatment of leukemia, lymphoma and as an antiviral (Murti and Agrawal, 2010; Christensen and Martin, 2016). The discovery of marine natural products has accelerated over the past two decades producing promising active anti-cancer compounds including sarcodictyin (*Stonlonigeran* coral), eleutherobin (*Eleutherobia* corals), bryostatins (bryozoan- *Bugula neritine*), cytarabine (sponge- *tectitethya crypta*) and eribulin (sponge- *Halichondria*) (Murti and Agrawal, 2010; Newman and Cragg 2012) and the high level of cytotoxicity exhibited by these compounds has made marine invertebrates a preferred source of bioactive natural products (Munro et al., 1999; Huyck, 2011).

Since the 1960s over 20 000 marine natural products have been discovered including various terpenoids, steroids, alkaloids, ethers, phenols and peptides (Simmons et al., 2005; Hu et al., 2011). These chemicals are generally secondary metabolites which are uniquely produced with a variety of bound halogen atoms, such as bromine and chlorine, which are believed to be readily available within the surrounding seawater (Simmons et al., 2005). Most of these biologically active compounds have been isolated from sponges (Faulkner, 2000; Taylor et al, 2007;

Leal et al., 2012; Mehbub et al., 2014), with more than 200 new compounds reported each year for the last decade, contributing to nearly 30% of all marine natural products discovered so far. SCUBA and new diving techniques, including remote operated machines, have made previously inaccessible depths routinely available aiding in the isolation of thousands of structurally unique bioactive compounds (Newman and Cragg, 2012). However, although promising activity against human infection and diseases has been shown in a number of these compounds from marine sponges as potential drug candidates, further development has been largely restricted due to limitations in natural product supply (Prosksch et al., 2002; Gerwick et al., 2012; Newman and Cragg, 2012; Mehbub et al., 2014).

Chemical extractions of natural products often yield low quantities below milligrams per kilogram of invertebrate mass, which may be sufficient for chemical characterization and preliminary evaluation, although not sustainable for pharmacological assays or clinical trials (Thoms and Schupp, 2005; Gerwick et al., 2012). An example of this was seen in the production of halichondrins isolated from the marine sponge *Halichondria okadai* which were identified as potent antitumor compounds with enormous potential (Hirata and Uemura, 1986). It was estimated that in order to obtain a 300mg sample of the halichondrin analogs over 1 ton of sponge biomass was needed to be harvested which, in addition to the environmental impact of harvesting vast amounts of sponge material from the ocean, was simply not feasible (Munro et al., 1999; Hart et al., 2000; Prosksch et al., 2002). Alternative strategies to address the supply problem have included chemical synthesis, ex situ culture and mariculture which have involved various challenges including molecular complexity and economic feasibility, inability to replicate optimum cultivation parameters and control of environmental parameters respectively (Proksch et al., 2003; Sipkema et al., 2004; Thoms and Schupp, 2005).

For many years the presence of microorganisms in marine invertebrates was known except considered to be of little significance until observations by Perry et al. (1988) suggested the toxin pederin isolated from the South American beetle *Paederus fucipes* shared similarities with the antiviral compound mycalamide A and B from the New Zealand sponge *Mycale hentscheli* (Perry et al., 1988). These findings showed remarkably close similarity between compounds from completely different taxa which led to the suggestion of a common microbial origin (Perry et al., 1988). Striking structural similarities between natural products from marine invertebrates such as sponges and known microbial metabolites further suggested that microorganisms were the true sources of such products, or are at least involved in their biosynthesis, which could have far reaching consequences for the development of sustainable fermentation-based drug candidates (Fenical, 1993; Bernan et al*.,* 1997; Prosksch et al., 2002; Clardy, 2005; Newman and Hill, 2005; Thomas et al., 2010).

1.7 Bioactive marine natural products of the Latrunculiidae sponge family

Sponges belonging to the family Latrunculiidae (Demospongiae, Poecilosclerida) are known for their production of bioactive pyrroloiminoquinone alkaloids (Antunes et al., 2005). The family comprises five genera, four of which, *Strongylodesma, Latrunculia, Cyclacanthia* and *Tsitsikamma* are endemic to the south-eastern coast of South Africa (Antunes et al., 2004; van Soest, 2015). Latrunculid sponges are characterised as large, encrusting, hemispherical, spherical or pedunculated sponges with slightly raised, lightened and rounded areas known as areolate porefields (Parker-nance unpublished data). These sponges are described to have a leathery texture and range in colour from brownish black with tinges of green or blue to dark brown and even pale beige (Parker-nance unpublished data). Latrunculid sponges are found in polar and warm temperate regions and their distribution ranges from coastal sublittoral zones which are exposed to sunlight and significant tidal flows to the bathybenthic or deep sea zone (Samaai and Kelly, 2002). Southern hemisphere latrunculid sponges are typically are found in cold waters among sheltered rocky reef environments down to a depth of 50m are seen to be abundant off the temperate South African coastline which is rich in species diversity and largely unexplored (Antunes, 2004).

Latrunculid sponges are known for their production of various pyrroloiminoquinone alkaloids including discorhabdins, makaluvamines and tsitsikammamines which are of great interest due to their therapeutic potential (Antunes et al., 2005). The unique fused ring skeletons of these alkaloids carry interesting properties and a variety of biological activities including inhibition of topoisomerase I and II, antifungal and antimicrobial properties, as well as cytotoxicity against various tumour cell lines (Sun et al., 1990; Ding et al., 1999; Antunes et al., 2005; Gunasekera et al., 2003; Dijoux et a., 2005; Davis et al., 2012). Endemic to the coast of South Africa, the sponge genus *Tsitsikamma* comprised of *Tstitsikamma favus, Tsitsikamma scurra* and *Tsitsikamma pedunculata* that produce pyrroloiminoquinone secondary metabolites (Antunes et al., 2004). Whilst discorhabdins are common throughout the Latrunculiidae sponges*,* *Tsitsikamma favus* has been found to be the only sponge species from which Tsitsikammamines have been isolated (Antunes et al., 2004; Walmsley, 2012). The presence of pyrroloiminoquinones has not only been limited to latrunculid sponges but has in fact, been reported in other non-sponge sources. A related bis-pyrroloiminiquinone, Wakayin, with structural similarity to the Tsitsikammamines, was isolated from the Fijian ascidian *Clavelina sp*, and other non-sponge isolated pyrroloiminoquinones such as Makaluvamine A, isolated from the myxomycete *Didymnium bahiense* and lymphostin isolated from *Streptomyces* spp. and Salinispora spp. have suggested a microbial biosynthetic origin (Nagata et al., 1997; Ishibashi et al., 2001; Copp et al., 2002; Hughs et al., 2009; Miyanaga et al., 2011).

A study by Walmsley et al. (2012) hypothesized one or a group of closely related sponge-associated microbial symbionts are the producers of the pyrroloiminiquinone secondary metabolites, tsitsikammamines. Denaturing gradient gel electrophoresis (DGGE) analyses revealed that *Tsitsikamma favus* contained a highly conserved bacterial community which was confirmed by both clonal libraries of full length 16S rRNA and 454 pyrosequencing Walmsley et al. (2012). *Tsitsikamma favus* was also dominated by a unique Betaproteobacterium with a microbial community that largely did not cluster with other sponge-associated sequences. Previous characterization of the bacterial communities associated with six latrunculid species in a study by Matcher et al. (2017) showed the unprecedented conservation of dominant Betaproteobacteria and Spirochaetae symbionts within the *Latrunculiidae* family of marine sponges. Furthermore, Matcher et al. (2017) provided experimental evidence supporting the proposal that the microbiomes associated with different latrunculid sponge taxa were species specific. The abovementioned studies provided motivation for this study and these reported findings emphasised the need to expand the datasets to incorporate more *Tsitsikamma* species, and to further investigate the unique and unusual bacterial communities associated with latrunculid sponges in comparison to their surrounding environment and outlier sponge species.

1.8 Hypothesis

Latrunculid sponge species producing related secondary metabolites are associated with conserved microbial communities.

1.8 Aim

The aim of the research presented in this thesis was to investigate and characterize the bacterial communities associated with South African latrunculid sponges.

1.10 Research Objectives

1. To collect and taxonomically identify latrunculid sponge species native to the Agulhas bioregion
2. To characterise the latrunculid sponge-associated bacterial communities and compare them with their surrounding environment
3. To compare the bacterial communities associated with latrunculid sponges versus outlier sponge species

Chapter Two

Methodology and materials

2.1 Sponge collection

Marine sponge specimens were collected by Self-Contained Underwater Breathing Apparatus (SCUBA) diving or Remote Operated Vehicle (ROV) provided by the African Coelacanth Ecosystem Programme (ACEP) Infrastructure Platform within the Agulhas Bioregion, South Africa. Specimens were collected between May 2009 and June 2016 from Evans Peak, RIY Banks reefs in Algoa Bay and Rheeders Reef in the Tsitsikamma Marine Protected Area (Fig. 2.1.). Sponge specimens were collected at depths of 20-50 m (Table 2.1.) each in a separate Ziploc bag. Seawater and sediment samples were collected from their immediate vicinity (Table 2.2.). Individual sponge samples were stored at 4°C before being processed in the laboratory within 3 hours of collection.

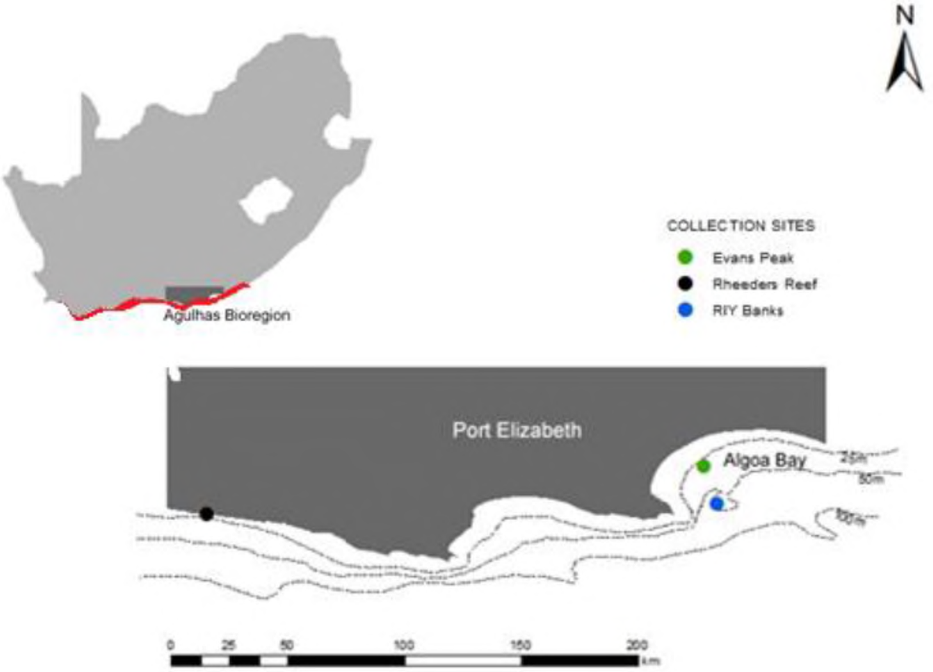


Figure 2.1: Geographic location of sponge collection sites within the Agulhas bioregion.

Table 2.1: Taxonomic identification and collection data for sponge specimens used in this study.

Taxonomic Identification	Collection number	Collection site	Collection date (year)	Depth (m)	DNA extraction method
Tsitsikamma favus	TIC2015-013	Evans Peak 33°99.817S;25°70.988E	Aug-15	20	1
	TIC2015-025	Evans Peak 33°84.548S;25°81.663E	Sep-15	30	1
	TIC2015-026	Evans Peak 33°84.548S;25°81.663E	Sep-15	30	1
	TIC2015-027	Evans Peak 33°84.548S;25°81.663E	Sep-15	30	1
	TIC2016-050A	Evans Peak 33°84.548S;25°81.663E	Jun-16	20	1
	TIC2009-002[a]	Evans Peak 33°50.578S;25°48.988E	May-09	30	2
	TIC2012-057	Evans Peak 33°50.578S;25°48.988E	Dec-12	30	2
	TIC2014-001	Evans Peak 33°50.578S;25°48.988E	Aug-14	30	2
Tsitsikamma michaeli	TIC2015-201	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2015-202	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2015-203	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2015-204	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2011-102	Evans Peak 33°50.578S;25°48.988E	Apr-11	30	2
Tsitsikamma nguni	TIC2015-003	Rheeders Reef 34°02.735S;23°90.468E	Jun-15	20	1
	TIC2015-006	Rheeders Reef 34°02.892S;23°92.928E	Jun-15	20	1
	TIC2010-2B	Evan's Peak 33°50.578S;25°48.988E	May-10	30	2
Tsitsikamma pedunculata	TIC2015-205	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2015-206	Evans Peak 33°84.418S;25°81.522E	Oct-15	30	1
	TIC2015-217	Evans Peak 33°84.548S;25°81.663E	Nov-15	34-38	1
	TIC2015-218	Evans Peak 33°84.548S;25°81.663E	Nov-15	34-38	1
Tsitsikamma sp. 005	TIC2009-070	Evans Peak 33°50.578S;25°48.988E	May-10	30	2
Mycale sp.	TIC2015-204B	Evan's Peak 33°84.42 S;25°81.522E	Oct-15	30	1
	TIC2010-030	Evans Peak 33°50.578S;25°48.988E	May-10	30	2
Cyclacanthia bellae	TIC2012-056	RIY Banks 33°59.960S;25°58.764E	Dec-12	25–30	1
Latrunculia algoaensis[b]	TIC2010-031	Evans Peak 33°50.578S;25°48.988E	May-10	30	1
Tethya rubra	TIC2015-221	Evans Peak 33°50.729S;25°48.998E	Nov-15	30	1
	TIC2015-222	Evans Peak 33°50.729S;25°48.998E	Nov-15	30	1
	TIC2016-001	RIY Banks 34°00.099S;25°51.37E	Feb-16	51	1
	TIC2016-003	RIY Banks 34°00.099S;25°51.373E	Feb-16	51	1

1. ZR Soil Microbe DNA MiniPrep Kit (Zymo D6001)
2. Guanidine thiocyanate (Walmsley et al., 2012)

a) Previously identified as *Tsitsikamma favus* by Walmsley et al. (2012).
b) Morphological description published in Samaai et al. (2012). Collection details in Samaai et al. (2012) are incorrect, corrected in Fig. 2.1 above.

Table 2.2: Collection data for the environmental (seawater and sediment) samples used in this study.

Identification	Sample key	Collection site	Collection date (year)	Depth (m)
Seawater	ESW1	Evans Peak	Aug-14	30
	ESW2	Evans Peak	Jun-16	20
	ESW3	Evans Peak	Jun-16	20
	ESW4	Evans Peak	Aug-14	30
	ESW5	Evans Peak	Nov-15	34
	RSW1	RIY Banks	Mar-16	51
	TSW1	Rheeders Reef	Jun-15	20
	TSW2	Rheeders Reef	Jun-15	20
Sediment	ESED1	Evans Peak	Nov-15	38
	ESED2	Evans Peak	Aug-14	39
	TSED1	Rheeders Reef	Jun-16	20

Each sponge specimen was rinsed with sterile artificial seawater buffer (ASW: 24.6 g NaCl, 0.67 g KCl, 1.36 g $CaCl_2.2H_2O$, 6.29 g $MgSO_4.7H_2O$, 4.66 g $MgCl_2.6H_2O$ and 0.18 g $NaHCO_3$ made up to 1 litre with distilled water) for the removal of transient microorganisms as described in Walmsley et al. (2012). A wedge, extending from the centre to the perimeter of individual sponge specimens, was aseptically cut for genomic DNA extraction. The sediment samples were preserved in RNA-*Later*™ (ThermoFisher Scientific, Johannesburg, South Africa) and stored at -80°C for further processing. Marine microbial biomass was collected through filtration of 2 L seawater through a 0.2 µm polyethersulfone (PES) filter. The filters were preserved in RNA-*Later*™ and stored at -80°C.

2.2 Morphological identification of sponge-specimens

Sponge specimens were morphologically identified by Dr Shirley Parker-Nance of the South African Environmental Observation Network (SAEON) Elwandle Coastal Node, Port Elizabeth. Voucher samples, preserved in ethanol, were deposited at the South African Institute for Aquatic Biodiversity (SAIAB) in Grahamstown, South Africa. The taxonomic identification of sponge specimens was based on the physical morphological characteristics including size, shape, colour, distribution and shape of the oscules and aerolate porefields, as well as the type of spicules found within the sponge tissue (Parker-Nance, Hilliar et al., Manuscript submitted). Photographic records of the *in situ*, freshly collected and preserved specimens were collected and archived with voucher samples. Type specimens and reference material were lodged at the SAIAB.

2.3 Genomic DNA extraction

Sponge samples (1 gram) were homogenized individually using a sterile mortar and pestle with 3 ml ASW for 5 min to release the sponge-associated microorganisms. The homogenized sponge tissue was transferred to separate 1.5 ml Eppendorf tubes and pelleted by centrifugation at 15 000 x g for 1 min to be used for gDNA extractions. Total genomic DNA (gDNA) was extracted from the homogenized sponge samples using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Cat. no. D6001) according to the manufacturers protocol yielding on average 100 µl purified gDNA for use in PCR amplification. Microbial DNA from each seawater and sediment sample collected was extracted using the PowerWater DNA Isolation kit (MoBio, San Diego, CA, USA, Cat. No. 14900-S) and the ZR Soil Microbe DNA MiniPrep kit (Zymo Research) respectively. Quality and quantity of extractions were assessed by measuring DNA concentrations and optical absorbance ratios (260 nm) using a NanoDrop lite spectrophotometer (Thermo Scientific), as well as by visualizing an aliquot of the extraction following agarose gel electrophoresis (1% TAE agarose gel).

2.4 Molecular identification of the partial 28S rRNA gene

The molecular identification of each sponge specimen was confirmed by analysis of the 28S rRNA gene sequences. The primers RD3A (forward) (5‘-GAC CCG TCT TGA AAC ACG A-3‘) and RD5B2 (reverse) (5‘-ACA CAC TCC TTA GCG GA-3‘) (Walmsley et al., 2012) were used to amplify the 625 nt D3 - D5 region of the 28S rRNA gene. Each PCR amplification was performed using 0.5 U Kapa HiFi HotStart Taq Polymerase (KAPA Biosystems) in a total reaction volume of 25 µl, including 0.3 µM of each primer, 0.75 µM dNTPs, 1X Buffer with $MgCl_2$, and 20 ng of DNA template. The thermal cycling parameters were as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles at 94 °C for 30 sec, 45 °C for 20 sec, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. PCR fragments were ligated into the pDrive cloning vector (Qiagen) and transformed into *Escherichia coli* DH5α competent cells. Recombinant plasmids were selected based on restriction analysis using *Bam* HI, *Hind* III and *Sma* I restriction enzymes to select for clones containing inserts, before Sanger sequencing.

2.5 Data curation and phylogenetic analysis of the partial 28S rRNA gene sequences

The 28S rRNA nucleotide sequence of each sponge specimen was aligned using ClustalW (Larkin et al., 2007) and examined for nucleotide polymorphisms between individual samples within the same species and between species. Phylogenetic trees based on the partial 28S rRNA gene sequences were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) 7.0.21 software (Kumar et al., 2016) and the phylogeny was inferred using the Maximum-Likelihood method, with 1000 bootstrap replicates. The analysis involved 29 nucleotide sequences with a total of 629 positions in the final dataset. Comparative sponge sequences were acquired from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). Resultant gene sequences were submitted to Genbank with the accession numbers MG203890, MG203892, KU695576, MG203888, KU695575, MG203888, MG203891, MG203893, MG203894, MG203895, KU695577, KU695579, KU695580, MG203897, MG203896 and KU695578.

2.6 16S rRNA Amplicon Pyrosequencing

For investigation into the bacterial diversity present within each sponge specimen, PCR amplicon libraries of the V4 - V5 hypervariable regions of the 16S rRNA gene were created using the primer pair E517F (5'-CAG CAG CCG CGG TAA-3') and E969-984 (5'-GTA AGG TTC YTC GCG T-3') (Wang *et al.,* 2007; Wang and Qian, 2009) with relevant multiplex identifier (MID) tags for 454 pyrosequencing. Amplification was performed using KAPA HiFi HotStart Taq (KAPA Biosystems), in a reaction volume of 25 µL as per the manufacturer's specifications. Each reaction included 5-10 ng extracted gDNA, 0.3 µM of each primer with PCR buffer containing 2mM additional $MgCl_2$, 10 µM dNTP's and 0.5 units KAPA HiFi HotStart DNA polymerase. Cycling parameters were as follows: Initial denaturation and enzyme activation at 98 °C for 5 min, 5 cycles of 98 °C for 45 sec, 45 °C for 30 sec, 72 °C for 45 sec, followed by 18 cycles of 98 °C for 30 sec, 50 °C for 30 sec, 72 °C for 45 sec. A final extension was done at 72 °C for 5 min. Difficulties in obtaining PCR products from the sponge DNA were frequently experienced possibly due to chemical compounds within the sponge samples that inhibited the PCR process. This was overcome by multiple attempts with adjustments to DNA template concentrations and annealing temperatures. PCR amplification library products were purified using Agencourt AMPure XP beads (Beckman Coulter). Amplicon libraries were sequenced using the GS Junior Titanium Sequencing platform (454 Life Sciences, Roche) as per the manufacturer's specifications.

2.7 Data curation and analysis of the 16S rRNA gene amplicons

Curation of the amplicon library sequence datasets was performed using the Mothur software v.1.34.4 (Schloss et al., 2009) as described in Matcher et al., 2017. Low-quality reads shorter than 250 nt in length, reads including homopolymeric runs greater than 7 nucleotides or ambiguous nucleotides were removed from the respective datasets. The UCHIME algorithm was used for the identification and removal of chimeric reads (Edgar et al., 2011). The Naïve Bayesian classifier was used against the SILVA Bacterial Database (Release version 123) for the classification of sequence reads and the removal of those classified as unknown, chloroplast, mitochondrial, or eukaryotic DNA. Sequence reads were clustered into operational taxonomic units at distance values of 0.03, and unique (i.e. distance value of zero) using a distance matrix (cut-off at 0.15) generated in Mothur. All OTUs with a relative percentage greater than 0.5% of the total bacterial reads were plotted as dominant bacterial populations. PRIMER7 (Quest Research Limited, Auckland, New Zealand) was used to generate the non-metric multidimensional scale (NMDS) plots using count table abundance data (generated using Mothur). This data underwent a square root transformation prior to Bray-Curtis resemblance and ANOSIM analysis (Quest Research Limited, Auckland, New Zealand).

Chapter Three

Results

3.1 Introduction

Sponge species differ in shape, colour, texture and size typical of their morphological plasticity, and even individuals of the same species can differ in their appearance between geographic areas and habitats (Erpenbeck et al., 2006; Wörheide et al., 2008). Sponge identification can be a difficult task given this variability and whilst fixed differences in macro- and micro- morphotraits typical of conventional morphological taxonomy have been the most reliable method of sponge taxonomy, inclusion of molecular identification has aimed to address these challenges and improve the reliability of sponge systematics.

The Latrunculiidae are a family of cold water sponges and four out of the five genera including *Strongylodesma*, *Latrunculia*, *Cyclacanthia* and *Tsitsikamma* include species endemic to the south-eastern coast of South Africa (Antunes et al., 2004; van Soest et al., 2015). Latrunculid sponges contain an abundance of bioactive secondary metabolites including makaluvamines, discorhabdins, damirones, batzellines and tsitsikammamines, which include a distinct pyrroloiminoquinone substructure (Antunes et al., 2004). These pyrroloiminoquinone secondary metabolites are of particular interest due to their potential in therapeutics and the South African coast provides a unique environment for the exploitation of these potent bioactive compounds (Antunes et al., 2005). The genus *Tsitsikamma* is known to be a rich source of the pyrroloiminoquinone bioactive secondary metabolites known as tsitsikammamines which mediate cytotoxicity as inhibitors of topoisomerase I (Antunes et al., 2004; Antunes et al., 2005; Walmsley et al., 2012). Tsitsikamamine A and B have consistently been isolated as the major secondary metabolites unique to *Tsitsikamma favus* sponges and have served as chemotaxonomic markers for this sponge species (Antunes et al., 2005; Walmsley et al., 2012; Kalinski and Dorrington unpublished data).

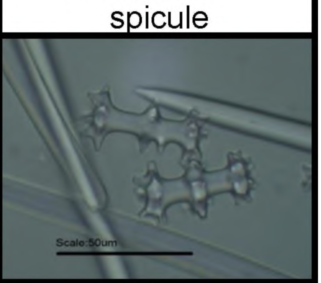
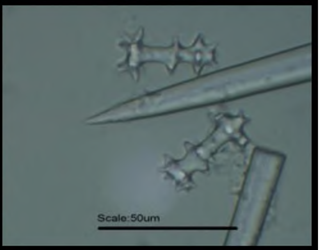
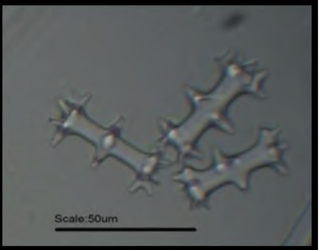
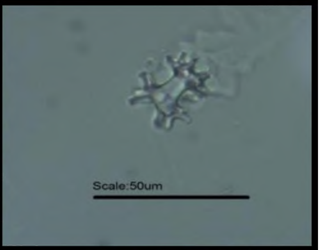
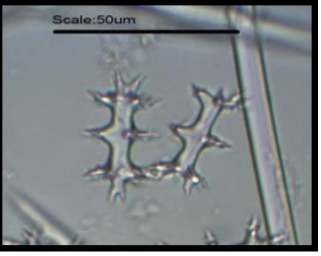
Microbial genome sequencing and genome mining have revealed the genomes of microbial organisms to have great capacity for secondary metabolite biosynthesis and many previously identified marine sponge secondary metabolites have been indicated to be of microbial origin (Ishibashi et al., 2001; Copp et al., 2002; Antunes et al., 2005; Piel, 2006; Muscholl-Silberhorn et al., 2008; Walmsley et al., 2012; Xi et al., 2012; Jordan and Moore et al., 2016). The isolation of the pyrroloiminoquinones Wakayin and Makaluvamine A from sources other than *Porifera* (Copp et al., 2002; Antunes et al., 2005), in addition to their structural similarity to tsitsikammamines has raised the possibility that pyrroloiminiquinones may be secondary metabolites produced by sponge-associated microbial symbionts.

Key studies by Walmsley et al. (2012) and Matcher et al. (2017), focused on characterising the microbial diversity associated with members of the *Tsitsikamma* genus, which provided motivation for this study. These studies emphasised the need to expand the datasets to incorporate more *Tsitsikamma* species to further investigate the unique and unusual bacterial communities associated with latrunculid sponges.

3.2 Sponge taxonomy

At the outset of this study, the genus *Tsitsikamma* comprised three species, namely *T. favus*, *T. pedunculata* and *T. scurra*. Eight *T. favus* and four *T. pedunculata* specimens were collected from Evans Peak in Algoa Bay at depths between 20m to 38m. Freshly collected *T. favus* sponge specimens were firm, hemispherical and dark brown in colour with a tough, leather-like ectosome and cylindrical lance-shaped oscules (Table 3.1.). The choanosome was divided into convoluted, honeycomb-like chambers reinforced by thick tracts which penetrate and divide the sponge interior. Once in ethanol the dark colouration intensified lending the preservative a deep brown/black colouration. *T. pedunculata* sponge specimens were pedunculated with a well-defined head and elongated stalk (Table 3.1.). Specimens were pink brown with sparse, cone shaped oscules on the head. The inner choanosome was soft, the stalk dense and once preserved in ethanol *T. pedunculata* remained a cream to tan colour.

Table 3.1: Voucher samples of five latrunculid sponge species collected for this study.

	1. Sponge specimen	2. *In situ*	3. Sponge spicule
Tsitsikamma favus			
Tsitsikamma michaeli (with yellow encrusting *Mycale* sp.)			
Tsitsikamma nguni			
Tsitsikamma pedunculata			
Cyclacanthia bellae			

Sponge specimen photographic images were taken after sponge collection, and *in situ* images were taken of each sponge species whilst in their natural habitat. Sponge photographic images and spicule analysis was performed by Dr Shirley Parker-Nance of the South African Environmental Observation Network (SAEON) Elwandle Coastal Node, Port Elizabeth.

In this study two new *Tsitsikamma* species, namely *T. michaeli* and *T. nguni*, were identified. The *T. michaeli* sponge specimens collected from Evans Peak at a depth of 30m, were smaller, rounded and dark green in colour with a firm resilient ectosome and small pointed oscules (Table 3.1.). The inner choanosome lacked reinforced tracts, was bright green and once in ethanol, was preserved to an olive to tan colour. *T. michaeli* specimens were often found encrusted with a yellow *Mycale* sp. sponge also known to be found on the surface of some *T. pedunculata* sponges. The *Mycale* sp. specimen was fibrous in texture and easily compressed with furrowed grooves on the surface of the sponge (Table 3.1.). *Tsitsikamma* sp. 005 specimen (Photographic and spicule image unavailable), provisionally considered to be a separate species belonging to the *Tsitsikamma* genus, was forest-green and brown with a tough ectosome similar in appearance to *T. michaeli.*

A second novel *Tsitsikamma* species, *T. nguni*, was also collected and identified during the course of this project. *T. nguni* sponge specimens were rigid, encrusting and irregular shaped with a dark grey/olive- brown exterior covered small knob shaped oscules (Table 3.1.). The softer, olive brown choanosome was divided by thick, cartilaginous-like reinforcing tracts dividing the sponge interior into uneven elongated chambers. Once preserved in ethanol *T. nguni* remained a uniform dark brown colour. Like *T. favus, T. nguni* sponge specimens shared similar partitioning however, the size and shape of the oscules and differing spicule sizes allowed for differentiation.

A specimen from the *Cyclacanthia* genus, *C. bellae*, was also collected. *C. bellae* specimens were compressible, slightly felt-like in texture and emerald green with small, crowded conical oscules (Table 3.1.). Specimens were thin, encrusting and the choanosome interior was brown, remaining so once preserved in ethanol. All species possessed the characteristic “chessman” spicules pertaining to Latrunculiidae shown by the straight shaft, thickened at regular intervals which grow into whorls of spined lobes (Table 3.1.).

Other sponge species, collected by other members of the research group that were included in this study include the *Tethya rubra* (TIC2015-221; TIC2015-222; TIC2016-001; TIC2016-003) (Waterworth et al., 2017), and *Latrunculia algoaensis*

(TIC2010-031) (Samaai et al., 2012; Matcher et al., 2017) specimens collected from Evans Peak and RIY Banks reefs. *T.rubra* specimens were spherical in shape, with an inner choanosome surrounded by a distinct cortex region consisting of large radial megascleres (Waterworth et al., 2017). These sponges were bright orange and contained distinct bright yellow embryos visible against the red coloured endosome. *T. rubra* specimens were firm and incompressible encrusted by ephibionts. The *L. algoaensis* specimen collected at Evans Peak was green, hemispherical in shape, with thick areolate porefields (Samaai et al., 2012).

3.3 Molecular identification of sponge specimens

Alignment of 28S rRNA sequences and phylogenetic analysis showed that all sponge specimens morphologically identified as species within the *Tsitsikamma* genus were clustered together (Fig. 3.1.). The 28S rRNA phylogenetic tree showed that *T. favus* and *T. nguni* are closely related, which is expected as no differences between their nucleotide sequences were observed in the partial 28S rRNA sequences used for this analysis. Similarly, *T. michaeli* and *T. pedunculata* also clustered together in a single clade, with no apparent discrepancies in their 28S rRNA sequences (Fig. 3.1.). *Tsitsikamma* sp. 005 (TIC2015-070), and *C. bellae* (TIC2015-056) cluster with the *Tsitsikamma* species. *Cyclacanthia* is more closely related to species within the *Tsitsikamma* genus, however *L. algoaensis* was more closely related to the *Tsitsikamma* sponges than the outlier *T. rubra* and *Mycale* spp. The outlier sponges *T. rubra* and *Mycale* sp., belonging to the families Tethyidae and Mycalidae respectively, form distinct external branches completely separate from the sponges belonging to Latrunculiidae, representing the different evolutionary lineages. *T. rubra* specimens were included in this study were found to be growing in close proximity to the *Tsitsikamma* sponges and like sponges belonging to the family Latrunculiidae, they have been shown to produce bioactive secondary metabolites (Waterworth et al., 2017) . The *Mycale* specimens were found to be encrusting sponges upon both *T. michaeli* and *T. pedunculata* sponge specimens.

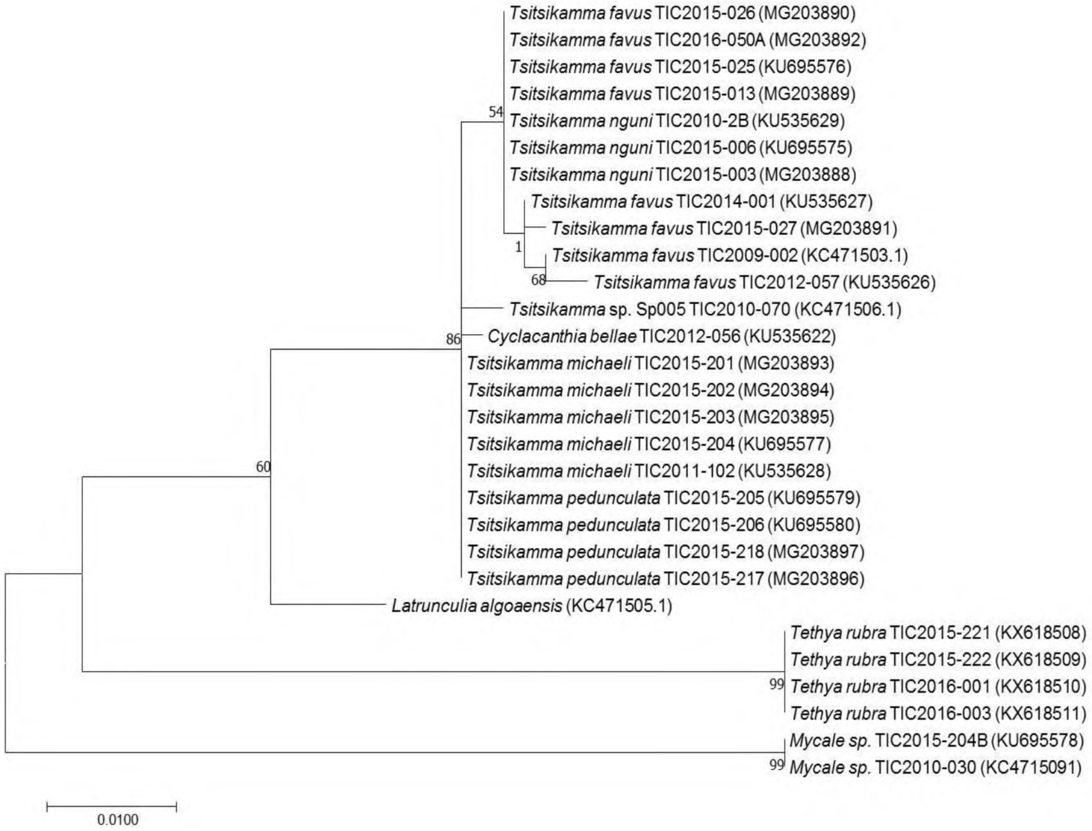


Figure 3.1: Phylogeny of sponge species based on partial 28S rRNA gene sequences. The phylogenetic analysis was generated using MEGA7 software (Kumar, Stetcher and Tamura, 2016) and evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 629 positions in the final dataset.

3.4 Phylogenetic characterisation of sponge-associated versus environmental microbial communities

A study by Walmsley et al. (2012) in particular, which focused on characterising the microbial diversity associated with *T. favus*, showed that specimens of this sponge species contained a distinct and conserved microbial population that was stable over time and dominated by a unique Betaproteobacterium species. The microbial community associated with this species was considered unusual and a study by Matcher et al. (2016) confirmed this finding by expanding the scope to six latrunculid species from the genera *Tsitsikamma*, *Cyclacanthia* and *Latrunculia*. The bacterial communities of 29 sponge specimens, 8 seawater and 3 sediment samples were analysed in this thesis to expand upon the dataset of 9 sponge specimens and 2 environmental samples used in the dataset by Matcher et al., (2016).

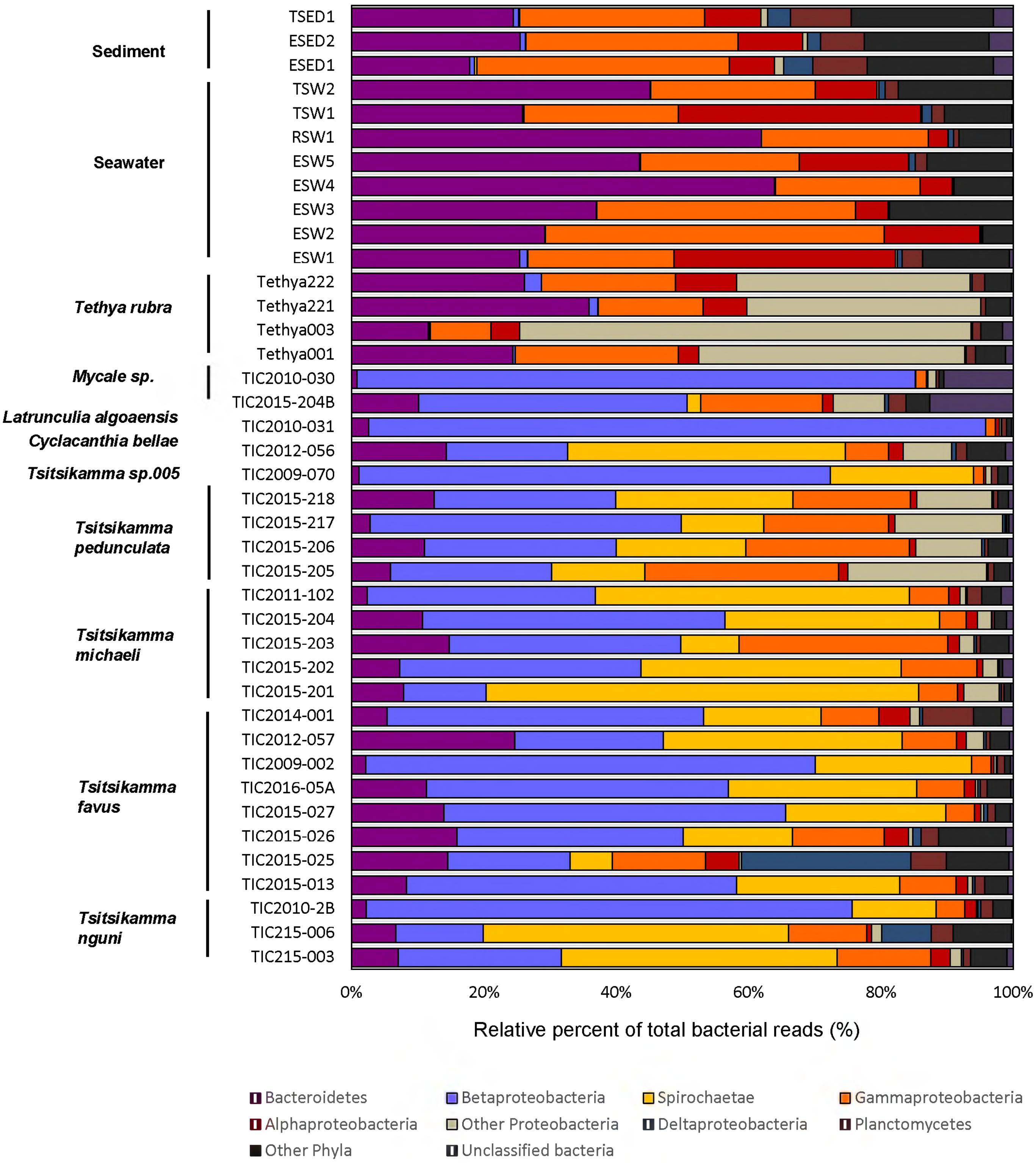


Figure 3.2: Phylogenetic classification (at the level of phylum and subphylum) of the bacterial communities found in sponges, seawater and sediment using Naïve Bayesian classification with the SILVA bacterial database (release version 123) as the reference. Phylogenetic classifications are indicated by a coloured key.

A total of 276 882 sequence reads, spanning the V4-V5 regions of the bacterial 16S rRNA gene of bacterial taxa associated with sponges and the surrounding seawater and sediment, were analysed. Phylogenetic classification of the bacterial communities illustrated a striking difference between the sponge-associated bacterial communities and those in the surrounding seawater and sediment (Fig. 3.2.). The environmental samples, comprising the seawater and sediment, were dominated by Gammaproteobacteria and Bacteroidetes with a high abundance of Alphaproteobacteria. The bacterial community profiles between the seawater and sediment samples were similar due to the presence of shared Bacteroidetes, Gammaproteobacteria and Alphaproteobacteria phyla, however the higher abundance of Planctomycetes present in the sediment samples served as a distinguishing feature between the two environments.

Dominance of the Betaproteobacteria, Spirochaetae and unclassified Proteobacteria ("Other Proteobacteria") phyla in the sponge samples illustrated the distinction of sponges from their environment. Betaproteobacteria were dominant in all three Latrunculiidae genera as well as the *Mycale* sp. sponge associated bacterial communities, accounting for 10% to 93% of the sequence reads obtained. This Betaproteobacterial phylum was absent from the water column and sediment. Distinct from the latrunculid and *Mycale* sp. sponges, *T. rubra*, was dominated by unclassified Proteobacteria with observed similarity with the surrounding environment (Fig. 3.2.). While Betaproteobacteria dominated the majority of *Tsitsikamma* bacterial assemblages, Spirochaetae were also found in high abundance, accounting for 6% to 65% of the reads. Spirochaetae were also found as a dominant phylum in the *Tsitsikamma* and *C. bellae* sponge associated microbiota, but absent in the *L. algoaensis* specimen. Bacteroidetes and Gammaproteobacteria were present to some extent in all sponge associated microbial communities with relatively high abundances of Gammaproteobacteria present in the *T. pedunculata* specimens. Reads classified as Bacteroidetes, Gammaproteobacteria and Alphaproteobacteria were ubiquitous among both sponge and environmental samples (Fig. 3.2.).

The *T. rubra* specimens were dominated by unclassified Proteobacteria ("Other Proteobacteria") based on the classification using SILVA Bacterial Database

(Release version 123) (Fig. 3.2.). However, analysis of the bacterial community sequence reads clustered into OTUs associated with two *T. rubra* populations in a study by Waterworth et al. (2017), identified the reads classified by the SILVA database (Release version 123) as Other Proteobacteria to be a Betaproteobacterium OTU. This finding highlighted the limitations when using a database for bacterial classification and formed part of the motivation to investigate the bacterial communities at the level of OTU in this study. Unlike Genbank, a publicly annotated sequence database, the SILVA Bacterial Database is regularly updated with curated classifications. A challenge with using an online database such as the SILVA Bacterial Database (Release version 123) is that the depth of the database is limited by the number of entries often resulting in the overlooked grouping of “unclassified bacteria”, due to the absence of corresponding sequences to match query sequences. It should be noted that classification according to taxonomic rankings does not necessarily accurately represent the potential diversity present within the particular environment.

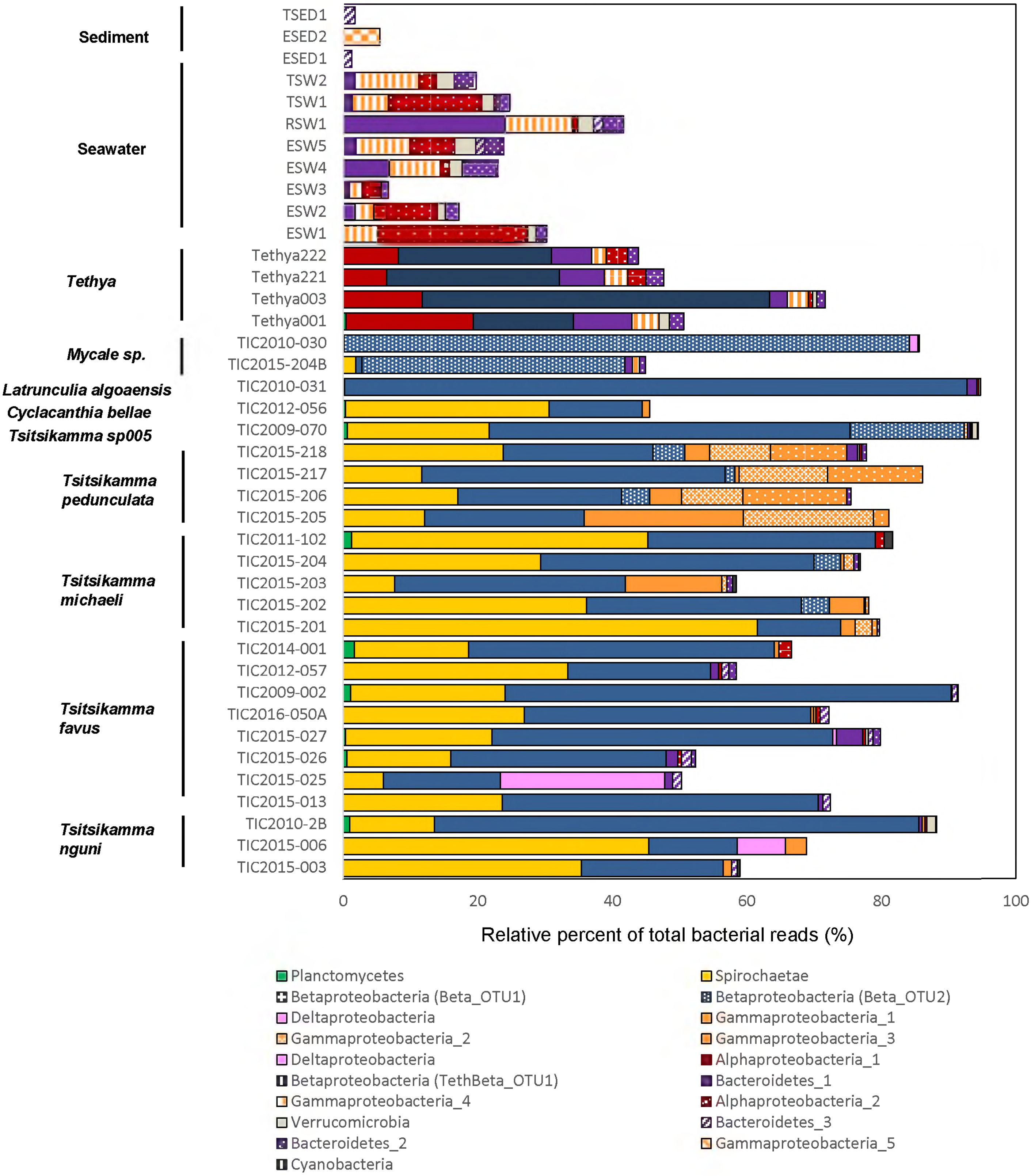


Figure 3.3: Classification of the top 20 dominant OTUs (at a distance of 0.03) found in sponges, seawater and sediment. Read abundance is indicated as the relative percentage of the total bacterial reads analysed for each sample. Classifications of the dominant bacterial OTUs are indicated by a coloured key.

3.5 Analysis of dominant bacterial Operational Taxonomic Units in sponge associated and environmental microbial communities

A total of 286 065 reads were assigned to OTUs at a distance value of 3%. The diversity of bacterial species associated with sponge species was characterized through classification of the top 20 dominant bacterial OTUs (clustered at a distance of 0.03) (Fig. 3.3.). The OTU analysis revealed a similar pattern to that which was observed in the phylogenetic classification. The seawater samples were dominated by the Alphaproteobacteria_2, Bacteroidetes_1 and Gammaproteobacteria_4 OTUs (Fig. 3.3.). The Alphaproteobacteria_2 OTU, accounting for 22% of the reads in the Evans Peak ESW1 seawater sample, was identified as the highly abundant aquatic *Pelagibacter ubique*. Alphaproteobacteria_2 OTU was present in all of the seawater samples, with high abundances in the seawater samples collected from Evans Peak. A low abundance of the Alphaproteobacteria_2 OTU was found to be present in a few sponge specimens from the *Tethya* and *Tsitsikamma* genera. Alphaproteobacteria_2 OTU was completely absent from the sediment samples. A separate Alphaproteobacteria_1 OTU was found to be present in high abundance in the *T. rubra* sponge specimens which, was not present in any other sponge or environmental samples (Fig. 3.3.).

The Gammaproteobacteria_4 OTU was present in all of the seawater samples and *T. rubra* sponge specimens, however absent in both the sediment and remaining sponge species. High abundances of three Gammaproteobacterial OTUs, Gammaproteobacteria_1, Gammaproteobacteria_2 and Gammaproteobacteria_3, were observed in the *T. pedunculata* sponge specimens (Fig. 3.3.). These Gammaproteobacterial OTUs were also found to be present in the remaining *Tsitsikamma* sponge species however in very low abundances. Gammaproteobacteria_1, Gammaproteobacteria_2 and Gammaproteobacteria_3 were absent from both the seawater and sediment samples, indicating they are sponge specific and different from the Gammaproteobacteria_4 OTU present in the seawater samples. Two Bacteroidetes OTUs, Bacteroidetes_1 and Bacteroidetes_2, were present in the seawater samples and *Tethya rubra* sponge specimens, however absent from the sediment samples (Fig. 3.3.). Bacteroidetes_1 and Bacteroidetes_2 were observed in few specimens belonging to the remaining sponge species however, at very low levels. Bacteroidetes_3 and Gammaproteobacteria_5 OTUs, which dominated the sediment samples, were observed to be sediment specific as they were absent from all of seawater samples and sponge specimens.

Numerically, the sponges belonging to Latrunculiidae were dominated by the same betaproteobacterial OTU (B_OTU1) accounting for 13% to 72% in *T. nguni* and 17% to 66% of the sequence reads from specimens of *T. favus* (Fig. 3.3.). Generally B_OTU1 was less abundant in *T. michaeli* and *T. pedunculata* specimens, but very dominant in the *Tsitsikamma* sp. 005 and *L. algoaensis* specimens (54% and 92%, respectively). B_OTU1 was absent in the sediment samples, however it was found to be present in one seawater sample at a low levels below the 0.5% read cut off for this analysis. It is believed that the seawater sample was contaminated by *T. favus* sponge material during sampling (Matcher et al., 2017).

A second Betaproteobacteria OTU (B_OTU2) was dominant in the two *Mycale* sp. specimens, accounting for 39% and 84% of the reads, respectively. B_OTU2 shared 95% sequence identity with B_OTU1. B_OTU2 reads were also detected in the *Tsitsikamma* sp. 005 specimen and specimens from *T. michaeli* and *T. pedunculata* (Fig. 3.3.). Since B_OTU2 was only found in *T. pedunculata* and *T. michaeli* sponge specimens that had been collected with the *Mycale* sp. encrusting sponge, it is likely that the presence of this OTU is due to contamination that occurred during processing of sponge specimens after collection. A Betaproteobacteria OTU (TethBeta_OTU1) was dominant in the *T. rubra* sponges. With the exception of a single water sample collected with the *T. favus* sponge specimen TIC2014-001, no reads corresponding to the three betaproteobacterial OTUs, B_OTU1, B_OTU2 and TethBeta_OTU1, found in the latrunculid and *T. rubra* sponge specimens respectively, were detected in the seawater and sediment samples indicating they are sponge specific (Fig. 3.3.). Furthermore, the Betaproteobacterial OTUs, B_OTU1 and TethBeta_OTU1, appear to be specific to latrunculid and Tethya sponges respectively.

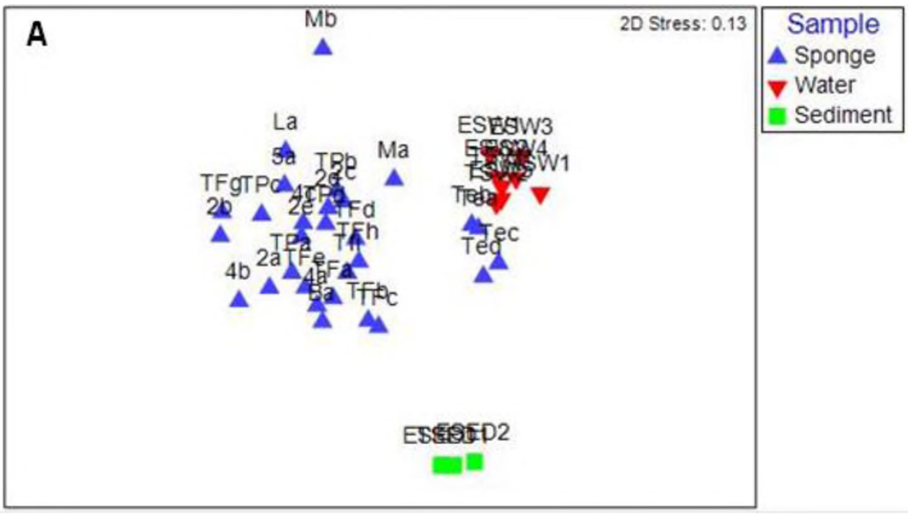
In the sponges, the Spirochaetae were represented by a single OTU that were dominant in *Tsitsikamma* and *Cyclacanthia* species (Fig. 3.3.). This OTU accounted for between 12% to 45% in *T. nguni* specimens, 6% to 36% in *T. favus* specimens,8% to 62% in *T. michaeli* specimens, 12% to 24% in *T. pedunculata* specimens and 30% in *C. bellae*. The shared OTU present in the *Tsitsikamma* and *Cyclacanthia* sponges represents a special relationship which may likely be representative of a specialized function in the bacterial communities associated with these sponges.

3.6 Nonmetric multidimensional scaling comparison of sponge associated vs environmental bacterial communities

A previous study by Matcher et al. (2017) showed that all latrunculid sponge microbial community profiles investigated were associated with distinct bacterial OTUs which motivated for the closer examination of the bacterial communities associated with the sponge, seawater ad sediment samples. To compare the sponge-associated bacterial communities with those of the environmental samples, the distribution of OTUs was analysed using nonmetric multidimensional scaling (NMDS) ordination plots followed by ANOSIM analysis at a distance of 0.03 and 0.00.

3.6.1 Comparison of sponge-associated bacterial communities with the environmental samples

An NMDS ordination plot of pairwise distances was generated to compare the bacterial communities associated with sponge specimens and environmental samples. The global R statistic as calculated using ANOSIM was 0.707 with a $p<0.05$ (0.001) demonstrating that the overall difference between the sponge-associated bacterial communities and the seawater and sediment was reliable and statistically highly significant (Fig. 3.4.). Pairwise comparisons showed that the bacterial communities of the seawater and sediment environmental samples were also significantly different (Fig. 3.4.).

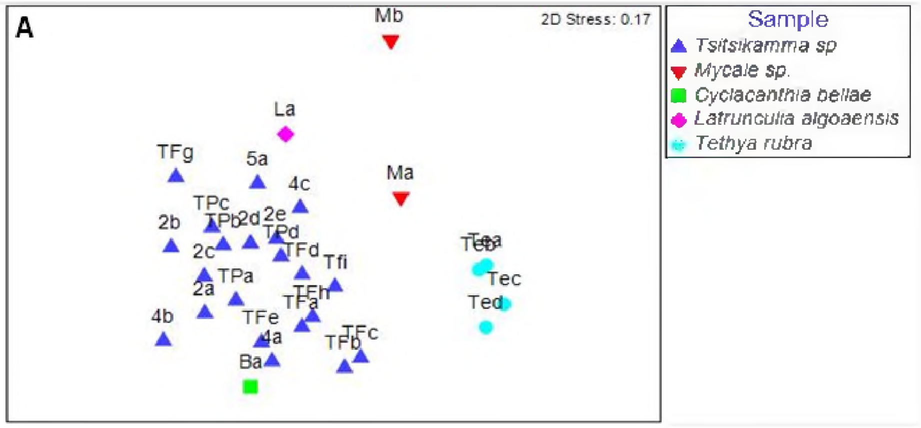


B	**0.03**	
Sample	**R value**	**P value**
Sponges vs Seawater	0.610	*0.001
Sponges vs Sediment	0.876	*0.001
Seawater vs Sediment	1.000	*0.006

Figure 3.4: Statistical ordination of bacterial OTUs clustered at a distance of 0.03 (**A**) associated with sponge specimens compared with the environmental samples using non-metric multidimensional scaling. Construction of Bray-Curtis resemblance matrix was based on partial 16S rRNA gene sequences following application of square root transformation to bacterial community abundance data. ESW: Seawater collected from Evans Peak; TSW: Seawater collected at Tsitsikamma MPA; RSW: Seawater collected at RIY Banks; ESED: Sediment collected at Evans Peak; TSED: Sediment collected at Tsitsikamma MPA; TF: *Tsitsikamma favus*; TP: *Tsitsikamma pedunculata*; 2: *Tsitsikamma michaeli;* 4: *Tsitsikamma nguni;* 5: *Tsitsikamma* sp. 005; Ba: *Cyclacanthia bellae*; LA*: Latrunculia algoaensis*; M: *Mycale* sp. and Tea-Ted: *Tethya rubra*. (**B**) Statistical representation of the bacterial communities at the level of species (0.03) associated with sponge specimens compared with the environmental samples. *(p < 0.05) statistically significantly different).

3.6.2 Comparative analysis of sponge associated bacterial communities

Having shown that the sponge associated bacterial communities were distinct from those in the associated seawater and sediment, the next step was to determine whether sponge-associated microbiomes were species specific. An NMDS plot ordination of pairwise distances was generated to compare the bacterial communities at the level of species (0.03) associated with latrunculid and outlier sponge species (Fig. 3.5.). Statistical analysis with a global R value of 0.792 and significance of p < 0.05 (0.001) demonstrated that overall, there was a significant difference between the sponge species associated bacterial communities.



B	**0.03**	
Sample	**R value**	**P value**
Tsitsikamma sp. vs *Mycale* sp	0.795	*0.004
Tsitsikamma sp. vs *C. bellae*	0.399	0.091
Tsitsikamma sp. vs *L. algoaensis*	0.524	*0.045
Tsitsikamma sp. vs *T. rubra*	0.911	*0.001
Mycale sp vs *C. bellae*	1.000	0.333
Mycale sp vs *L. algoaensis*	1.000	0.333
Mycale sp vs *T. rubra*	1.000	0.067
C. bellae vs *T. rubra*	1.000	0.200
L. algoaensis vs *T. rubra*	1.000	0.200

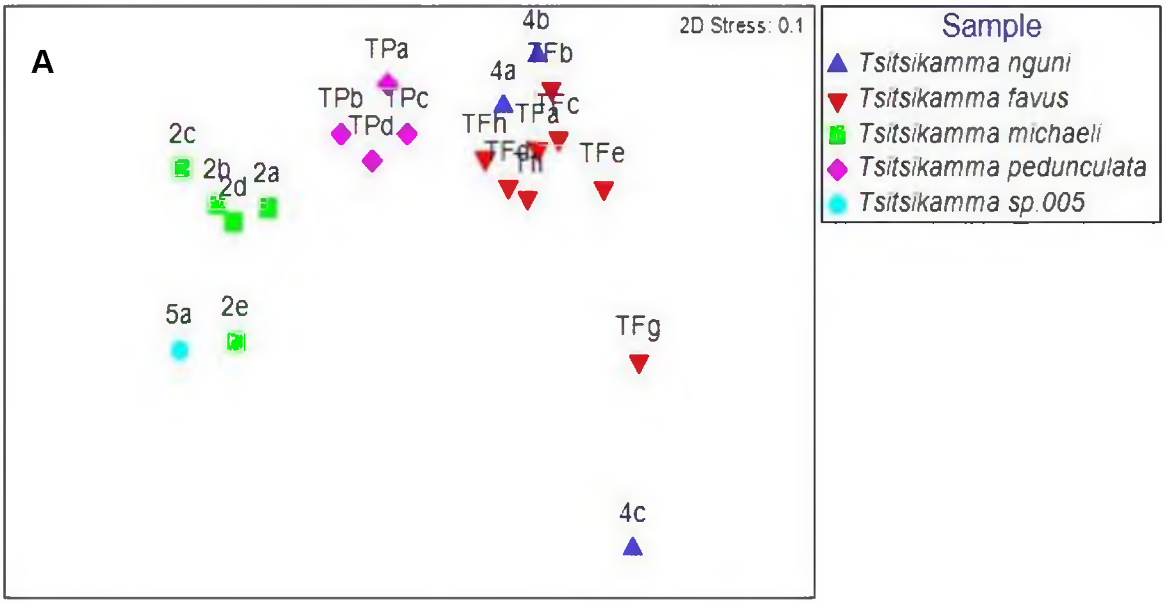
Figure 3.5: Statistical oordination of bacterial OTUs clustered at a distance of 0.03 (**A**) associated with latrunculid sponge species compared with the outlier sponge species using non-metric multidimensional scaling. Construction of Bray-Curtis resemblance matrix was based on partial 16S rRNA gene sequences following application of square root transformation to bacterial community abundance data. TF: *Tsitsikamma favus*; TP: *Tsitsikamma pedunculata*; 2: *Tsitsikamma michaeli;* 4: *Tsitsikamma nguni;* 5: *Tsitsikamma* sp. 005; Ba: *Cyclacanthia bellae*; LA*: Latrunculia algoaensis*; M: *Mycale* sp. and Tea-Ted: *Tethya rubra*. (**B**) Statistical representation of the bacterial communities at

the level of species (0.03) associated with latrunculid sponge species compared with the outlier sponge species. *(*p* < 0.05) statistically significantly different).

A previous study by Matcher et al. (2017) provided experimental evidence supporting the proposal that the microbiomes associated with different latrunculid sponge taxa were species specific. Pairwise comparisons were used to test the significance of the ANOSIM statistic between the sponge species associated bacterial communities. The results showed that the bacterial communities associated with the *Tsitsikamma* species were significantly different $p < 0.05$ (0.001) from the *Mycale* sp. sponge, *L. algoaensis* and *T. rubra* (Fig. 3.5.). The *Tsitsikamma* species were identified as not significantly different $p > 0.05$ from *C. bellae* however, the R value of 0.399 in addition to the low permutations lead to this result as statistically unreliable and was interpreted with caution.

3.6.3 Comparative analysis of *Tsitsikamma* sponge species associated bacterial communities

Matcher et al. (2017) showed that there were unique OTUs (0.00) associated with specific latrunculid sponge species. An NMDS ordination plot of pairwise distances showed that there was a significant difference with $p < 0.05$ (0.001) between unique OTUs associated with different *Tsitsikamma* species and the R value 0.718 confirmed the reliability of this result (Fig. 3.6.). A pairwise comparison between *Tsitsikamma* species revealed the bacterial communities associated with species within the *Tsitsikamma* genus were not uniformly different. The bacterial communities associated with both *T. nguni* and *T. favus*, which were not significantly different ($p > 0.05$, Low R statistic supported by high permutations) from one another, were significantly different ($p < 0.05$) from those associated with *T. michaeli* and *T. pedunculata*. Conversely, the bacterial communities associated with *Tsitsikamma* sp. 005 were not significantly different ($p > 0.05$) from *T. nguni*, *T. favus*, *T. michaeli* and *T. pedunculata*. The bacterial communities associated with *T. michaeli* and *T. pedunuculata* were significantly different ($p >0.05$) (Fig3.6.)



B	**0.00**	
Sample	**R value**	**P value**
T. nguni vs *T. favus*	0.304	0.085
T. nguni vs *T. michaeli*	0.815	0.018*
T. nguni vs *T. pedunculata*	0.667	0.029*
T. nguni vs *Tsitsikamma* sp. 005	1.000	0.250
T. favus vs *T. michaeli*	0.972	0.002*
T. favus vs *T. pedunculata*	0.676	0.002*
T. favus vs *Tsitsikamma* sp. 005	1.000	0.111
T. michaeli vs *T. pedunculata*	0.875	0.008*
T. michaeli vs *Tsitsikamma* sp. 005	0.240	0.333
T. pedunculata vs *Tsitsikamma* sp. 005	1.000	0.200

Figure 3.6: Statistical ordination of bacterial communities at the level of (A) species (0.03) and (B) unique (0.00) associated with *Tsitsikamma* sponge species using non-metric multidimensional scaling (NMDS). Construction of Bray-Curtis resemblance matrix was based on partial 16S rRNA gene sequences following application of square root transformation to bacterial community abundance data. (A: 2D stress value: 0.15; B: 2D stress value: 0.10). Statistically significant clustering ($p < 0.05$) was determined using ANOSIM in PRIMER7 (Quest Research Limited, Auckland, New Zealand). TF: *Tsitsikamma favus*; TP: *Tsitsikamma pedunculata*; 2: *Tsitsikamma michaeli;* 4: *Tsitsikamma nguni;* 5: *Tsitsikamma* sp005; Ba: *Cyclacanthia bellae*; LA*:* *Latrunculia algoaensis*; Ma: *Mycale* sp. and Tea-Ted: *Tethya rubra*.

3.7 Phylogenetic analysis of unique sponge associated bacterial OTUs

Previous characterization of the bacterial communities associated with six latrunculid species in a study by Matcher et al. (2017) showed the unprecedented conservation of two dominant bacterial symbionts within the *Latrunculiidae* family of marine sponges. Betaproteobacteria OTUs observed in the different latrunculid sponges were closely related and their phylogenetic relationship followed that of their hosts whereas the Spirochaetae OTU was observed to be present in only two genera, *Tsitsikamma* and *Cyclacanthia*. Given the production of pyrroloiminoquinone secondary metabolites, their potent biological activity and the unusual dominant bacterial symbionts associated with sponges belonging to the Latrunculiidae family, it was decided to determine whether the bacterial conservation was also true for the sponges investigated in this study.

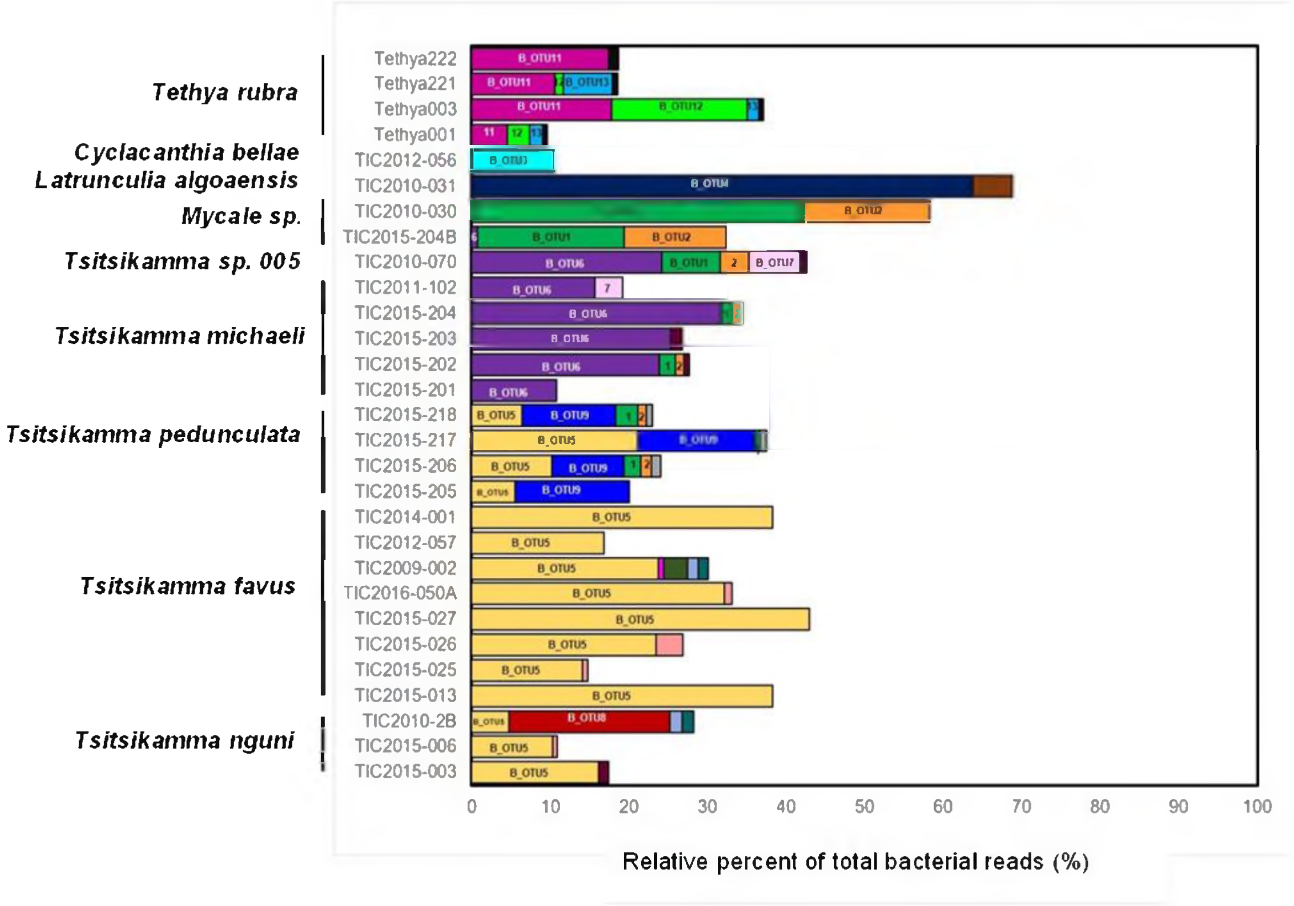


Figure 3.7: Dominant, unique (0.00) Betaproteobacteria OTUs from each sponge specimen. Read abundance is indicated as the relative percentage of the total bacterial reads analyzed for each sponge.

All *T. favus* sponges were associated with a common dominant unique OTU, B_OTU5. B_OTU5 was also dominant in the two closely related *T. nguni* sponge specimens (TIC2015-003 and TIC2015-006), collected at Rheeder's reef at Stormsriver mouth but not in the *T. nguni* specimen TIC2010-2B from Evans Peak, which harbored a second dominant betaproteobacterial OTU, B_OTU8 (Fig. 3.7.). Betaproteobacterium B_OTU5 was also dominant in *T. pedunculata* along with a second dominant betaproteobacterial OTU, B_OTU9 found only in the T. pedunculata sponges.

The dominant Betaproteobacterium in *T. michaeli* sponges was B_OTU6, which was also present in *Tsitsikamma* sp. 005 as well as the *Mycale* sp. found as an encrusting sponge on *T. michaeli* (TIC2015-204). *L. algoaensis* and *C. bellae* specimens were associated with betaproteobacterial OTUs, B_OTU4 and B_OTU3, respectively, neither of which were not identified in any *Tsitsikamma* sponges (Fig. 3.7.).

B_OTU1 and B_OTU2, representing different betaproteobacterial species from that associated with the latrunculid sponges, were the dominant OTUs associated with the two *Mycale* sp. sponges. This OTU was present at low levels in *Tsitsikamma* sp. 005, two *T. michaeli* sponge specimens (TIC2015-202 and TIC2015-204) and two *T. pedunculata* sponge specimens (TIC2015-206 and TIC2015-218) (Fig. 3.7.). The *Mycale* sp. TIC2010-030 and TIC2015-204B were found to be encrusting sponges on TIC2010-070 *Tsitsikamma* sp.005 and TIC2015-204 *T. michaeli* sponge specimens respectively. *Mycale* sp. sponges were also growing upon *T. pedunculata* sponge specimens TIC2015-206 and TIC2015-218 and the *T. michaeli* sponge TIC2015-202. The presence of B_OTU1 and B_OTU2 in the *Tsitsikamma* sponge specimens therefore could likely be due to cross-contamination during the removal of the encrusting *Mycale* sponge tissue as *Tsitsikamma* sponges not harboring the *Mycale* sp. did not contain B_OTU1 and B_OTU2 (Fig. 3.7.). B_OTU6, which is dominant in *T. michaeli* and *Tsitsikamma* sp. 005 sponge samples, was present at very low levels in one *Mycale* sp. sponge specimen (TIC2015-204B) which could be due to cross-contamination during the removal of the encrusting *Mycale* tissue.

T. rubra sponges were associated with three betaproteobacterial OTUs including B_OTU11 (dominant in all four specimens), B_OTU12 and B_OTU13, found in three of the four specimens (Fig. 3.7.). The *T. rubra* sponges were collected from two different reefs in Algoa Bay and a previous study has shown that sponges from the different locations have distinct microbiomes (Waterworth et al., 2017). However, the association with their betaproteobacterial OTUs does not appear to be location dependent (Fig. 3.7.). Each *T. rubra* betaproteobacterial OTU was also not found in any other sponge species.

Analysis of the phylogenetic relationship between the dominant betaproteobacterial OTUs identified in this study and other sponge-associated Betaproteobacteria revealed the presence of two large monophyletic clusters, designated Group I and II (Fig. 3.8). Both sponge-associated and free-living bacterial species were included in Group I, which included members of the *Nitrospira* genus known as ammonia-oxidizing sponge-associated taxa and for their adaptability to different ecological niches (Prosser, Head, & Stein, 2014). A Betaproteobacterium OTU represented by the clone Sp02-5 from the 16S rRNA clone library generated in a previous study of *T. favus* specimen TIC2009-002 (Walmsley et al., 2012), clustered within this *Nitrospira* genus.

Betaproteobacterial OTUs identified in this study were included in Group II, which is comprised of uncultured sponge-associated bacterial taxa. The latrunculid OTUs conserved in *T. favus*, *T. nguni*, and *T. pedunculata* sponges form a cluster with the dominant OTU *T. favus* clone Sp02-1 from the 16S rRNA clone library generated by Walmsley et al., (2012) and two uncultured proteobacteria from *Latrunculia apicalis*. B_OTU8 present in *T. nguni* and B_OTU5 present in *T. nguni*, *T. favus* and *T. pedunculata*, form a cluster and are closely related to B_OTU4 from *L. algoaensis* with a sequence identity greater than 99%. Similarly, B_OTU7 present in *Tsitsikamma* sp. 005 and B_OTU6 present in *Tsitsikamma* sp. 005 and *T. michaeli*, are also very closely related, clustering together with B_OTU3 which is the dominant OTU present in *C. bellae* (Fig. 3.8). In a separate clade with Betaproteobacteria associated with *Crambe crambe* and *Antho chartacea*, the *T. rubra* betaproteobacterial OTUs were all closely related to uncultured betaproteobacterial sequences from *Tethya californiana* and the dominant betaproteobacterial OTUs from the *Mycale* sp. were found to be most closely related to an uncultured betaproteobacterial sequence from *Mycale acerata* (Fig. 3.8.)

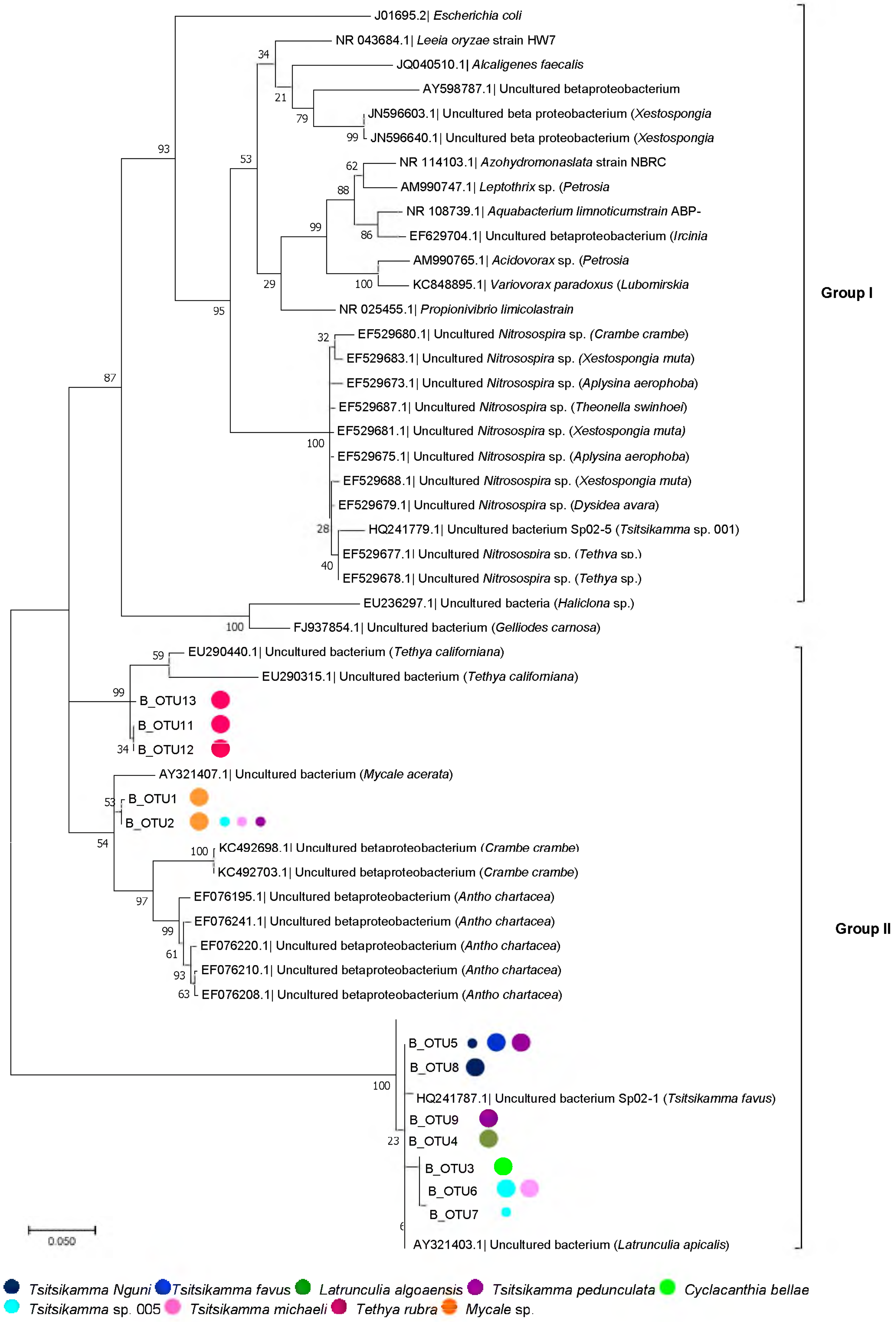

J01695.2| Escherichia coli
NR 043684.1| Leeia oryzae strain HW7
JQ040510.1| Alcaligenes faecalis
AY598787.1| Uncultured betaproteobacterium
JN596603.1| Uncultured beta proteobacterium (Xestospongia
JN596640.1| Uncultured beta proteobacterium (Xestospongia
NR 114103.1| Azohydromonaslata strain NBRC
AM990747.1| Leptothrix sp. (Petrosia
NR 108739.1| Aquabacterium limnoticumstrain ABP-
EF629704.1| Uncultured betaproteobacterium (Ircinia
AM990765.1| Acidovorax sp. (Petrosia
KC848895.1| Variovorax paradoxus (Lubomirskia
NR 025455.1| Propionivibrio limicolastrain
EF529680.1| Uncultured Nitrosospira sp. (Crambe crambe)
EF529683.1| Uncultured Nitrosospira sp. (Xestospongia muta)
EF529673.1| Uncultured Nitrosospira sp. (Aplysina aerophoba)
EF529687.1| Uncultured Nitrosospira sp. (Theonella swinhoei)
EF529681.1| Uncultured Nitrosospira sp. (Xestospongia muta)
EF529675.1| Uncultured Nitrosospira sp. (Aplysina aerophoba)
EF529688.1| Uncultured Nitrosospira sp. (Xestospongia muta)
EF529679.1| Uncultured Nitrosospira sp. (Dysidea avara)
HQ241779.1| Uncultured bacterium Sp02-5 (Tsitsikamma sp. 001)
EF529677.1| Uncultured Nitrosospira sp. (Tethya sp.)
EF529678.1| Uncultured Nitrosospira sp. (Tethya sp.)
EU236297.1| Uncultured bacteria (Haliclona sp.)
FJ937854.1| Uncultured bacterium (Gelliodes carnosa)
EU290440.1| Uncultured bacterium (Tethya californiana)
EU290315.1| Uncultured bacterium (Tethya californiana)
B_OTU13
B_OTU11
B_OTU12
AY321407.1| Uncultured bacterium (Mycale acerata)
B_OTU1
B_OTU2
KC492698.1| Uncultured betaproteobacterium (Crambe crambe)
KC492703.1| Uncultured betaproteobacterium (Crambe crambe)
EF076195.1| Uncultured betaproteobacterium (Antho chartacea)
EF076241.1| Uncultured betaproteobacterium (Antho chartacea)
EF076220.1| Uncultured betaproteobacterium (Antho chartacea)
EF076210.1| Uncultured betaproteobacterium (Antho chartacea)
EF076208.1| Uncultured betaproteobacterium (Antho chartacea)
B_OTU5
B_OTU8
HQ241787.1| Uncultured bacterium Sp02-1 (Tsitsikamma favus)
B_OTU9
B_OTU4
B_OTU3
B_OTU6
B_OTU7
AY321403.1| Uncultured bacterium (Latrunculia apicalis)
93
34
21
79
99
53
62
88
99
86
29
100
95
87
32
100
28
40
100
59
99
34
53
54
100
97
99
61
93
63
100
23
6
0.050
Group I
Group II
● Tsitsikamma Nguni ● Tsitsikamma favus ● Latrunculia algoaensis ● Tsitsikamma pedunculata ● Cyclacanthia bellae
● Tsitsikamma sp. 005 ● Tsitsikamma michaeli ● Tethya rubra ● Mycale sp.

Figure 3.8: Phylogenetic relationship between dominant Betaproteobacteria OTUs and other sponge-associated Betaproteobacteria present in their respective sponge hosts. Related Betaproteobacteria sequences were obtained from GenBank with the corresponding accession numbers, and the phylogenetic analysis was generated using MEGA7 software (Kumar, Stetcher and Tamura, 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 50 nucleotide sequences. Bootstrap values, calculated based on 1,000 replicates, are indicated next to the branches (Felsenstein, 1985). The 16S rRNA gene sequence of Escherichia coli (JO1695) was used to root the tree. The host of each OTU is indicated by a colour-coded circle with large versus small circles indicating relative dominance.

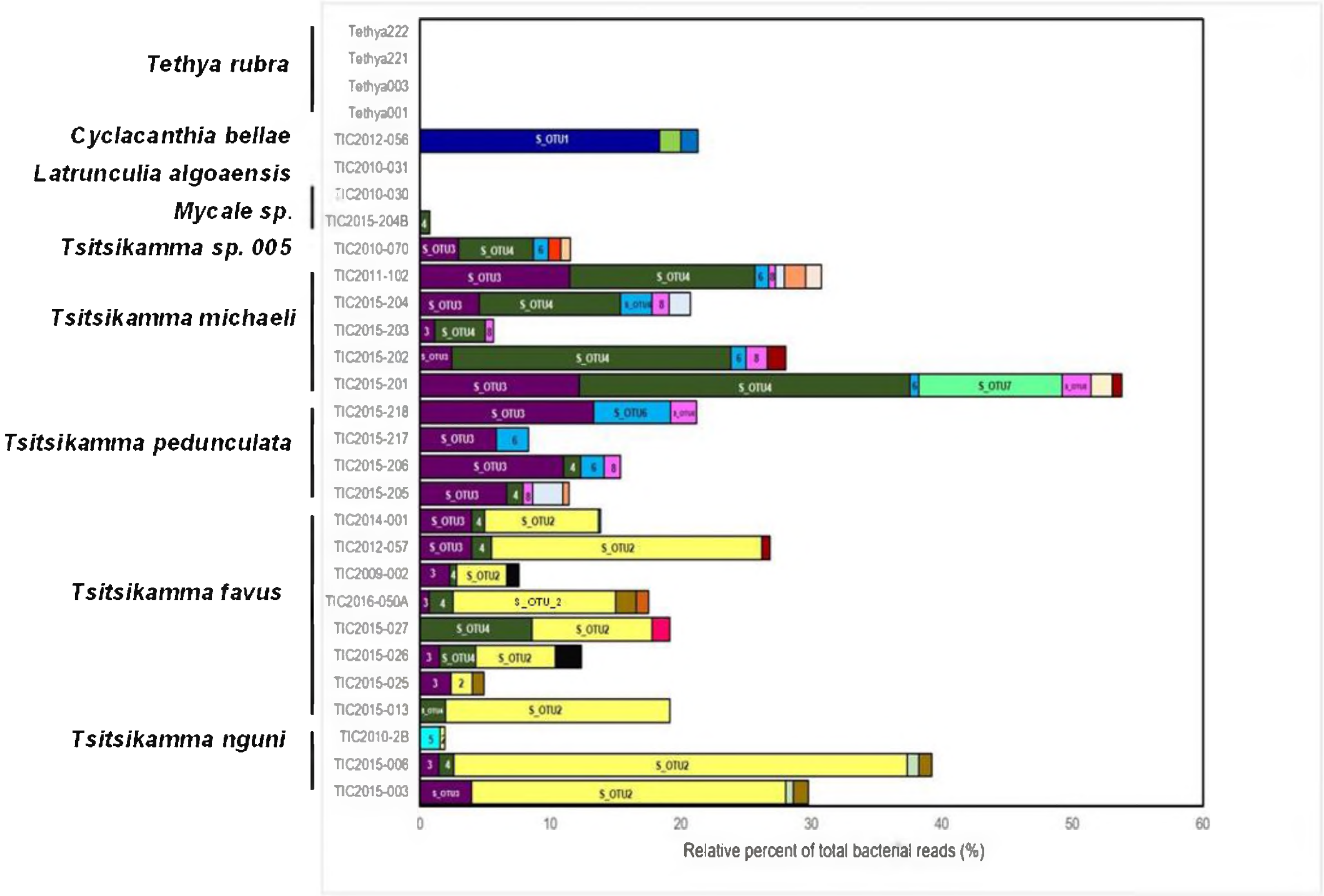


Figure 3.9: Dominant, unique (0.00) Spirochaetae OTUs from each sponge specimen. Read abundance is indicated as the relative percentage of the total bacterial reads analyzed for each sponge.

Sequence reads classified within the Spirochaetae phylum formed a significant proportion of the bacterial populations within *Tsitsikamma* sponge species and in the *C. bellae*. A single unique Spirochaetae taxon S_OTU1, was dominant in and unique to *C. bellae.* In *Tsitsikamma* sponges, there were three unique Spirochaetae OTUs, with the relative abundance and distribution of one OTU appearing to be species related (Fig 3.9.). S_OTU4 was present but not dominant in all of the *Tsitsikamma* species. This OTU was dominant in *T. michaeli* sponges and in the closely related *Tsitsikamma* sp.005 specimen. A very small number of S_OTU4 reads were observed in one *Mycale* sp. sponge specimen (TIC2015-204B) which, as mentioned above, could be due to cross-contamination from the removal of the encrusting *Mycale* tissue from the *T. michaeli* (TIC2015-204) sponge sample.

The dominant Spirochaetae in all *T. favus* sponge specimens, S_OTU2, was also dominant in the *T. nguni* specimens, TIC2015-003 and TIC2015-006, collected in the Tsitsikamma Marine National Park (MPA) (Fig 3.9.). However, while S_OTU2 was present in *T. nguni* TIC2010-2B, S_OTU5 was dominant. S_OTU2 was absent in the remaining *Tsitsikamma* sponge species. S_OTU3 was present, but not dominant, in the majority of *Tsitsikamma* sponges, with the dominance of S_OTU3 being confined to *T. pedunculata* sponges (Fig 3.9.). S_OTU5 was dominant in the Algoa Bay *T. nguni* sponge, TIC2010-2B*,* but not in the specimens collected in the Tsitsikamma MPA. These differences mirror similar observations with betaproteobacterial OTUs in the *T. nguni* sponges suggesting that the sponge populations in the Tsitsikamma MPA and Algoa Bay may have descended from different lineages or may be in the process of evolving into two new species.

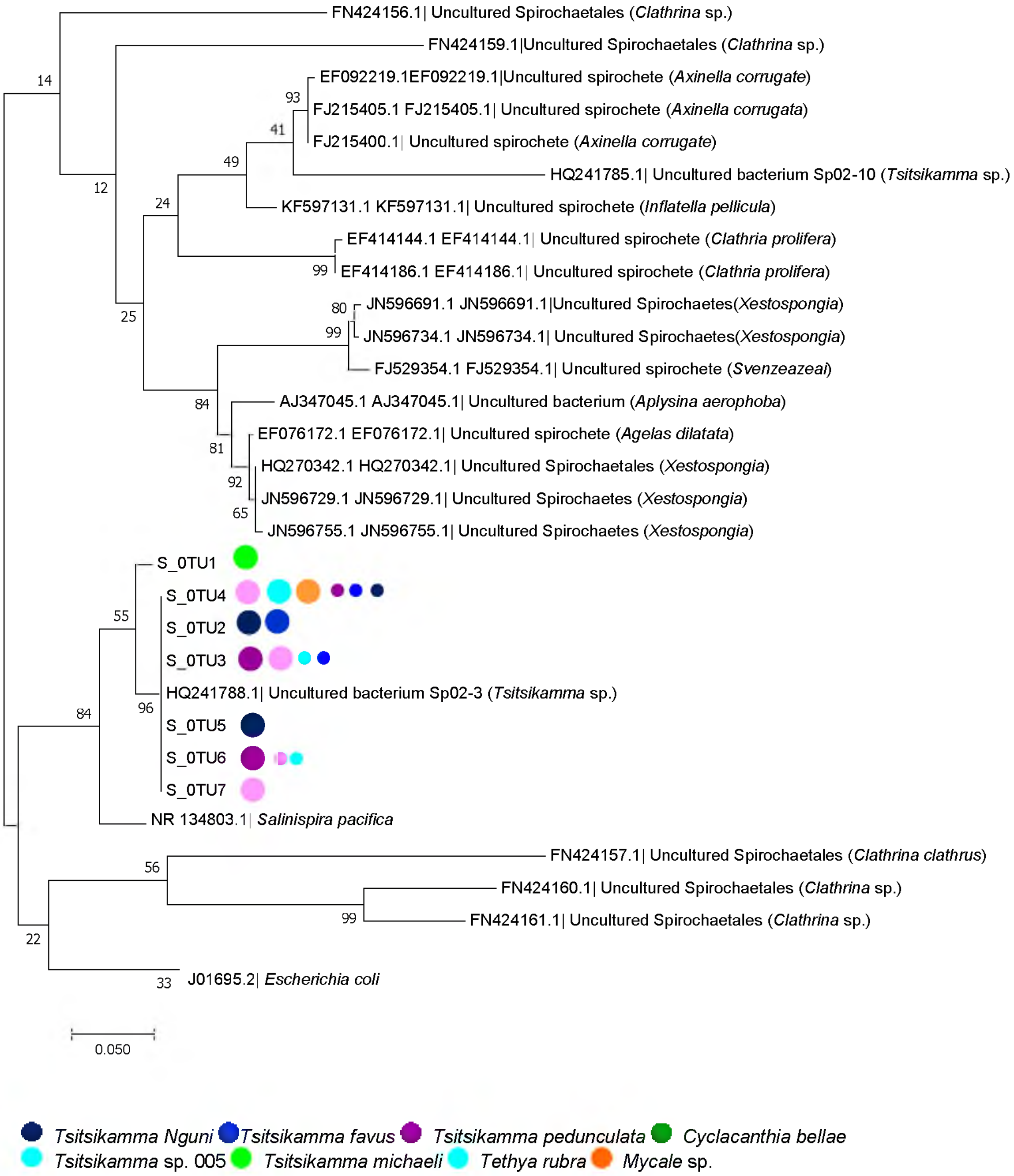


Figure 3.10: Phylogenetic relationship between dominant latrunculid Spirochaetae OTUs and other Spirochaetes predominantly associated with sponges. Related Spirochaete sequences were obtained from GenBank with the corresponding accession numbers, and the phylogenetic analysis was generated using MEGA7 software (Kumar, Stetcher and Tamura, 2016).The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The

tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. Bootstrap values, calculated based on 1,000 replicates, are indicated next to the branches (Felsenstein, 1985). The host of each OTU is indicated by a colour-coded circle with large versus small circles indicating relative dominance.

The phylogenetic tree (Fig. 3.10) revealed that based upon their partial 16S rRNA sequences, the *Tsitsikamma* Spirochaetae OTUs comprising of S_OTU3, S_OTU6, S_OTU2, S_OTU5, S_OTU4 and S_OTU7 are extremely closely related, indicated by the clustering with the dominant Spirochaetae 16S rRNA gene sequence (Sp02-3) obtained from a clone library of *T. favus* (Walmsley et al., 2012). Belonging to a different genus within the family Latrunculiidae, the sponge specimen *C. bellae* harboured a dominant Spirochaetae, S_OTU1, which was shown to be less closely related to the *Tsitsikamma* OTUs. A BLAST analysis of the Sp02-3 16S rRNA sequence showed a strain of *Salinispira pacifica*, with a 93% sequence identity, to be the closest relative which was isolated from hypersaline microbial mats (Ben Hania et al., 2015). The nearest phylogenetic relative to the latrunculid sponge-associated spirochaete OTUs was associated with sponges belonging to the genus *Clathrina* with >90% sequence identity. The distinct and separate group of closely related OTUs observed in the latrunculid sponges, highlights a very different interaction which may represent a well adapted symbiotic relationship between members of a new family of specialized sponge-bacterial symbionts and their hosts.

Chapter Four

Discussion

Sponges produce a plethora of chemical compounds with highly diverse carbon skeletons and the biological properties of alkaloids bearing a pyrroloiminoquinone ring system have attracted considerable attraction due to their potential in pharmaceutical applications and therapeutics (Goey et al., 2016; Lin et al., 2017; Botić et al., 2017). Isolation of pyrroloiminoquinones, wakayin and makaluvamine, from phylogenetically unrelated species including ascidians, myxomycetes and actinomycetes indicated these metabolites are not confined to sponges, tentatively suggesting the biosynthetic origin of latrunculid pyrroloiminoquinones may likely be microbial (Ishibashi et al., 2001; Copp et al., 2002; Hughes et al., 2009; Miyanga et al., 2011 and Davis et al., 2012).

The aim of the research presented in this thesis was to investigate the conservation of bacterial communities associated with South African latrunculid sponge species native to the Agulhas bioregion. It was hypothesised that latrunculid sponge species producing related secondary metabolites would be associated with conserved microbial communities. To test this hypothesis, the diversity of microbiomes associated with latrunculid sponges, other sponge species and their environment, was investigated. This study focused on the bacterial communities associated with 23 latrunculid sponge specimens representing three endemic genera, *Tsitsikamma, Cyclacanthia* and *latrunculia* (Fig. 4.1) all of which are known to produce pyrroloiminoquinone alkaloids (Antunes et al., 2005). Six additional outlier sponge specimens representing two species: *Mycale* sp., a member of the Mycaliidae family classified within the same order (Poecidosclerida) as the Latrunculiidae and *T. rubra,* a member of the Tethyidae family within the Tethyda order (Fig. 4.1). In addition, 11 environmental samples, comprised of both seawater and sediment, were included in this study.

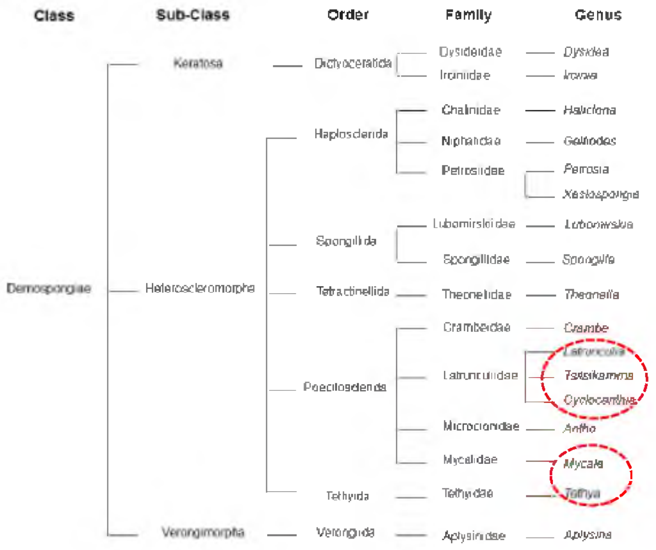


Fig. 4.1: Taxonomic relationship between sponge species relevant to this study (taken from Matcher et al., 2017).

4.1 Taxonomic identification of sponges

Taxonomy is crucially important in marine natural product studies as taxonomic uncertainties amongst the Porifera can lead to consequential confusion regarding sponge sources of interesting chemistry. The first objective was to collect and identify latrunculid and outlier sponge specimens native to the Agulhas bioregion. The sponge specimens were identified using traditional taxonomic methods coupled with molecular based techniques. The study identified two new *Tsitsikamma* species, namely *T. nguni* and *T. michaeli*. Morphologically, *T. favus* and *T. nguni* specimens were found to be highly similar with slight differences in their porefields and spicules that set the two apart (Parker-Nance, Hilliar et al., Manuscript submitted. Appendix A.). A review of archived voucher samples found that this morphological similarity has resulted in their identification as a single species (Parker-Nance, personal communication). The close relationship between these two sponge species was also reflected in their 28S rRNA gene sequences, which were identical in the region used for molecular taxonomy.

A comparison of the pyrroloiminoquinone compounds produced by *T. favus* and *T. nguni* confirmed the two species are very closely related, both producing maklauvamines and tsitsikammamines but in different amounts (Jarmo Kalinski, Dorrington Laboratory, personal communication). Interestingly, there were three specimens identified as *T. favus*, which had significantly different 28S rRNA gene sequences compared to those of other *T. favus* and *T. nguni* sponges. These differences were not reflected in the diversity of their overall microbial communities. Further chemical analysis on these specimens may provide a means of resolving their classification.

Morphologically, *T. michaeli* resembles *T. pedunculata* however, is distinct by colour and the absence of a reinforced stalk (Parker-Nance, Hilliar et al., Manuscript submitted. Appendix A.). A close phylogenetic relationship was observed between morphologically distinct specimens, *T. michaeli* and *T. pedunculata*. *T. michaeli* and *T. pedunculata* also clustered together in a single clade with no polymorphisms in the 28S rRNA sequence surveyed in this study. Based on the chemical analysis, *T. pedunculata* was found to produce a suite of metabolites that are distinct from those of *T. favus*, *T. nguni* and *T. michaeli* and all four *Tsitsikamma* species were found to produce a compound with the same mass as makaluvic acid C (180.1) (Jarmo Kalinski, Dorrington Laboratory, personal communication of unpublished data). Collectively, novel, uncharacterised brominated compounds likely to be pyrroloiminoquinones were isolated from all *Tsitsikamma* sponge species and differences in the chemistry associated with each species motivated the further investigation into the associated bacterial communities.

Tsitsikamma sp. 005 was morphologically identified as a potentially novel species belonging to the *Tsitsikamma* genus, which was confirmed by phylogenetic analysis using the 28S rRNA partial gene sequence. *Tsitsikamma* sp. 005 was observed to be more closely related to *T. michaeli* and *T. pedunculata* than *T. favus* and *T. nguni* specimens. However, as only one *Tsitsikamma* sp. 005 specimen was used in this study analysis of multiple specimens would be required to better provide insight into the classification and phylogeny of this species with relation to other members in the *Tsitsikamma* genus. In relation to the *Tsitsikamma* genus, phylogenetic analysis of the remaining latrunculid sponges in this study revealed *C. bellae* to be a closer relative than *L. algoaensis*. *L. algoaensis* however, was more closely related to the *Tsitsikamma* and *C. bellae* sponges than the outlier genera, *Mycale and Tethya*. Due to the limitations of the 28S rRNA gene sequence separation below the level of genus in this study, PCR amplification of the larger 28S rRNA gene fragment, as reported in Thacker et al. (2013), was attempted however did not result in suitable products to be used in further phylogenetic analyses. To overcome this limitation, further attempts to PCR amplify the longer 28S rRNA gene fragment with adjusted PCR parameters should be attempted as well as a multi-loci approach with amplification of multiple gene markers to better aid sponge specimen identification, especially between closely related latruculid sponges.

4.2 Characterisation of sponge-associated microbial communities

Following the identification of sponge specimens, the next objective was to characterise sponge-associated bacterial communities. The data showed that latrunculid sponge microbiomes were different from those of the sympatric sponges and that sponge associated bacteria were distinct from those in the environment. Statistical oordination and ANOSIM analysis of bacterial OTUs revealed that latrunculid sponges were highly significantly different from the surrounding environment. Similarities between the bacterial communities associated with Tethya sponges and the environment were observed. However, closer analysis identified an Alphaproteobacteria OTU that was absent from both the seawater and sediment. Different Gammproteobacteria and Bacteroidetes OTUs were specific to the sediment. Conservation of a high abundance of other different Gammaproteobacteria OTUs in the *T. pedunculata* sponge specimens warrants further investigation considering *T. pedunclata* sponges produce a suite of secondary metabolites distinct from those produced by other *Tsitsikamma* sponge species.

4.3 Closely related betaproteobacterial species dominate sponge microbiomes

Taxa belonging to the Betaproteobacteria phylum dominated the bacterial communities associated with all sponges but were absent in the environmental samples, leading to the conclusion that these bacteria are sponge-specific. The data revealed the conservation of a single dominant Betaproteobacterial OTU within the microbiomes of latrunculid sponge species including *T. nguni, T. favus, T. michaeli, T. pedunculata, Tsitsikamma sp.005, C. bellae* and *L. algoaensis*. A different, but closely related Betaproteobacterium species was conserved in the *T. rubra* and *Mycale* sp. sponges.

Analysis of OTUs (clustered at a distance of 0.00) revealed that, remarkably, each sponge species hosts a unique Betaproteobacterium strain that is conserved in multiple specimens of the same species. Phylogenetic analysis of unique Betaproteobacteria OTUs showed that these bacteria mirror the phylogeny of their host species. A study by Waterworth et al. (2017) provided evidence showing that this Betaproteobacterial symbiont is vertically transmitted from one *T. rubra* sponge generation to the next. reported to not only be found in the sponges but also within the embryos of several *Tethya* sponge specimens. This may also be the case for the Betaproteobacterial OTUs conserved in the latrunculid species.

The latrunculid betaproteobacterial OTUs were phylogenetically closely related to dominant OTUs in a 16S rRNA clone library derived from *L. apicalis* collected near Antarctica. This shows that the presence of a dominant Betaproteobacterium species is restricted to latrunculid sponges collected from the same geographical location (Matcher et al., 2017). Bacterial communities dominated by Betaproteobacterial symbionts are also found in sponges belonging to the Crambeidae, Microcionidae and Mycalidae families (Matcher et al., 2017). The two dominant Betaproteobacterial OTUs (B_OTU1 and B_OTU2) identified in the *Mycale* sp. clustered with 16S rRNA clone sequences derived from the Antarctic sponge *Mycale acerata*, so it is likely that *M. acerata* sponge also harbours a dominant Betaproteobacterium. Related Betaproteobacteria are found in other sponge species include *Crambe-crambe* which, is dominated by a single Betaproteobacterial symbiont (Croué et al., 2013) and *Antho chartacea* in which several closely related OTUs have been reported from Taylor et al. (2007).

The general conservation of dominant Betaproteobacteria in the microbiomes of many sponge families raises interesting questions about their function as sponge symbionts. A recent metagenomic study revealed a dominant Betaproteobacterium, with a reduced genome from *Amphimedon queenslandica* sponge, native to the Great Barrier Reef (Gauthier et al., 2016). A reduced genome is a common feature among endosymbiotic bacteria and the data suggest that the Betaproteobacterium is likely to be an obligate symbiont, which may provide an important functional role in ammonia assimilation for the host sponge (Gauthier et al., 2016).

The functional roles of conserved bacteria within sponge hosts is a constantly speculated topic in the investigation of sponge – symbiont interactions and based on BLAST sequence analysis of the 16S rRNA genes, dominant Betaproteobacterial strains identified in this study were associated with the ammonia oxidizing Nitrosomonadaceae family. The presence of nitrifying microbial symbionts has been suggested in the mesohyl of several sponge species which are able to excrete nitrate and/ or nitrite, and it is postulated that Nitrospira are involved in the nitrification of sponges (Jimenez and Ribes, 2007; Bayer et al., 2008 and Hoffman et al., 2009). The Nitrosomonadaceae strains in the latrunculid sponges in this study were numerically dominant suggesting these symbionts may play a role in the bioavailability of nitrogen for their sponge hosts.

4.4 A conserved Spirochete species in *Tsitsikamma* and *Cyclacanthia* sponges

In *Tsitsikamma and Cycalcanthia species,* there is a dominant Spirochaetae OTU that is not found in the other sponge species including *L. algoaensis* suggesting that at least in the context of this study, this species is specific to these two latrunculid genera. Phylogenetic analysis revealed that *Tsitsikamma* Spirochaetae OTUs are closely related. These OTUs formed a distinct clade clustering with a Spirochaetae clone previously isolated from *T. favus* TIC2009-009 (Walmsley et al., 2009). S_OTU1, the dominant OTU from *C. bellae* was found to be less closely related than the *Tsitsikamma* OTUs. BLAST analysis showed that the closest relative to the Spirochaetae clone previously isolated from *T. favus* was a strain of *Salinispira pacifica* isolated from hypersaline microbial mats (Ben Hania et al., 2015). The *Tsitsikamma* and *Cyclacanthia* Spirochaetae OTUs are phylogenetically closely related however, only distantly related to other sponge associated Spirochaetae.

The occurrence of Spirochaetae species within sponge microbiomes have been reported by several studies (Hentschel et al., 2002; Taylor et al., 2007 and Walmsley et al., 2012). Studies by Isaacs et al. (2009), Neulinger et al. (2010) and Fiore et al. (2013) reported Spirochete symbionts in *Xestospongia muta*, *Clathria prolifera* and *Clathrina clathrus* sponge species. In particular, Spirochaetae were found to be highly abundant throughout the mesohyl of two morphotypes of the calcareous sponge, *Clathrina clathrus,* and similarities between the sequences identities of the Spirochaetae associated with *C. clathrus* and four additional Spirochaetae OTUs from another *Clathrina* species based on 16S rRNA gene sequences, indicated all of the associated OTUs were distantly related to each other (Neulinger et al., 2010). Species in the family Latrunculiidae from a study by Matcher et al., (2017) are currently the only reported sponges with a conserved, dominant Spirochete.

4.5 Comparison of bacterial communities associated with the sponges and the surrounding environment

Previous research by Matcher et al. (2017) identified distinct bacterial OTUs which were conserved in the microbial community profiles associated with latrunculid sponges of the same genera, collected in geographically distinct locations (Algoa Bay and Tsitsikamma National Park). These reported findings motivated the closer examination into the bacterial communities associated with these sponges, raising the question whether they were consistently different from the bacterial communities associated with their surrounding environment and outlier sponges belonging to the families Tethyidae and Mycalidae. Statistical oordination and ANOSIM analysis of bacterial OTUs revealed that the latrunculid sponges were highly significantly different ($p < 0.05$) from the surrounding environment, including both the seawater and sediment. Furthermore, an investigation into the bacterial communities associated with individual sponge species revealed the *Tsitsikamma* sponge associated bacterial communities were significantly different ($p < 0.05$) from both the *Mycale* and *Tethya* outlier species. Comparatively, the bacterial communities associated with species belonging to the family Latrunculiidae, showed *Tsitsikamma* sponge associated bacterial communities were significantly different ($p < 0.05$) from the *L. algoaensis* associated bacterial communities. The low R statistic and corresponding low permutations for the bacterial community statistic between

Tsitsikamma species and *C. bellae* was considered statistically unreliable and interpreted with caution.

Association of unique OTUs (0.00) with specific latrunculid sponge species was reported by Matcher et al. (2017) which motivated the investigation into the bacterial communities associated with *Tsitsikamma* sponge species in this study. Statistical oordination and ANOSIM analysis of bacterial OTUs showed that there was a significant difference ($p > 0.05$) between unique OTUs associated with the different *Tsitsikamma* species which further supports the data by Matcher et al. (2017).

4.6 Conclusions

The data presented in this study revealed that bacterial communities associated with latrunculid sponges were significantly different from those associated with the environment and outlier sponge species. Conservation of bacterial communities associated with the same genera collected in geographically distinct locations including Algoa Bay and Tsitsikamma National Park were observed. The bacterial communities associated with latrunculid sponges appear host specific and conservation of Betaproteobacteria and Spirochete populations in this expanded study remained consistent with the findings in previous studies by Walmsley et al., (2012) and Matcher et al., (2017). The Betaproteobacteria associated with latrunculid sponges appear to have co-evolved with their hosts from an ancient symbiosis, and conserved Spirochete OTUs found to be exclusively in *Tsitsikamma* and *Cyclacanthia* suggests a symbiosis that may have been established more recently. Conservation of a dominant Spirochete symbiont in the latrunculid sponges suggests these bacteria may be involved in a specialized function within these closely related sponge genera. The conservation of dominant bacterial symbionts within sponges belonging to the *Latrunculiidae* family raises interesting questions regarding the interaction of this symbiosis and whether potential involvement by these symbionts in the biosynthesis of the associated pyrroloiminoquinone secondary metabolites that characterize this sponge family exists.

References

Abdelmohsen, U. R., Bayer, K. and Hentschel, U. (2014). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat. Prod. Rep.* 31: 381-399. doi: 10.1039/c3np70111e.

Althoff, K., Schütt, C., Steffen, R., Batel, R. and Mueller, W. E. G. Evidence for a symbiosis between bacteria of the genus rhodobacter and the marine sponge halichondria panicea: Harbor also for putatively toxic bacteria? (1998). *Mar. Biol.* 130: 529–536.

Antunes, E. M., Beukes, D. R, Kelly, M., Samaai, T., Barrows, L. R, Marshall, K. M., Sincich, C. and Davies-Coleman, M. T. (2004). Cytotoxic pyrroloiminoquinones from four new species of South African latrunculid sponges. *J Nat Prod*. 67:1268-1276

Antunes, E. M., Copp, B. R., Davies-Coleman, M. T., and Samaai, T. (2005). Pyrroloiminoquinone and related metabolites from marine sponges. *Natural Products Reports.* 22:62-72.

Bansal, A. K. and Meyer, T. E. (2002). Evolutionary Analysis by Whole-Genome Comparisons. *J. Bacteriol.* 184:2260-2272.

Bayer, K., Schmitt, S., & Hentschel, U. (2008). Physiology, phylogeny and *in situ* evidence for bacterial and archeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environmental Microbiology*, *10*, 2942–2955.

Beer, S. and Ilan, M. (1998). In situ measurements of photosynthetic irradiance responses of two Red Sea sponges growing under dim light conditions. *Mar. Biol.* 131:613–617.

Ben Hania, W., Joseph, M., Schumann, P., Bunk, B., Fiebig, A., Spröer, C., Klenk, H. P., Fardeau, M. L. and Spring, S. (2015). Complete genome sequence and description of Salinispira pacifica gen. nov., sp. nov., a novel spirochaete isolated form a hypersaline microbial mat. Standards in Genomic Sciences. 10:7.

Bergmann, W. and Feeney, R.J. Contributions to the study of marine products. XXXII. (1951). The nucleosides of sponges *I. J. Org. Chem.* 16:981–987. doi: 10.1021/jo01146a023.

Bergquist, P. R. (1978). Sponges. Hutchinson & Co. London.

Bernan V. S., Greenstein, M. and Maiese, W. M. (1997). Marine microorganisms as a source of new natural products. *Adv Appl Microbiol.* 43: 57-90.

Bewley, C. A., Holland, N. D. and Faulkner. D. J. (1996). Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia*. 52:716–722.

Botić, T., Defant, A., Zanini, P., Žužek, M. C., Frangež, R., Janussen, D., Kersten, D., Knez, Ž., Mancini, I. and Sepčić, K. (2017). Discorhabdin alkaloids from Antaarctic *Latrunculia* spp. sponges as a new class of cholinesterase inhibitors. *European Journal of Medicinal Chemistry*. 136:294-304.

Borchiellini, C., Chombard, C., Lafay, B. et al. (2000). *Hydrobiologia.* 420:15.

Boury-Esnault, N., De Vos, L., Donadey, C. and Vacelet, J. (1990) Ultrastructure of choanosome and sponge classification. In: Rützler K (ed) New perspectives in sponge biology. Smithosonian Institution Press, Washington, D.C. pp 237–244

Brain, C. B., Prave, A. R., Hoffman, K.-H., Fallick, A. E., Botha, A., Herd, D. A., Sturrock, C., Young, I., Condon, D. J. and Allison, S. D. (2012). The first animals: Ca. 760 my sponge-like fossils from Namibia. *South African Journal of Science*, 108:658–666.

Carella, M., Agell, G., Cárdenas, P. and Uriz, M. J. (2016). Phylogenetic Reassessment of Antarctic Tellidae (Demospongiae, Tetractinellida) Reveals New Genera and Genetic Similarity among Morphologically Distinct Species. PLoS ONE 11(8):e0167011. https://doi.org/10.1371/journal.pone.0167011

Carte, B. K. (1996). Biomedical potential of marine natural products. *Bioscience.* 46:271-287.

Chombard, C., N. Boury-Esnault & S. Tillier, 1998. Reassesment of homology of morphological charcters in tetractinellid sponges based on molecular data. Syst. Biol. 47: 351–366.

Christen, R., A. Ratto, A. Baroin, R. Perasso, K. G. Grell & A. Adoutte, 1991. An analysis of the origin of metazoans, using comparisons of partial sequences of the 28S rRNA, reveals an early emergence of triploblasts. Embo J. 10: 499–503.

Christensen, A. and Martin, G. D. A. (2017). Identification and bioactive potential of marine microorganisms from selected Florida coastal areas. *Microbiology Open*. 6, e00448.

Clardy, J. (2005). Using genomics to deliver natural products from symbiotic bacteria. *Genome biology*. 6:232.

Copp, B. R., Ireland, C. M., and Barrows, L. R. (2002). Wakayin: a novel cytotoxic pyrroloiminoquinone alkaloid from the ascidian Clavelina species. *Journal of Organic Chemistry*. 56:4596-4597.

Costello, M. J. and Chaudhary, C. (2017). Marine Biodiversity, Biogeography, Deep-Sea Gradients, and Conservation. *Current Biology*. 27:511-527.

Croué, J., West, N. J., Escande, M. L., Intertaglia, L., Lebaron, P., & Suzuki, M. T. (2013). A single betaproteobacterium dominates the microbial community of the crambescidine-containing sponge *Crambe*. *Scientific Reports*, *3*, 2583.

Dams, E., Vandenberghe, A. and de Wachter, R. (1982). Nucleotide sequence of three Poriferan 5S ribosomal RNAs. *Nucl. Acids Res.* 10: 5297-5302.

Davis, R. A., Buchanan, M. S., Duffy, S., Avery, V. M., Charman, S. A., Charman, W. N., ... Quinn, R. J. (2012). Antimalarial activity of pyrroloiminoquinones from the Australian marine sponge Zyzzya sp. *Journal of Medicinal Chemistry*. *55*: 5851-5858.

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match. *Biology Letters*. *10*(9), 20140562. http://doi.org/10.1098/rsbl.2014.0562

Dias, D. A., Urban, S. and Roessner, U. (2012). A Historical overview of natural products in drug history. *Metabolites*. 2:303-336.

Dijoux, M. G., Schnabel, P. C., Hallock, Y. F., Boswell, J. L., Johnson, T. R., Wilson, J. A., Ireland, C. M., van Soest, R., Boyd, M. R., Barrows, L. R. and Cardellina, J. H. (2005). Antitumour activity and distribution of pyrroloiminiqionones in the sponge genus *Zyzzya*. *Bioorg Med Chem.* 13(21):6035-44.

Ding, Q., Chichak, K. and Lown, J. W. (1999). Pyrroloquinoline and pyridoacridine alkaloids from marine sources. *Current Medicinal Chemistry*. 6:1-27.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. *27*:2194-2200.

Ereskovsky, A. V., Gonobobleva, E. and Vishnyakov. A. (2005). Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarca). *Mar. Biol.* 146:869-875.

Erpenbeck, D., Breeuwer, J. A. J. & van Soest, R. W. M. (2005) Implications from a 28S rRNA gene fragment for the phylogenetic relationships of halichondrid sponges (Porifera: Demospongiae). *Journal of Zoological Systematics and Evolutionary Research*. 43: 93-99.

Erpenbeck, D., Breeuwer, J. A. J., Parra, F. J. & van Soest, R. W. M. (2006) Speculation with spiculation? Three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Molecular Phylogenetics and Evolution*. 38: 293-305.

Erpenbeck, D. and Wo¨rheide, G. (2007). On the molecular phylogeny of sponges (Porifera*)* in: Zhang, Z.-Q. & Shear, W.A. (Eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy*. Zootaxa*, 1668: 1–766.

Faulkner, D. J. (1984). Marine natural products: metabolites of marine invertebrates. *Nat. Prod. Rep*. 1:551-598.

Faulkner, D. J. Marine natural products. *Nat Prod Rep*. (2000). 17:7-55.

Fenical, W. and Jensen, P.R. (2006). Developing a new resource for drug discovery: Marine actinomycete bacteria. *Nat. Chem. Biol*. 2:666–673.

Fenical, W. (1993). Chemical studies of marine bacteria: developing a new resource. *Chem Rev*. 93:1673-1683.

Fiore, C., Jarett, J. K., & Lesser, M. P. (2013). Symbiotic prokaryotic communities from different populations of the giant barrel sponge, *Xestospongia muta*. *Microbiology Open*. *2*:938-952.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology,* 3: 294-299.

Friedrich, A. B., Merkert, H., Fendert, T., Hacker, J. Proksch, P. and Hentschel, U. (1999). Microbial diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*) analyzed by fluorescence *in situ* hybridisation (FISH). *Mar. Biol.* 134**:**461-470.

Funayama, N. (2010). The stem cell system in demosponges: Insights into the origin of somatic stem cells. *Develop. Growth Differ*. 52:1-14. doi: 10.1111/j.1440-169X.2009.01162

Garate, L., Sureda, J., Agell, G. and Uriz, M. J. (2017). Endosymbiotic calcifying bacteria across sponge species and oceans. *Scientific Reports*. 7:1-14.

Gauthier, M. E. A, Watson, J. R. and Degnan, S. M. (2016). Draft genomes shed light on the dual bacterial symbiosis that dominates the microbiome of the coral reef sponge Amphimedon queenslandica. *Front Mar Sci*. 3:196.

Gazave, E., Lapébie, P., Renard, E., Vacelet, J., Rocher, C., Ereskovsky, A. V., et al. (2010). Molecular Phylogeny Restores the Supra-Generic Subdivision of Homoscleromorph Sponges (Porifera, Homoscleromorpha). PLoS ONE 5(12): e14290. https://doi.org/10.1371/journal.pone.0014290

Gerwick, W. H. and Moore, B. S. (2012). Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology. *Chemistry and Biology.* 19:1631.

Goey, A. K. L., Chau, C. H., Sissung, T. M., Cook, K. M., Venzon, D. J., Castro, A., Ransom, T. R., Henrich, C. J., McKee, T. C., McMahon, J. B., Grkovic, T., Cadelis, M. M., Copp, B. R., Gustafson, K. R. and Figg, W. D. (2016). Screening and biological effects of marine pyrroloiminoquinone alkaloids: potential inhibitors of the HIF-1α/p300 interaction. *J. Nat. Prod.* 79:1276-1275.

Gunasekera, S. P., Zuleta, I. A., Longley, R. E., Wright, A. E. and Pomponi, S. A. (2003). Discorhabdins S, T, and U, new cytotoxic pyrroloiminoquinones from a deep-water Caribbean sponge of the genus Batzella. *Journal of Natural Products*. 66: 1615-1617.

Halanych, K. M. (1991). 55 ribosomal RNA sequences inappropriate for phylogenetic reconstruction. *Mol. Biol. Evol.* 8: 249-253.

Handelsman J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev*. 68:669-685.

Hart, J. B., Lill, R. E., Hickford, S. J. H., Blunt, J. W. and Munro, M. H. G. (2000). The halichondrins: Chemistry, biology, supply and delivery. In: Fusetani N (ed) Drugs from the sea. Karger, Basel. pp 134-153.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C. and Hacker, J. (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol Ecol*. 35:305-312.

Hentschel, U., Hopke, J., Horn, M., Friedrich, A. B., Wagner, M., Hacker, J. and Moore, B. S. Molecular evidence for a uniform microbial community in sponges from different oceans. (2002). *Appl. Environ. Microbiol.* 68:4431–4440.

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J. and Horn, M. (2003) Microbial diversity of marine sponges. In: Mueller WEG (ed) Sponge (Porifera). Springer, Berlin Heidelberg New York.

Hentschel, U., Piel, J., Degnan, S. M. and Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol.* 10:641-54.

Hill, M., Hill, A., Lopez, N. and Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). *Mar. Biol.* 148:1221-1230.

Hirata, Y. and Uemura, D. (1986). Halichondrins- antitumour polyether macrolides from a marine sponge. *Pure Appl. Chem.* 58(5): 701-710.

Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H. T., ... Kuypers, M. M. (2009). Complex nitrogen cycling in the sponge *Geodia barretti*. *Environmental Microbiology*. 11:2228-2243.

Hooper, J. N. A., and R. W. M. van Soest. 2002. Systema Porifera: a guide to the classification of sponges. Kluwer Academic/Plenum Publishers, New York, NY. pp 15-18.

Hu, J-F., Fan, H., Xiong, J. and Wu, S. (2011). Discorhabdins and pyrroloiminoquinone-related alkaloids. *Chem. Rev.* 117:5465-5491.

Hughes, C. C., MacMillan, J. B., Gaudêncio, S. P., Jensen, P. R., and Fenical, W. (2009). The ammosamides: Structures of cell cycle modulators from a marine-derived Streptomyces species. *Angewandte Chemie International Edition*. 48:725-727.

Huyck, T. K., Gradishar, W., Manuguid, F. and Kirkpatrick, P. (2011). Eribulin mesylate. *Nature Rev. Drug Discov.* 10:173–174.

Isaacs, L., Kan, J., Nguyen, L., Videau, P., Anderson, M. A., Wright, T. L and Hill, R. T. (2009). Comparison of the bacterial communities of wild and captive sponge Clathria prolifera from the Chesapeake Bay. *Mar Biotechnol.* 11:758-770.

Ishibashi, M., Iwasaki, T., Imai, S., Sakamoto, S., Yamaguchi, K., and Ito, A. (2001). Laboratory culture of the myxomycetes: Formation of fruiting bodies of Didymium bahiense and its plasmodial production of makaluvamine A. *Journal of Natural Products*. 64:108-110.

Jeong, I.-H., Kim, K.-H., and Park, J.-S. (2013). Analysis of bacterial diversity in sponges collected off Chujado, an Island in Korea, using barcoded 454 pyrosequencing: analysis of a distinctive sponge group containing Chloroflexi. *J. Microbiol.* 51:570–577. doi: 10.1007/s12275-013-3426-9.

Jimenez, E., & Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnology and Oceanography*. *52*:948-958.

Jones, W. C. (1984). Spicule dimensions as taxonomic criteria in the identification of haplosclerid sponges from the shores of Anglesey. *Zoological Journal of the Linnean Society*. 80:239-259.

Jones, C. G., Lawton, J. H., and Shachak, M. (1997). Positive and negative effects of organisms as physical ecosystem engineers. *Ecology*. 78:1946-1957.

Jordan, P. A. and Moore, B. S. (2016). Biosynthetic pathway connects cryptic ribosomally synthesized posttranslationally modified peptide genes with pyrroloquinoline Alkaloids. *Cel. Chem. Bio*. 23:1504-1514.

Kelly-Borges, M., P. R. Bergquist & P. L. Bergquist, 1991. Phylogenetic relationships within the order Hadromerida (Porifera, Demospongiae, Tetractinomorpha) as indicated by ribosomal RNA sequence comparisons. Biochem. Syst. Ecol. 19: 117–125.

Kelly-Borges, M., Pomponi, S. A. (1994). Phylogeny and classification of lithistid sponges (Porifera: Demospongiae): a preliminary assessment using ribosomal DNA sequence comparisons. *Molecular Marine Biology and Biotechnology*. 3:87-103.

Keren, R., Mayzel, B., Lavy, A., Polishchuk, I., Levy, D., Fakra, S. C., Pokroy, B. and Ilan, M. (2017). Sponge-associated bacteria mineralize arsenic and barium on intracellular vesicles. *Nature communications*. 8:1-12.

Komiya H. et al. (1983). Nucleotide sequences of 5s rRNAs from sponge *Halichondria japonica* and tunicate *Halocynthia roretzi* and their phylogenetic positions. *Nucleic Acids Res*. 11:1969-1974.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution.* 33:1870-1874.

Lafay, B., Boury-Esnault, N., Vacelet, J., and Christen, R. (1992). An analysis of partial 28S ribosomal RNA sequences suggests early radiations of sponges. *Biosystems*. 28:139-51.

Lafi, F. F., Fuerst, J. A., Fieseler, L., Engels, C., Wei Ling Goh, W. and Hentschel, U. (2009). Widespread distribution of poribacteria in Demospongiae. *Appl. Environ. Microbiol*. 75:5695–5699.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T.J., Higgins, D. G. (2007). ClustalW and ClustalX version 2, Bioinformatics, 23(21), 2947 – 2948. DOI: 10.1093/bioinformatics/btm404

Leal, M. C., Puga, J., Serodio, J., Gomes, N. C. M. and Calado, R. (2012) Trends in the discovery of new marine natural products from invertebrates over the last two decades - where and what are we bioprospecting? *PLoS ONE* **7**, e30580.

Leys, S. P., Yahel, G., Reidenbach, M. A., Tunnicliffe, V., Shavit, U., Reiswig, H. M. (2011). The Sponge Pump: The Role of Current Induced Flow in the Design of the Sponge Body Plan. 6:1-17. PLoS ONE6(12): e27787. https://doi.org/10.1371/journal.pone.0027787

Lin, S., McCauley, E. P., Lorig-Roach, N., Tenney, K., Naphen, C. N., Yang, A-M., Johnson, T. A., Hernadez, T., Rattan, R., Valeriote, F. and Crews, P. (2017). Another look at pyrroloiminoquinone alkaloids- perspectives on their therapeutic potential from known structures and semisynthetic analogues. *Mar. Drugs*. 15:1-18.

Macintyre, L., Zhang, T., Viegelmann, C., Martinez, I. G., Cheng, C., Dowdells, C., Abdelmohsen, U. R., Gernert, C., Hentschel, U., and Edrada-Ebel, R. (2014). Metabolomic tools for secondary metabolite discovery from marine microbial symbionts*. Mar. Drugs*. 12:3416-3448.

Maloof, A. C., Rose, C. V., Beach, R., Samuels, B. M., Calmet, C. C., Erwin, D. H., Poirier, G. R., Yao, N. and Simons, F. J. (2010). Possible animal-body fossils in pre-Marinoan limestones from South Australia. *Nat Geosci*. 3(9):653–659. http://dx.doi.org/10.1038/ngeo934

Malve, H. (2016). Exploring the ocean for new drug developments: Marine pharmacology. *Journal of Pharmacy & Bioallied Sciences*. *8*(2):83–91. http://doi.org/10.4103/0975-7406.171700

Matcher, G., Dorrington, R., Henninger, T., and Froneman, P. (2011). Insights into the bacterial diversity in a freshwater-deprived permanently open Eastern Cape estuary, using 16S rRNA pyrosequencing analysis. *Water Sa. 37*:381-390.

Mehbub, M. F., Lei, J., Franco, C., and Zhang, W. (2014). Marine sponge derived natural products between 2001 and 2010: Trends and opportunities for discovery of bioactives. *Marine Drugs*. *12*:4539-4577.

Miyanaga, A., Janso, J. E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustaì Quio, A. S., Fielding, E. N., Carter, G. T., Jensen, P. R., Feng, X., Leighton, M., Koehn, F. E. and Moore, B. S. (2011). Discovery and assembly-line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in Salinispora bacteria. *Journal of the American Chemical Society*. 133:13311-13313.

Mohan, G., Thangappanpillai, A. K. and Ramasamy, B. (2016). Antimicrobial activities of secondary metabolites and phylogenetic study of sponge endosymbiotic bacteria, *Bacillus* sp. at Agatti Island, Lakshadweep Archipelago. *Biotechnology reports*. 11:44-52.

Moitinho-Silva, L., Nielsen, S., Amir, A., Gonzalez, A., Ackermann, G. L., Cerrano, C., … Thomas, T. (2017). The sponge microbiome project. *GigaScience*. 10:1-7. http://doi.org/10.1093/gigascience/gix077.

Moran, N. A. (2002). Microbial minimalism: genome reduction in bacterial pathogens. *Cell* 108:583-586. http://dx.doi.org/10.1016/S0092 -8674(02)00665-7.

Montalvo, N. F., Mohamed, N. M., Enticknap, J. J. and Hill. R. T. (2005). Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek*. 87:29-36

Montaser, R. and Luesch, H. (2011). Marine natural products: a new wave of drugs? *Future Medicinal Chemistry*. 3:1475-1489. http://doi.org/10.4155/fmc.11.118

Müller, W. E. G. (2003). The Origin of Metazoan Complexity: Porifera as Integrated Animals, *Integrative and Comparative Biology*. (43):3-10. https://doi.org/10.1093/icb/43.1.3

Munro, M. H., Blunt, J. W., Dumdei, E. J., Hickford, S. J., Lill, R. E., Li, S., Battershill, C. N. and Duckworth, A. R. (1999). The discovery and development of marine compounds with pharmaceutical potential. *J. Biotechnol.* 70:15-25.

Muricy, G. and Ribeiro, S. M. (1999). Shallow-water Haplosclerida (Porifera, Demospongiae) from Rio de Janeiro state, Brazil (Southwestern Atlantic). *Beaufortia* 49: 47-60.

Murti, Y. and Agrawal, T. (2010). Marine derived pharmaceuticals development of natural health products from marine biodiversity. *International Journal of Chem Tech Research.* 2:2198-2217.

Muscholl-Silberhorn, A., Thiel, V. and Imhoff, J. F. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microb. Ecol.* 55:94-106.

Nagata, H., Ochiai, K., Aotani, Y., Ando, K., Yoshida, M., Takahashi, I. and Tamaoki, T. J. (1997). Lymphostin (LK6-A), a novel immunosuppressant from Streptomyces sp. KY11783: Taxonomy of the producing organism, fermentation, isolation and biological activities. *The Journal of Antibiotics*. 50:537-542.

Neulinger, S. C., Stöhr, R., Thiel, V., Schmaljohann, R., and Imhoff, J. F. (2010). New phylogenetic lineages of the Spirochaetes phylum associated with *Clathrina* species (Porifera). *The Journal of Microbiology*. 48:411-418.

Newman D. J, Cragg, G. M. (2012). Natural Products as Sources of New Drugs over the 30 Years from 1981 to 2010. *Journal of natural products*. 75:311-335.

Newman, D. J. and Hill, R. T. (2005). New drugs from marine microbes: the tide is turning. *J. Ind. Microbiol. Biotechnol. 33*:539-544.

Nguyen, M., Liu, M. and Thomas, T. (2013). Ankyrin-repeat proteins from sponge symbionts modulate amoebal phagocytosis. *Mol. Ecol.* 23:1635-1645.

Olson, J. B. and McCarthy, P. J. (2005). Associated bacterial communities of two deep-water sponges. *Aquat. Microb. Ecol.* 39: 47–55.

Perry, N. B., Battershill C. N., Blunt J. W., Fenwick G. D., Munro M. H. G., and Bergquist P. R. (1988), Occurrence of variabilin in New Zealand sponges of the order Dictyoceratida. *Biochem. Syst. Ecol.* 15: 373-376.

Piel, J. (2006). Bacterial symbionts: Prospects for the sustainable production of invertebrate-derived pharmaceuticals. *Curr. Med. Chem.* 13:39–50.

Proksch, P., Edrada, R. and Ebel, R. (2002). Drugs from the seas-Current status and microbiological implications. *Appl. Microbiol. Biotechnol.* 59: 125-134.

Prosser, J. I., Head, I. M., and Stein, L. Y. (2014). The Family *Nitrosomonadaceae*. In Rosenberg, E., DeLong, E. F. Lory, S., Stackebrandt, E. and Thompson, F. *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria* Berlin, Heidelberg: Springer Berlin Heidelberg. 901-918.

Reiswig, H. M. (1975). Bacteria as food for temperate-water marine sponges. *Can. J. Zool.* 53:582–589.

Riisgård, H, U and Larsen, P. S. (2010). Particle-capture mechanisms in suspension-feeding invertebrates. *Marine Ecology - Progress Series*. 418:255-293.

Rzhetsky, A. (1995). Estimating substitution rates in ribosomal RNA genes. *Genetics*. 141:771-783.

Samaai, T. and Kelly, M. (2002) Family Latrunculiidae. In Hooper J.N.A. and Van Soest R.W.M. (eds) Systema Porifera. A guide to the supraspecific classification of sponges. Volume 1. New York: Kluwer Academic/Plenum Publishers, pp. 708–719.

Samaai, T., Janson, L., & Kelly, M. (2012). New species of *Latrunculia* from the Agulhas shelf, South Africa, with designation of a type species for subgenus *Biannulata* (Demospongiae, Poecilosclerida, Latrunculiidae). Zootaxa. *3395*: 33-45.

Santavy, D. L., and Colwell, R. R. (1990). Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater. *Mar. Ecol. Prog. Ser.* 67:73-82.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environment Microbiology. 75*: 7537-7541.

Schmidt, E., Obraztsova, A., Davidson, S., Faulkner, D. J. and Haygood, M. G. (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge Theonella swinhoei as a novel δ-proteobacterium, “Candidatus Entotheonella palauensis”. *Marine Biology*. 136: 969-977.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., et al. (2012). Assessing the complex sponge microbiota: core, variable and species specific bacterial communities in marine sponges. *ISME J.* 6:564–576. doi: 10.1038/ismej.2011.116.

Sciscioli, M., Lepore, E., Gherardi, M. and Liaci, L. S. (1994). Transfer of symbiotic bacteria in the mature oocyte of Geodia cydonium: (Porifera, Demospongiae): an ultrastructural study. *Cah. Biol. Mar.* 35:471-478.

Sethman, I., Wo¨rheide, G., 2008. Micron 39, 209–228.

Sharp, K. H., B. Eam, D. J. Faulkner, and M. G. Haygood. (2007). Vertical transmission of diverse microbes in the tropical sponge Corticium sp. *Appl. Environ. Microbiol.* 73:622–629.

Simister, R. L., Deines, P., Botte, E. S., Webster, N. S. and Taylor, M. W. (2012). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environ. Microbiol.* 14:517–524.

Simmons, T. L., Andrianasolo, E., Mcphail, K., Flatt, P. and Gerwick, W. H. (2005). Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics.* 4:333-342.

Sipkema, D., de Caralt, S., Morillo, J. A., Al-Soud, W. A., Sorensen, S. J. Smidt, H. and Uriz, M. J. (2015). Similar sponge-associated bacteria can be acquired via both vertical and horizontal transmission. *Environ. Microbiol.* 17:3807-3821.

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M, Neal, P. R., Arrieta, J. M. and Herndl, G. (2006). Microbial diversity in the deep sea and the underexplored 'rare biosphere'. *Proc Natl Acad Sci USA.* 103:12115-12120.

Steger, D., Ettinger-Epstein, P., Whalan, S., Hentschel, U., de Nys, R. and Wagner, M. (2008). Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges. *Environ Microbiol.* 10:1087-1094

Sun, H. H., Sakemi, S., Burres, N. and McCarthy, P. (1990). Isobatzellines A, B, C, and D. Cytotoxic and antifungal pyrroloquinoline alkaloids from the marine sponge Batzella sp. *J. Org. Chem.* 55:4964-4966.

Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D. and Clardy, J. (1981). Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria. *J Am Chem Soc.* 103: 2469-71.

Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution.*10:512-526.

Taylor, M. W., Radax, R., Steger, D., and Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology and Biotechnological Potential. *Microbiology and Molecular Biology Reviews.* *71*(2): 295-347. http://doi.org/10.1128/MMBR.00040-06

Taylor, M. W., Tsai, P., Simister, R. L., Deines, P., Botte, E., Ericson, G., Schmitt, S. and Webster, N. S. (2013). 'Sponge-specific' bacteria are widespread (but rare) in diverse marine environments. *The ISME Journal*. *7*:438–443.

Thacker, R. W., Hill, A. L., Hill, M. S., Redmond, N. E., Collins, A. G., Morrow, C. C., Spicer, L., Carmack, C. A., Zappe, M. E., Pohlmann, D., Hall, C., Diaz, M. C. and

Bangalore, P. V. (2013). Nearly complete 28S rRNA gene sequences confirm new hypotheses of sponge evolution. *Integr. Comp. Biol.* 53:373-387.

Thomas, T., Rusch, D., DeMaere, M. Z., Yung, P. Y., Lewis, M., Halpern, A., Heidelberg, K. B., Egan, S., Steinberg, P. D. and Kjelleberg, S. (2010). Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *Int. Soc. Mic. Ecol.* 4:1557-1567.

Thoms, C. and Schupp, P. (2005). Biotechnological potential of sponges and their associated bacteria as producers of new pharmaceuticals (Part II). *Journal of International Biotechnology Law.* 6:257-264.

Unson, M. D., Holland, N. D. and Faulkner, D. J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar. Biol.* 119:1-11.

Uriz, M. J. (2006). Mineral skeletogenesis in sponges. *Can. J. Zool.* 84:322-356.

Uriz, M. J., Turon, X., Becerro, M., and Agell, G. (2003). Siliceous spicules and skeleton frameworks in sponges: origin, diversity, ultrastructural patterns and biological functions. *Microsc. Res. Tech.* 62: 279-299.

Vacelet J, Donadey C (1977) Electron-microscope study of association between some sponges and bacteria. *J Exp Mar Biol Ecol.* 30:301–314.

Vacelet, J., Boury-Esnault, N., Fiala-Medioni, A and Fisher, C. R. (1995). A methanotrophic carnivorous sponge. *Nature.* 377:296.

Van Soest, R. (2015). Latrunculiidae Topsent, 1922. In R. W. M. Van Soest, N. Boury-Esnault, J. N. A. Hooper, K. Rützler, de Voogd N. J., de Alvarez Glasby B., E. Hajdu, A. B. Pisera, R. Manconi, C. Schoenberg, D. Janussen, K. R. Tabachnick, M. Klautau, B. Picton, M. Kelly, J. Vacelet, M. Dohrmann, M. –C. Díaz & P. Cárdenas. World Porifera database. Accessed through: World Register of Marine Species at http://www. marinespecies.org/aphia.php?p=taxdetails&id=131671.

Walmsley, T. A., Matcher, G. F., Zhang, F., Hill, R. T., Davies-Coleman, M. T. and Dorrington, R. A. (2012). Diversity of bacterial communities associated with the Indian Ocean sponge Tsitsikamma favus that contains the bioactive pyrroloiminoquinones, Tsitsikammamine A and B. *Marine Biotechnology.* 14:681-691.

Wang, Y. and Qian, P. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomics studies. PLoS ONE, 4, e7401.

Wang, Q., Garrity, G. M. Tiedje, J. M. and Cole, J. R. (2007). Naïve Bayesian Classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environment Microbiology. 73:5261-5267.

Waterworth, S. C., Jiwaji, M., Kalinski, J-C. J., Parker-Nance, S. and Dorrington, R. A. (2017). A place to call home: An analysis of the bacterial communities in two Tethya rubra Samaai and Gibbons 2005 populations in Algoa Bay, South Africa. *Mar. Drugs*. *15*(4):95. doi:10.3390/md15040095

Webster, N. S., and Hill. R. T. (2001). The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-proteobacterium. *Mar. Biol*. 138**:**843-851.

Webster, N. S. and Taylor, M. W. (2012). Marine sponges and their microbial symbionts:love and other relationships. *Environ Microbiol*. 14:335-346. http://dx.doi.org/10.1111/j.1462-2920.2011.02460.

Webster, N. S., Taylor, M. W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. and Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environmental Microbiology, 12: 2070-2082.

Webster, N. S. and Thomas, T. (2016). The sponge hologenome. *MBio*. 7:e00135-16.

Wehrl, M., Steinert, M. and Hentschel, U. (2007). Bacterial uptake by the marine sponge Aplysina aerophoba. *Microb. Ecol*. 53:355-365.

Weisz, J.B., Lindquist, N., and Martens, C.S. (2008) Do associated microbial abundances impact marine desmosponge pumping rates and tissue densities? Oecologia 155:367– 376.

Weinheimer, A. J. and Spraggins, R. L. (1969). The occurrence of two new prostaglandin derivatives (15-*epi*-PGA and its acetate, methyl ester) in the Gorgonian *Plexaura homomalla* chemistry of Coelenterates. XV. *Tetrahedron Lett.* 10, 5185-5188.

Wilkinson, C. R. (1978). Microbial associations in sponges. II. Numerical analysis of sponge and water bacterial populations. *Mar. Biol.* 49:169–176.

Wilkinson, C. R. (1983). Net primary productivity in coral reef sponges. *Science.* 219:410–412.

Wilkinson, C. R., Garrone, R. and Vacelet, J. (1984). Marine sponges discriminate between food bacteria and bacterial symbionts: electron microscope radioautography and *in situ* evidence. *Proc. R. Soc. Lond.* 220:519-528.

Wörheide, G., Erpenbeck, D. and Menke, C. (2008). The Sponge Barcoding Project: aiding in the identification and description of poriferan taxa. Porifera Research: Biodiversity, Innovation & Sustainability. Rio de Janeiro: Museu Nacional de Rio de Janiero Book Series, 123âA ¸S128.

Xi, L., Ruan, J. and Huang, Y. (2012). Diversity and biosynthetic potential of culturable actinomycetes associated with marine sponges in the China seas. *Int. J. Mol. Sci.* 13:5917-5932.

Appendix A

New species in the genus *Tsitsikamma* (Poecilosclerida, Latrunculiidae) from South Africa.

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Abstract

Three species of the genus *Tsitsikamma* (Latrunculiidae) are known from the South African coast: *Tsitsikamma favus* collected from the Garden Route National Park, Tsitsikamma Marine Protected Area (MPA), *T. pedunculata* collected off Cape Recife in St. Francis Bay and *T. scurra* from a wreck site in a small bay west of Hout Bay on the west coast of South Africa. Two new species: *Tsitsikamma michaeli*, a characteristic small green purse-like species from Algoa Bay and *Tsitsikamma ngunii,* from The Garden Route National Park, Tsitsikamma MPA are described. Supplementary morphological characteristics, spicule morphology and distribution records are supplied for *T. favus* and *T. pedunculata* from Algoa Bay. The phylogenetic relationship of these five *Tsitsikamma* species is investigated.

Key words:
28S rDNA, Algoa Bay, Latranculiidae, South Africa, St. Francis Bay, Tsitsikamma, Western Indian Ocean

Introduction

The family Latrunculiidae Topsent, 1922 consists of seven genera; *Bomba* Reiswig and Samaai, 2016, *Cyclacanthia* Samaai, Govender and Kelly, 2004, *Latrunclava* Kelly, Reiswig and Samaai, 2016, *Latrunculia* du Bocage, 1869, *Sceptrella* Schmidt, 1870, *Strongylodesma* Lévi, 1969 and *Tsitsikamma* Samaai and Kelly, 2002. The genus *Latrunculia* incorporates three sub-genera *Biannulata* Samaai et al., 2006, *Latrunculia* du Bogage, 1969 (Samaai et al. 2003, 2006, 2012) and *Uniannulata* Kelly, Reiswig and Samaai, 2016 (Kelly et al. 2016). The genera *Cyclacanthia, Latrunculia, Strongylodesma* and *Tsitsikamma* have known South African species. The genus *Tsitsikamma*, named after the type locality Tsitsikamma, a Marine Protected Area that forms part of The Garden Route National Park, includes three known species; *Tsitsikamma favus* from Tsitsikamma MPA, *T. pedunculata* from St. Francis Bay, western Indian Ocean, and *T. scurra* collected west of Hout Bay on the Atlantic side of the Cape Peninsula.

The two different morphologically growth forms within this genus are also phylogenetically distinct (Fig. 6). The ‘favus group’ represent thick encrusting or hemispherical species with large attachment areas and a choanosome structurally reinforced by interior tracts (Samaai and Kelly 2002). The ‘pedunculata group’ represents species that are attached to the bottom by a narrow peduncle supporting a rounded head without any reinforcing tracts subdividing the interior. In general, the genus is characterized by cone shaped oscules and areolate porefields distributed over the sponge surface. The interior choanosome is airy and contrasts heavily with the densely spiculate ectosome and, when present, the internal tracts that penetrate and divide the interior choanosome into smaller chambers. Anisostyle megascleres and chiadiscorhabd microscleres are the characteristic skeletal components.

Material and methods

Collections were made using SCUBA or Remote Operated Vehicle (ROV). All new collections are from the Agulhas Ecoregion, along the Western Cape and Eastern Cape Province coasts of South Africa, between depths of 18-40 meters. All sponges collected were preserved in 70 % ethanol or frozen. Photographic records were collected of *in situ*, freshly collected and preserved specimens where possible. Type specimens and reference material are lodged with the South African Institute for Aquatic Biodiversity (SAIAB). All specimens were collected by Shirley Parker-Nance except where otherwise indicated. Table 1 provides supplementary collection locality information.

Chiadiscorhabd microsclere morphology changes as the spicules develop (Samaai and Kelly 2002) and vary intra and interspecifically. Measurement of the spicule shaft length and width, the apical whorl and manubrium optical diameter and the total length of the spicules were made to quantify differences. The largest of 40 megascleres and 20 microsclere present in 20 images taken from permanent prepared slides were used to define size attributes. A distinction is made between the smaller apical terminal whorls and a larger manubrium or basal whorl and both are provided for the microscleres measured.

Sequences were constructed of approximately 629 base pair fragments of 28S nuclear ribosomal genes using primers SP18cF and SP18dR. Multiple sequences were aligned using Clustal Omega (Sievers et al. 2011) and molecular phylogenetic analysis was done by the maximum likelihood method based on the Jukes-Cantor model (Junks and Cantor, 1969). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) were obtained by applying Neighbor-Join and BioNJ algorithms using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, the analysis involved 13 nucleotide sequences, there were a total of 599 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al 2016). Table 2 provides catalogue information of material used in phylogenetic tree contraction.

Taxonomy

Class Demospongiae Sollas, 1885
Order Poecilosclerida Topsent, 1928
Family Latrunculiidae, Topsent, 1922
Genus *Tsitsikamma* Samaai and Kelly, 2002
The favus group
    Tsitsikamma favus Samaai and Kelly, 2002
    Tsitsikamma ngunii Parker-Nance, sp. nov.
    Tsitsikamma scurra Gibbons, Kelly and Davies-Coleman, 2003
The pedunculata group
    Tsitsikamma pedunculata Samaai, Gibbons, Kelly and Davies-Coleman, 2003
    Tsitsikamma michaeli Parker-Nance, sp. nov.

Tsitsikamma favus Samaai and Kelly, 2002

(Fig. 1a-p)

Tsitsikamma favus Samaai and Kelly, 2002: p. 718, Fig. 6 A-G. Samaai, Gibbons, Kelly & Davies-Coleman, 2003: p. 19.

Type-locality

Western Cape Province, Garden Route National Park, Tsitsikamma, Rheeders Reef, BMNH1997.7.3.2, 1995, 22 m depth, collected by Philip Coetzee, Holotype; BMNH, several specimens from Rheeders Reef.

Western Cape Province, Garden Route National Park, Tsitsikamma, The Knoll, Tsitsikamma National Park, as per Samaai and Kelly, 2002, Paratypes.

Material examined

Western Cape Province, Garden Route National Park, Tsitsikamma, The Knoll: SAIAB 141112, 2 May 1993, 18 m depth, collected by Colin Buxton, 3 specimens.

Western Cape Province, Garden Route National Park, Tsitsikamma, Rheeders Reef: TIC1994-219, 25 May 1994, 30 m depth, collected by John Allen and Steve Brower, 9 specimens; SAIAB141356, 18 March 1995, 22 m depth, collected by Rob Palmer, Brad Carté and Philip Coetzee, 2 specimens, material collected at same locality and time as type material; TIC1994-216A and TIC1994-218A, 25 May 1994, 25-30 m depth, collected by John Allen and Steve Brower.

Eastern Cape Province, Algoa Bay, Bell Buoy Reef, Shark Alley: TIC2016-054, 5 June 2016, 9-10 m depth.

Eastern Cape Province, Algoa Bay, Whitesands Reef: TIC2001-080, 18 May 2001, 20 m depth; TIC2002-476, 20 March 2002, 15 m depth, collected by Scripps.

Eastern Cape Province, Algoa Bay, Evans Peak: TIC2009-004A, TIC2009-005A, TIC2009-007A and TIC2009-008, 15 May 2009, 25-30 m depth; TIC2009-070A, May 2009, 30 m depth; TIC2010-003 to 004, 10 October 2010, 25-33 m depth; TIC2014-001, 3 August 2014, 22-30 m depth, 10 specimens; TIC2015-025 and TIC2015-026, 6 September 2015, 30 m depth; TIC2016-050, 2 June 2016, 15-20 m depth, collected by Thomas Bornman, Shaun Deyzel and Shirley Parker-Nance, several specimens.

Eastern Cape Province, Algoa Bay, RIY Banks: TIC1999-535 and TIC1999-536, 23 February 1999, 28m, collected by CRRF; TIC2012-053 to 055, 057 and 058, 14 December 2012, 25-30 m depth.

Additional material included

Western Cape Province, Plettenberg Bay, Grootbank Reef: 0CDN7241-G, 22 March 2000, 10-13 m, collected by CRRF, identified by Michelle Kelly.

Eastern Cape Province, Algoa Bay, Table Top Reef: 0CDN6068-Y, 4 October 1998, 16 m depth, collected by CRRF, identified by Michelle Kelly.

Eastern Cape Province, Algoa Bay, White Sands Reef, 0CDN6384-X, 14 February 1999, 14 m depth, collected by CRRF, identified by Lori J Bell Colin.

Description

Firm, dark brown hemispherical sponge, up to 15 cm high and 20 cm in diameter, sessile with a large area of attachment. Surface smooth and firm although undulant presenting a folded appearance is some specimens (Fig. 1a-c). Oscules are lance, triangular or volcano shaped with a wide base, laterally flattened and tapering to a small opening, 2-8 mm high and 1.5-8 mm wide at the base, laterally flattened in collected and preserved specimens, are located on the upper half of the sponge. The stalked areolate porefields, mushroom shaped *in situ*, are 1-

10 mm high and 2-14 mm in diameter (Fig. 1d). Chiadiscorhabds line the outer surface of the extosome mostly arranged perpendicular to the underling megascleres (Fig. 1e). The extosome 660(430-1120) µm thick, reinforced by styles is generally thinner than the tracts that penetrate and divide the interior choanosome. The tracts are 1200(740-1780) µm and form elaborate chambers of 5110(3610-8460) µm in diameter, of soft choanosome (Fig.1f).

Megascleres are irregularly arranged within the choanosome to form the elliptical shaped meshes 255-300 µm in diameter. Slightly sinuous styles, hastate, mucronate or blunt, occasionally tylote are present in two categories; long thick centrally thickened styles 570(420-788) x 14(9-19) µm and long very thin styles 598(449-907) x 10(3-16) µm (Fig. 1g-j) (n = 520). Short thick anisotrongyles 494(139-751) x 13(8-21) µm, may be present (Fig.1k) (n = 42). Microcleres consist of chiadiscorhabds with a total length of 53(40-68) µm. The shaft is 43(29-59) µm long and 9(6-14) µm thick. Chiadiscorhabds, generally have three, but can have up to five complete whorls, with many intermediate forms. The terminal end may or may not have a similar tubercle arrangement (Fig. 1l-p). Typically the manubrium is 26(19-37) µm and the apical whorl is 24(17-34) µm (n = 280) in optical transverse section. Chiadiscorhabds with the terminal tubercles arranged in such a way to give a flattened appearance are also present (basal whorl in Fig. 1o).

Distribution: Plettenberg Bay, Tsitsikamma Marine Protected Area and Algoa Bay, 9-33 m.

Discussion

Examined material compares well with the type description given by Samaai and Kelly (2002). Re-examination of the duplicate type material and additional material collected from a wider geographical distribution and depth range confirm intraspecific variation in the shape and size of the chiadiscorhabds. Deviation from the more symmetrically formed microscleres or isochiadiscorhabds, as described by Samaai and Kelly (2002), to chiadiscorhabds with apical and basal whorl that differ in size and tubercle arrangement seem to occur particularly in specimens collected over the summer months, in Algoa Bay. This may be a response to various environmental factors such as localized upwelling (Uriz et al. 2003).

Live or freshly collected specimens are dark brown, olive or army green in colour and may be heavily encrusted with soft corals, hydroids, ascidians and other encrusting sponges with the oscules and porefields protruding through the surface epibionts. As freshly collected specimens are preserved, the extract dye the preservative (70%) a deep brown colour, this intensifies to almost black while the tissue lightens, long exposure to the stained preservative darkens the tissue again. Successive preservative changes (long-term curated specimens) removes the pigment and the specimens are beige in colour. Frozen material may be dark slate green to tan externally, the tracks are prominently tan and the choanosome dark brown.

Tsitsikamma ngunii **Parker-Nance, sp. n.**

(Fig. 2)

Type material examined

Western Cape Province, Garden Route National Park, Tsitsikamma, Rheeders Reef: TIC2015-003B, 8 June 2015, 20 - 21 m depth, Holotype; TIC2015-006B, 9 June 2015, 20 - 21 m depth, Paratype.

Material examined

Western Cape Province, Garden Route National Park, Tsitsikamma, Rheeders Reef: TIC2015-003A, TIC2015-006A, 8 June 2015, 20-21 m depth.
Western Cape Province, Garden Route National Park, Tsitsikamma, The Knoll: TIC1993-100, 2 May 1993, 18 m, collected Colin Buxton.

Description

Thick large encrusting or sessile hemispherical or convex cushions, dark slate-coloured when alive but black brown to black in preservative. The sponge is very firm and rigid, 3-6 cm high and 3-10 cm in diameter (Fig. 2a-d). The upper third to half of the sponge surface is dominated by small short, blunt rounded knob shaped or button-like oscules, 2-5 mm high and 2.5-5 mm wide at the base. The surface surrounding the upper osculate area, the shoulder and upper side of the sponge, has well-spaced small round almost slightly elevated or sessile porefields. These gradually merge to form larger round porefields that join together to form irregular or blotch shaped structures. In general porefields are 1-4 mm high and 3-14mm in diameter. The ectosome is 780(430-1560) µm thick guarded externally by a prominent palisade of microscleres arranged perpendicularly to the prominent inner style layer. The softer choanosome is divided into small uneven circular to oval shaped chambers 6640(2290-19770) µm in diameter by reinforcing tracts, 1410(530-3200) µm thick (Fig. 2e). Sand particles and shell fragments may be incorporated in the sponge tissue.

Megascleres are slightly sinuous or curved, hastate or mucronate styles, in two size categories. The thick styles are robust and conspicuously centrally thickened 555(428-672) x 14(10-19) µm while the thinner styles may be very long 561(449-832) x10(3-14) µm (Fig. 2f-g). Occasionally short thick strongyles or anisostrongyles are present 463(287-552) x 14(7-21) µm (Fig. 2h). Microscleres are chiadiscorhabds generally with three whorls, although intermediate partial whorls or tubercular projections are not uncommon and spicules with two intermediate whorls also present. Chiadiscorhabds are 51(40-60) µm in total length, with a shaft measuring 42(34-54) x 9(6-13) µm. The basal whorl (manubrium) measures 25(18-37) µm and the apical whorls 23(16-32) µm in optical transverse section (Fig. 2i-l). Whorls are constructed of acanthoses cone shaped tubercles arranged in groups of two to four, the apical whorl and manubrium differ in size and generally in arrangement of the tubercles.

Etymology

The Nguni is a cattle breed unique to southern Africa with characteristic dappled colour and blotchy patterns on the hide. This uneven patterning evident in the shape of the elaborate blotch-shape areolate porefields is typical of this species and most prevalent in larger specimens.

Distribution

Tsitsikamma Marine Protected Area, 18-21m.

Discussion,

In life the sponge appears a dark slate or grey colour. Freshly collected specimens consist of the dark olive brown exterior with dark brown surface structures. The interior tracts are light olive cartilaginous with softer withdrawn olive brown choanosome. Preserved specimens are a uniform dark brown in colour with the preservative (70% ethanol) a deep rich brown almost black colour. *Tsitsikamma favus* and *T. ngunii* have similar partitioning of the choanosome and although that of *T. ngunii* forms larger more regular chambers with generally thicker tracks and the sponge is notably firmer, it is the slender stalked porefields of *T. favus* in contrast to the large sessile often elaborate porefields and generally smooth clean surface appearance that sets *T. ngunii* apart.

Tsitsikamma pedunculata **Samaai, Gibbons, Kelly and Davies-Coleman, 2003**

(Fig. 3)

Type Locality

Eastern Cape Province, St. Francis Bay, Thunderbolt Reef off Cape Recife, 25 February 1999, -34.05237, 25.68925, 40 m, collected by P.L. Colin, CRRF 0CDN 6451-V, BMNH 2003.1.10.2 (Samaai et al. 2003).

Material examined

Eastern Cape Province, Algoa Bay, Evans Peak. TIC2015-205, TIC2015-206 (30 October 2015, 34-38 m, collected by Ryan Palmer and Shirley-Parker-Nance, SeaEye ROV from the uKwabelana research vessel, TIC2015-216, TIC2015-217, TIC2015-218, TIC2015-219, 12 November 2015, 30-34 m, collected by Ryan Palmer and Shirley-Parker-Nance, SeaEye ROV from the uKwabelana research vessel, Algoa Bay / St. Francis Bay, TIC2002-550, 5 November 2002, specific collection site unknown.

Description

Characteristic dirty pink, pink brown pedunculate species with well-defined head up to 7 cm in diameter on a narrow stalk, 1-3 cm wide and up to 7 cm long. Living sponges appear dirty pink although this is often obscured by epibionts, especially the yellow encrusting *Mycale* (*Mycale*) sponge also found growing on other members of this genus (Fig. 3a-b). Freshly collected material is a dusty pink to pink brown to dark purple while preserved material has an olive green, cream to tan colour (Fig. 3c). Small well-spaced cone-shaped oscules 1.5-2 mm high and 1.5-3 mm in diameter are present over the upper part of the head gradually replaced by small to bigger elevated areolate porefields, 1-4.5 mm high and 2-7.5 mm in diameter, toward the base where the stalk is attached. In preserved specimens the oscules retain their shape but the upper border of the porefields contracts inwards giving it a button like appearance. The resistant ectosome 818(200-1800) µm encapsulate soft choanosome with delicate tracts (Samaai et al. 2003) (Fig. 3d-e). The stalk consists of densely arranged spicules and has a vascular appearance in section (Fig. 3f).

Spicules

The skeletal structure of the sponge consists of two classes of styles slightly sinuous, robust centrally thickened, acerate, conical, hastate or somewhat blunt even mucronate styles, 636(541-788) x 15(12-17) µm and thin conspicuously sinuous and sometimes conspicuously thickened centrally styles 673(562-798) x 11(4-15) µm, n = 160 (Fig. 3g-h). Chiadiscorhabds have only two terminal whorls and a total length of 29(26-34) µm and a shaft length of 19(16-24) x 7(5-9) µm. The large manubrium measures 23(19-27) µm and the conspicuously smaller apex whorl is 19(12-24) µm in optical transverse section. The tubercles are acanthose and arranged in a pincushion-like manner on the apical head of the microsclere (Fig. 3i-l).

Distribution. Algoa Bay and St. Francis Bay deep reef systems, 34-40 m deep.

Discussion

The shape of the sponge, the long peduncle, round head, colour and the shape of the microscleres set this species well apart from any other species in this genus. All specimens collected were attached to rock on the sides of medium profile reef adjacent to sandy gullies at a depth between 33-40 m.

Tsisikamma michaeli Parker-Nance, sp. n.

(Fig. 4)

Type material

Holotype. Eastern Cape Province, Algoa Bay. Evans Peak, TIC2015-210, 12 November 2015, 30-34 m, Paratype. Eastern Cape Province, Algoa Bay, Evans Peak, TIC2009-009, 15 May 2009, 25-30 m.

Material examined

Eastern Cape Province, Algoa Bay; Evans Peak. TIC2010-001, TIC2010-002, 10 October 2010, 25-33 m; TIC2015-201A, TIC2015-202A, TIC2015-203, TIC2015 – 204, 30 October 2015, 30-34 m, TIC2011-102, Algoa Bay/St. Francis Bay specific collection site unknown, April 2011, 30 m, TIC2002-552, 5 November 2002.

Description

Small olive green, purse shaped sponge up to 5 cm high (2cm stalk and 3 cm rounded head) or sessile, 5-10 cm in diameter. In some cases the large sponge may be loosely subdivided into sections (Fig. 4a-c). The soft delicate, bright green, interior choanosome is encapsulated by a protected firm resilient green ectosome 1000(200-1500) µm thick (Fig. 4d-e). The attachment area or short stalk is represented by a thickening of the ectosome. No reinforced tracts are present though the interior. Small cone shaped oscules, 2.5-4 mm high and 1.8-5.5 mm wide at the base narrows to a point and may be laterally flattened in preserved material. The particularly large stalked cauliform porefields are 3-7mm high and 3-6.5 mm wide, with the porefields over spilling the supporting stalk. The freshly collected sponge is a dark to olive green with light cream tipped oscules and darker brown green areolate porefields. The interior choanosome is bright green. Preserved specimens are olive to tan in colour.

Spicules. The skeletal component for this species consists of acerate, hastate or blunt styles that are prominently centrally thickened to a great extent 713(537-935) x 21 (12-30) µm. The balance of styles are long and slender 622(439-769) x 9(4-13) µm and short thick anisostrongyles (Fig. 4f-h). The microsclere are chiadiscorhabds 38(34-44) µm in length with 3 to 4 whorls. The shaft is 30(19-37) x 6(4-8) µm. The terminal whorls consist of a clearly larger manubrium 19(14-23) µm and apical whorls 16(13-21) µm in optician transection. The acanthose tubercle arranges in sets of two to four radiating from the terminal ends (Fig. 4i-n).

The sponge is often encrusted by a yellow sponge *Mycale* (*Mycale*) sp. also found on the surface of *T. pedunculata* (Fig. 3b-c and Fig. 4b, d).

Etymology

Tsitsikamma michaeli is named in recognition of the numerous contributions made by Michael T Davies-Coleman to our ever growing knowledge of the South African marine fauna. He is proof that one man can make a difference.

Distribution

Algoa Bay, 33-38 m.

Discussion

Sac or purse like sponge, green colour with well-spaced oscules and porefields is characteristic. This species resembles *T. pedunculata* but differs in the colour and the absence of a reinforced stalk. The microsclere of these two species have similarly composed terminal whols, *T. pedunculata* does not have a median whorl while *T. michaeli* may have up to two median whorls.

***Tsitsikamma scurra* Samaai, Gibbons, Kelly and Davies-Coleman, 2003**
(Figure 5)

Type Locality
Holotype. Western Cape Province, Hout Bay. 28m, CRRF 0CDN 7364-O, BMNH 2003.1.10.3, 31 March 2000, near the wreck of British "The Maori", collected by Pat. L. Colin; Paratype, SAM H-4971, 25 January 2003, 28m, near the wreck of British "The Maori, collected by L. West.

Material examined
Western Cape Province, west of Hout Bay, TIC2003-465, TIC2003-462, 25 January 2003, 28m, near the wreck of British "The Maori, collected by L. West.

Description
Globular, thick encrusting sponge with narrow long stalked areolate porefields and small thin cone-shaped oscules distributed over the folded surface. The porefields may appear strap-like in preserved specimens and are mounted on a small globular swellings forming the surface of the sponge (Fig. 5a-d). The porefields are 7-9 mm high, 2.5-5 mm in diameter, the pear-shaped oscules present on the examined material were 2-5.5 mm high and 1-1.5 mm in diameter. The extosome 530(380-880) µm is thin with a fine sand papery feel and seems to continue and fold within the interior of large specimens to form smaller subunits or internal chambers 150(100-230) µm in diameter (Fig. 5e). The choanosome is soft and may contain varying amount of sand, shell and other foreign material (Fig. 5d).

Sinuous styles in two size classes, large thick anisostyles 702(480-884) x 19(14-27) µm which are conspicuously centrally thickened sinuous sometimes with basal bend as well as long thin megascleres 692(518-821) µm (Fig. 5f-h). The chiadiscorhabds are 43(41-48) µm in total length with a shaft length of 37(19-41) µm. The median whorl is polar and situated closer to the apex whorl 20(19-22) µm than to the slightly larger manubrium 22(19-25) µm, between the apical whorl and the centre of the spicule, but more centrally situated in larger microscleres. The acanthose tubercle are generally arranges in pairs have similar pairwise arrangement (Fig. 5i-k).

Distribution
West of Hout Bay, a local area known as Maori Bay along the Western Cape Province coast at a depth of 28m.

Discussion
The specimens examined compared well with the description given by Samaai et al. 2003 although Samaai et al. (2003) describe the colour in life of the type specimen as lime green while colour photographs taken of the freshly collected specimen indicate a more brownish colouration. Preserved specimens are a medium to dark brown colour in ethanol. *Tstsikamma scurra* differs from all other known *Tsitsikamma* species in the folded globular thick encrusting growth structure with thin sandpaper-like ectosome. Epifauna may be present on

the sponge surface and the interior may contain a substantial amount of sand particles and shell fragments.

Phylogeny

In an attempt to understand and explain the relationships of the species within this genus further, 28S nuclear ribosomal gene analysis was done to construct a molecular phylogenetic tree. Although little information to support species autonomy was obtained within the genus *Tsitsikamma*. The "favus growth form" consisting of *T. favus*, *T. ngunii* and *T. scurra* and the "pedunculated growth form" consisting of *T. pedunculata* and *T. michaeli* form two district clades within the tree (Fig. 6).

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Conflicts of Interest: The authors declare that they have no conflict of interest.

References

Boury-Esnault N, and Rützler K (1997) Thesaurus of Sponge Morphology. Smithsonian Contributions to Zoology. 596, Smithsonian Institution Press, Washington, DC, 55 pp.

Genbank (www.ncbi.nlm.nih.gov/Genbank)

Jukes TH and Cantor CR (1969). Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, Academic Press, New York, 21-132.

Kelly M, Sim-Smith C, Stone R, Samaai T, Reiswig H, and Austin W (2016) New taxa arrangements within the family Latrunculiidae (Demospongiae, Poecilosclerida). Zootaxa, 4121 (1): 1-48.

Kumar S, Stecher G, and Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.

Samaai T, Gibbons MJ, Kelly M and Davies-Coleman M (2003) South African Latrunculiidae (Porifera. Demospongiae. Poecilosclerida): descriptions of new species of Latrunculia du Bocage, *Strongylodesma* Lévi, and *Tsitsikamma* Samaai and Kelly. Zootaxa, 371: 1-26.

Samaai T, Govender V and Kelly, M (2004) *Cyclacanthia* n.g. (Demospongiae. Poecilosclerida. Latrunculiidae incertea sedis), a new genus of marine sponges from South African waters, and description of two new species. Zootaxa, 725: 1-18.

Samaai T, Janson, L and Kelly, M (2012) New species of Latrunculia from the Agulhas shelf, South Africa, with designation of a type species for subgenus Biannulata (Demospongiae, Poecilosclerida, Latrunculiidae). Zootaxa, 3395: 33-45.

Samaai T. and Kelly M (2002) Family Latrunculiidae Topsent, 1922. In Hooper, JNA, and van Soest, RWM (Eds). Systema Porifera. Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York, 708–719.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD and Higgins DG 2011. Fast, scalable generation of high-quality protein multiple sequence alignment using Clustal Omega. Molecular Systems Biology, 7: 539.

Sink K, Holness S, Harris L, Majiedt P, Atkinson L, Robinson T, Kirkman S, Hutchings L, Leslie R, Lamberth S, Kerwath S, von der Heyden S, Lombard A, Attwood C, Branch G, Fairweather T, Taljaard S, Weerts S, Cowley P, Awad A, Halpern B, Grantham H, Wolf T. 2012. National Biodiversity Assessment 2011. Technical Report. Volμme 4. Marine and Coastal Component. South African National Biodiversity Institute, Pretoria, 325 pp.

Uriz M-J, Turon X, Becerro, M and Agell G 2003. Siliceous Spicules and Skeleton Framework in Sponges. Origin, Diversity, Ultrastructure Patterns, and Biological Function. Microscopy research and Technique, 6: 279-299.

Figure 1: Tsitsikamma favus a) in situ TIC2016-054; b) collected specimens TIC2016-054 scale: 5cm, c) collected SAF1995-001 scale: 5cm, d) section through preserved specimen SAF1995-001 scale: 1cm, e) isochiadiscorhabds arrangement on the surface of the ectosome, f) section of ectosome with underlying choanosome TIC1995-109 scale: 1000µm g-h) thin sinuous style, i) large sinuous centrally thickened style, j) occasionally tylote styles, k) rare short thick strongyles, (all megascleres TIC1993-006 scale: 100 µm), ; l-o) isochiadiscorhabds TIC2010-004 SEM scale: 20 µm and p) acanthose tubercles TIC2010-003 scale: 20 µm scale .

Figure 2: Tsitsikamma nguni: a-b) in situ TIC2015-006, TIC2015-003; c) collected specimens TIC2015-003 scale: 5cm, d) preserved specimen TIC1993-100 scale: 5cm, e) section of ectosome with underlying choanosome TIC2015-003A scale: 1000µm f-g) sinuous style, two size classes sometime tylote, h) short thick strongyles, (scale: 100 µm), i) collection of chiadiscorhabds scale: 20 µm and j-k) acanthose tubercles visible on SEM image chiadiscorhabds, l) developing chiadiscorhabds (TIC2015-006A scale: 20 µm scale) .

Figure 3: Tsitsikamma pedunculata: a-b) in situ (b, scale = 6 cm); c) collected specimens showing vascular stalk scale and covered with sponge epibiont Mycale (Mycale) sp., d) section of ectosome with underlying choanosome scale: 1000µm, e) outer section of ectomose with chiadiscorhabds in a dense layer externally, f) secton through the stalk showing luman of vascular-interior, g-h) various sinuous style (scale: 100 µm), i-j) collection of SEM images of chiadiscorhabds scale: 20 µm and k-l) light microscope image of chiadiscorhabds (scale: 20 µm scale) .

Figure 4: Tsitsikamma michaeli: a-c) in situ laser point distance = 6 cm; d) collected specimens with yellow encrusting Mycale (Mycale) sp. short stalk visible TIC2010-001 scale = 5 cm. e) section through the ectosome with underlying choanosome TIC2009-009 scale = 1000 µm, f) robust centrally thickened sinuous style TIC2015-210 scale = 100 µm, g) thin slightly ventrally thickened sinuous style TIC2015-203 scale = 100 µm, I-l) SEM images of chiadiscorhabds TIC2010-001 scale = 20 µm and m-n) light microscope images of chiadiscorhabds TIC2010-001 and TIC2010-009 scale = 20 µm.

Figure 5: Tsitsikamma scurra: a-b) freshly collected TIC2000-484 and TIC2003-465, c-d) preserves specimens TIC2003-462 scale = 2.5 cm and TIC2003-465 scale = 5cm, e) section of ectosome with underlying choanosome TIC2003-462 scale = 1000µm, f-g) robust centrally thickened sinuous style TIC2003-465 scale: 100 µm, g) thin slightly centrally thickened sinuous style TIC2003-465 scale = 100 µm, i-k) light microscope image of chiadiscorhabds i-j, TIC2003-465 k, TIC2003-462 scale: 20 µm scale .

Figure 6: Molecular phylogenetic based on 28S nuclear ribosomal gene. Numbers below branches indicate bootstrap values for Maximum Likelihood.

Table 1: Locality for specimens when available.

Table 2: List of species, specimen numbers and GenBank accession numbers