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Microbial Associations of Four Species of Algal Symbiont-Bearing Foraminifera from the Florida Reef Tract, USA

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Microbial Associations of Four Species of Algal Symbiont-Bearing Foraminifera from the Florida Reef Tract, USA

by

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DEDICATION

I would like to dedicate this thesis to four outstanding individuals; first, my parents for encouraging me to pursue my goals and being inspiring examples. Secondly, my fantastic fiancé, Jared, who has always supported me and was instrumental in the completion of my degrees. And lastly, my canine companion, Lady, who has (literally) been by my side through the entire writing process.

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ABSTRACT

Marine microbiome research is a rapidly expanding field of study, as scientists investigate the functions of microbial associations in eukaryotic organisms. Foraminifera are among the most abundant shelled organisms in the oceans, yet little is known of their associated microbiomes. This study investigated microbes associated with four species of Foraminifera that host three kinds of algal endosymbionts. The Order Miliolida, Family Soritidae, was represented by three species: *Archaias angulatus* and *Cyclorbiculina compressa*, which both host chlorophyte symbionts, and *Sorites orbiculus*, which hosts dinoflagellate symbionts. The fourth species, *Amphistegina gibbosa*, belongs to the Order Rotaliida and hosts diatom endosymbionts. Bacterial DNA extraction was attempted from 5−8 specimens per species followed by amplification and amplicon sequencing of the V4 variable region of the 16S rRNA gene. Three *Ar. angulatus* specimens shared 177 Operational Taxonomic Units (OTUs), and six *C. compressa* specimens shared 58 OTUs, of which 31 OTUs were found in all specimens of both species. Four *S. orbiculus* specimens shared 717 OTUs dominated by Proteobacteria, notably Amoebophilaceae. The three soritid species shared 26 OTUs, predominantly representing the bacterial families Rhodobacteraceae and Flavobacteriaceae. Since *S. orbiculus* shared 84% of the OTUs shared by *Ar. angulatus* and *C. compressa*, which host similar endosymbionts, phylogenetic relatedness of host taxa clearly had more influence on core microbiomes than the algal-symbiont taxon. The microbiomes of three normal-appearing and five partly-bleached specimens of *Am. gibbosa* varied widely, sharing only six OTUs, four of which represented

Proteobacteria. All four species shared only four OTUs, three of which may have been contaminants. As the first known microbiome study to include western Atlantic/Caribbean benthic foraminifers that host algal endosymbionts, the results for *Am. gibbosa* revealed quite similar results to a recent study of the microbiome of *Am. lobifera*, a closely related Indo-Pacific taxon.

CHAPTER 1. INTRODUCTION TO THE THESIS

Rationale

As genomic research has become more affordable and thus more widespread, the scientific community now recognizes that most organisms are "holobionts", hosting diverse assemblages of bacteria, archaea, fungi, and in some cases, symbiotic algae (McFall-Ngai et al., 2013). The unique assemblage of microbes hosted within and on the surface of the organism is known as a microbiome (McFall-Ngai et al., 2013). Studies of marine-invertebrate microbiomes have shown that the diversity and composition of bacteria in these organisms is specific to each organism and may play a vital role in maintaining the health of the host (Rosenberg et al., 2007; McFall-Ngai et al., 2013).

Foraminifers are the most abundant shelled organisms in the oceans, yet their microbiomes are virtually unknown. This study examined the microbial associations of four common Western Atlantic and Caribbean species of foraminifers that host algal symbionts. *Amphistegina gibbosa* d'Orbigny, Order Rotaliida, Family Amphisteginidae hosts diatom endosymbionts. *Sorites orbiculus* (Forskål), Order Miliolida, Family Soritidae, Subfamily Soritinae, hosts dinoflagellate symbionts. *Archaias angulatus* (Fichtel & Moll) and *Cyclorbiculina compressa* (d'Orbigny), both Order Miliolida, Family Soritidae, Subfamily Archaiasinae, host chlorophyte symbionts (e.g., Hallock, 1999 and references therein). Subsequently in this paper, the genus name *Amphistegina* will be abbreviated *Am*. to distinguish it from *Archaias*, which will be abbreviated *Ar*.

Using the data from the analyses of the microbiomes associated with these four species, three hypotheses regarding these foraminifers and their core microbial assemblages are tested. The first hypothesis is that foraminiferal microbiome compositions will be strongly influenced by host species, and thus more closely related host species will have a more similar microbiome composition. The second hypothesis is that foraminifers with similar algal symbionts will have a more similar microbiome composition than those with different algal symbionts. The last hypothesis is that visibly bleached specimens of *Am. gibbosa* will have an altered composition of microbes compared to specimens exhibiting normal coloration.

This thesis is organized into three chapters. Chapter 1 introduces the thesis topic and provides a review of pertinent literature. Chapter 2 is a stand-alone manuscript that has been prepared for publication in a peer-reviewed journal. Chapter 3 summarizes additional results and observations not included in Chapter 2, and provides suggestions for future research.

Background Information

What is a Microbiome?

One of the best known microbiome studies, *The Human Microbiome Project*, explains the term "microbiome" as the collective genome of the microbial symbionts that live both within and on the surface of a host (Turnbaugh et al., 2007). This study brought microorganisms to public attention and revealed that, even in higher animals like humans, metabolic functions are a blend of both host and microbial traits (McFall-Ngai et al., 2013). Microorganisms are hypothesized to play a wide variety of roles and research continues to reveal that multicellular organisms require associations with microbes to survive (McFall-Ngai et al., 2013). An animal's relationship with microbes is not only necessary, but evolutionarily advantageous, as the host's microbial

symbionts may perform useful functions or help the holobiont respond more rapidly to changing conditions (Reshef et al., 2006; Rosenberg et al., 2007; McFall-Ngai et al., 2013).

Advances in technology, such as next generation sequencing, are now allowing the analysis of the composition of marine-invertebrate microbiomes (McCauley et al., 2016). These culture-free, DNA-based techniques are especially important to the study of microbiomes of marine organisms because <1% of marine bacteria can be cultured using traditional methods (Rosenberg et al., 2007). However, DNA-based techniques still have their drawbacks: they only provide relative abundances of microbial composition and they can be biased towards bacterial species with DNA that is easier to extract (Rosenberg et al., 2007). Despite these limitations, DNA-based techniques are expanding scientific knowledge surrounding the microbiomes of marine organisms and helping to reveal the possible functions of these microbial associations.

Microbiome Function in Sponges and Corals

Perhaps the most widely studied marine organisms with respect to microbiomes are sponges and corals. In sponges, microbes including bacteria, archaea, fungi, and microalgae can comprise up to 40% of a sponges' total volume (Webster & Taylor, 2011). These sponge microbial communities are distinct from those of the surrounding seawater and show similarity among related sponge species even in different geographic locations (Webster & Taylor, 2011). The microbial symbionts of sponges are hypothesized to protect from fouling, disease, and predation, and there is mounting evidence for their role in nitrogen fixation, carbon fixation, and nutrient acquisition for the host sponge (Webster & Taylor, 2011).

However, microbial communities can be disrupted by changes in ocean conditions, such as warming water temperatures, and thus lead to decline in host health (Thurber et al., 2009;

Blanquer et al., 2016). Many sponge and coral diseases and mortality events have been linked to anomalously high water temperatures; new research provides evidence that the warm conditions do not harm the organism directly, but alter their microbial composition, leading to a decline in host health and increased vulnerability to pathogens (Thurber et al., 2009; Pootakham et al., 2018). Sponge microbiomes may even be used as a proxy for sponge health, as microbiome changes can be observed in the sponge tissue before a disease is visually evident (Blanquer et al., 2016).

Comparable microbiome functions have also been hypothesized in corals. In cases of coral bleaching, changes in the coral microbiome have been detected in samples taken before visual indicators of bleaching were observed, suggesting that shifts in conserved coral microbiota can be used as an indicator for large-scale bleaching events (Bourne et al., 2007). There is mounting evidence that a coral's microbiome can function as a barrier to disease in many ways; microbes isolated from corals have been observed to produce antibacterial, algicidal, antifouling, and cytotoxic compounds, allowing the microbes to disrupt cell-to-cell communication of coral pathogens as well as to competitively exclude organisms from the host's surface (Krediet et al., 2013; Pootakham et al., 2018).

Research into coral microbial communities has also shown that coral microbiomes play a crucial role in biogeochemical cycling, both within the coral host and throughout the entire reef ecosystem (Sharp & Ritchie, 2012; Hernandez-Agreda et al., 2017). The coral-associated microbes help cycle important particulate and dissolved organic compounds containing essential elements such as nitrogen, sulfur, and carbon (Sharp & Ritchie, 2012; Hernandez-Agreda et al., 2017).

There is also a hypothesis that coral microbiomes may assist the coral to adapt to changing ocean conditions. The "coral probiotic hypothesis" suggests that corals can alter the composition of their microbiome to better adapt to new conditions as the environment changes (Reshef et al., 2006; Rosenberg et al., 2007). This hypothesis has gained support from research documenting microbial shifts after an environmental stress event or pathogen invasion. Host mediated shifts in microbial compositions to select for microbes with more beneficial characteristics may allow corals to adapt to changing ocean conditions or develop an "immunity" to infection by specific pathogens (Rosenberg et al., 2007). Restructuring the coral microbial assemblage can provide the coral with a mechanism for much more rapid and versatile adaptation than can be achieved through genetic mutation of the coral host (Reshef et al., 2006; Rosenberg et al., 2007; McFall-Ngai et al., 2013).

The probiotic hypothesis can also be applied to other invertebrates, plants, and animals, and has thus been termed the "hologenome theory of evolution"; the genome of the host interacts with the genome of the host's microbial community to provide greater adaptive potential to the holobiont (Rosenberg et al., 2007). With increasing evidence that microbial associations play a large role in the health, nutrient cycling, and adaptation of corals, researchers hypothesize that microbial associates serve similar functions in many other organisms (McFall-Ngai et al., 2013). Thus, the expansion of microbiome studies to include other marine invertebrates, such as bivalves and ascidians, and protists such as foraminifers are important topics of study. Microbiomes are hypothesized to play many roles on both the organism and ecosystem levels, and as such, microbial processes may be important players in controlling the resilience and adaptation of reef systems to changing ocean conditions (Sharp and Ritchie, 2012).

Research into microbiomes in marine invertebrates, mainly focusing on corals, has found that many species maintain a "core microbiome" that is distinct from the microbial composition of their surroundings (Rosenberg et al., 2007; McCauley et al., 2016). This means that, even though coral microbiomes are taxonomically diverse and can vary with location or environmental conditions, different species have a specific set of microbial taxa, or "core microbiome", with which they are consistently associated (Rosenberg et al., 2007; McCauley et al., 2016). The factors that control the composition of an organism's microbiome are still being explored, but studies of corals and sponges have revealed evidence for microbiome species-specificity. Both coral and sponge species have shown microbiome similarity, even between different seasons or geographic locations (Webster & Bourne, 2007; Littman et al., 2009; Webster & Taylor, 2011; Reveillaud et al., 2014; Chu & Vollmer, 2016). Chu & Vollmer (2016) proposed an explanation for this strong species specificity, that is, different species of coral may offer different niches or host-derived nutrients favoring specific microbes.

Many studies have identified possible factors that can alter or impact the core microbiome of a species, including temperature stress, seawater pH, bleaching, disease, macroalgal growth, preferred habitat, ecological strategy, and algal symbionts. Microbial research has provided possible mechanisms for many long-standing observations of patterns in invertebrate health and disease (Bourne et al., 2007, 2013; Webster et al., 2010, 2016; Sharp & Ritchie, 2012; Thurber et al., 2012; Roder et al., 2015; Blanquer et al., 2016).

Anomalously high water temperatures have long been correlated with disease outbreaks and die-offs of marine organisms; new evidence suggests that temperature increases affect the microbial community of the hosts, causing the observed decline in organism health (Blanquer et al., 2016). Multiple studies have identified shifts in the diversity and/or abundance of core

microbiomes correlated with increased water temperature in species of foraminifers, crustose coralline algae (CCA), sponges, and corals (Webster et al., 2010, 2016; Sharp & Ritchie, 2012; Blanquer et al., 2016). In corals, increasing temperature has been observed to reduce the antibiotic activity of the core microbiome, leading to a higher diversity of bacteria in the coral and possible pathogen invasion (Sharp & Ritchie, 2012). Stressors like heat may also inhibit or alter the normal nutrient production or cycling ability of microbial associates, driving a shift in bacterial community (Thurber et al., 2009). Taking a mathematical model-based approach, Mao-Jones et al. (2010) predicted a temperature threshold above which a coral's core microbiome cannot protect against pathogen invasion, leading to two alternate stable states: beneficial microbes dominate and the coral remains healthy, or pathogenic microbes dominate and the coral succumbs to bleaching or disease. This idea of temperature-induced alternate microbial states provides a mechanism for observations of pathogen dominance in corals following thermal stress (Mao-Jones et al., 2010). The model also predicts that once a temperature threshold has been reached, pathogens may persist even after temperatures return to normal. This has implications for reef health with the growing threat of ocean warming; once a shift to a pathogenic state is induced, it may be much harder to reverse (Mao-Jones et al., 2010).

Coral bleaching has become one of the most prevalent afflictions of tropical coral reefs; over the past few decades bleaching events have increased in frequency and scale (Bourne et al., 2007). Samples of corals taken before, during, and after bleaching events showed a tight correlation between microbiome disruption and bleaching (Littman et al., 2011; Lins-de-Barros et al., 2012; Pootakham et al., 2018). Corals that were visibly bleached showed a higher diversity of microbes than before bleaching; if the coral recovered, the bacterial diversity then decreased (Bourne et al., 2007). Shifts in microbiome diversity can be detected before the visible

characteristics of the bleaching are observed, showing that microbial assemblage changes precede the onset of bleaching (Bourne et al., 2007). This has led some researchers to view microbial shifts as an "early warning system" for large-scale bleaching events (Bourne et al., 2007).

There is growing evidence for the role of microbiomes in protection from pathogens, so it is not surprising that diseases in marine organisms can be correlated with a perturbation of the microbiome. Microbiomes of corals can consist of several thousand different operational taxonomic units (OTUs) of bacteria, which is part of the reason why past researchers have had difficulty identifying causative agents of coral diseases among such diverse, conserved microbial communities (Gignoux-Wolfsohn & Vollmer, 2015). Although it is often still difficult to pinpoint a single bacterial taxon as the sole cause of a disease, clear differences have been observed in the composition of healthy and diseased coral microbiomes (Krediet et al., 2013; Gignoux-Wolfsohn & Vollmer, 2015). Heightened diversity of bacteria in coral microbiomes has been linked to White Band Disease in corals (Gignoux-Wolfsohn & Vollmer, 2015), sponge disease and die-off (Blanquer et al., 2016), unknown lesions in corals (Meyer et al., 2014), and many other instances of disease in corals and sponges. There are two main hypotheses regarding the disruption of the microbiome and its contribution to disease: the first suggests that when the antimicrobial activity of the associated microbes is hindered (by increase in temperature, pH reduction, or other environmental factors), outside pathogens are able to colonize the corals and cause disease (Rosenberg et al., 2007). The second hypothesis states that normal constituents of the host's own microbiome may opportunistically grow beyond their normal populations and lead to disease if the health of the host is compromised by outside stressors or if there is an increase in nutrients (Bourne et al., 2007). As methods continue to improve, more studies are

finding evidence for the second hypothesis. Members belonging to the core microbiome may switch from being beneficial/commensal to pathogenic when the holobiont experiences changes in nutrient conditions, environmental factors, or competition with other microbes (Kline et al., 2006; Krediet et al., 2013; Meyer et al., 2014).

Another interesting factor that may contribute to an organism's core microbiome is the presence and species of an algal symbiont. When corals bleach, losing their symbiotic dinoflagellates, the reactive oxygen species produced by the photosynthetic activity of the dinoflagellate are reduced (Bourne et al., 2007). These reactive oxygen species may act as a barrier to bacterial species entering into the host tissue; the loss of dinoflagellate may provide an additional mechanism for alteration of the microbiome associated with the holobiont (Bourne et al., 2007).

Furthermore, the algal symbionts likely have their own unique relationship with microbes, thus contributing to the diversity and composition of the holobiont (Bourne et al., 2013; Ainsworth et al., 2015). Studies of invertebrates and protists with and without algal symbionts revealed that the presence of a symbiont conferred a significant difference in the host's microbial structure (Bourne et al., 2013). The microbiome of juvenile corals differs significantly based on the type of *Symbiodinium* dinoflagellate that initially colonizes the corals (Sharp & Ritchie, 2012). Ainsworth et al. (2015) hypothesized that algal symbiosis contributes a unique assemblage of microbes to the holobiont, and that the microbes may even help facilitate the interaction between the host organism and algal symbiont. Those researchers further speculated that the unique combination of bacteria, algae, and host provides the holobiont with access to metabolic pathways and nutrients that the individual organisms could not access independently (Ainsworth et al*.,* 2015). Research on coral eggs and antibiotic potential revealed

that only eggs with incorporated *Symbiodinium* showed antibiotic activity (Sharp and Ritchie, 2012), leading to speculation that algal symbionts may be able to produce signaling molecules that can influence the composition of bacteria and contribute to the ability of the holobiont to produce antibiotic compounds. The role of algal symbionts in determining the composition of the holobiont microbiome is a topic in need of further investigation.

Why Symbiont-Bearing Foraminifera?

The Foraminifera are an important phylum of protists in the world's oceans, second only to coccolithophores as carbonate producers, thus playing a major role in oceanic carbon cycling. However, very little is known about the microbial assemblages of foraminifers. Only a handful of studies have included foraminifers in microbiome studies, even though Bourne et al. (2013) reported that photosymbiont-bearing foraminifers can have a more diverse microbiome than even corals. Symbiont-bearing foraminifers are of particular interest because, as previously discussed, studies indicate that algal symbionts, such as the dinoflagellates found in corals, may have an effect on the microbiome of the organism (Ainsworth et al., 2015). Because different species of foraminifers have symbiotic relationships with different types of algae, including diatoms, dinoflagellates, and green algae (Lee, 2006), study of their microbiomes provides an opportunity to further scientific knowledge regarding the role of algal symbionts in structuring core microbiome assemblages.

As previously noted, very few studies have utilized foraminifers in microbiome research. The earliest paper located that addresses this topic is Bourne et al. (2013). This paper explored the microbiome of 16 species of coral-reef associated invertebrates and protists, including corals, bivalves, bryzoans, ascidacians, and sponges, as well as foraminifers. Of these sixteen species,

eleven hosted algal symbionts and five did not. When the microbiomes of algal symbiont-bearing organisms were compared with those of organisms lacking algal symbionts, the study found that the presence of a symbiont significantly altered the composition of an organism's microbiome. The study also found that, when compared to the reef invertebrates, the three foraminiferal species sampled had the highest diversity in their microbial composition. Additionally, the only foraminifer in that study that hosted a diatom symbiont (*Heterostegina depressa*) had the highest species richness of all the organisms studied. These results indicate that the symbiont type may influence microbiome composition in foraminifers and provide evidence for the importance of exploring this topic further.

Webster et al. (2016) examined the effects of climate change on coral-reef invertebrate and protist microbiomes. The study included two coral species, one urchin species, one crustose coralline algal species, and two foraminiferal species. These organisms were exposed to experimental treatments in which water temperature was increased, pH was decreased, or a combination of both treatments. The results of the study showed that the foraminifers demonstrated the greatest microbiome shift when exposed to lower pH at a higher temperature. In addition, their findings showed the microbiomes of the foraminifers and the crustose coralline algae were the most sensitive to increases in temperature. The study highlighted previous research that indicates that microbiome shift is strongly correlated to decreasing host health in many marine organisms. The authors called for future studies to investigate the significance of microbiome shift in foraminiferal health, and to use the "holobiont approach" when assessing invertebrate health in the face of climate change.

Most recently, Prazeres et al. (2017) studied the microbiomes of the foraminiferal species *Amphistegina lobifera* Larsen across inner-, mid-, and outer-shelf sampling locations on the

Great Barrier Reef, Australia, to investigate the role of environmental conditions in shaping the foraminiferal microbiome. The researchers found 30 core bacterial OTUs shared by all *A. lobifera* samples throughout all three sites. Analysis of microbial taxonomic identities showed that the most abundant bacterial taxon was Proteobacteria. The authors also observed variation in the diversity of microbial communities among the three different sites; Actinobacteria was more common in the inner-shelf samples, while Bacteroidetes and Firmicutes were more common on mid- and outer-shelf sites. Although they found differences in microbiome composition among sites, the authors were unable to determine if the observed differences were driven by, or a response to, the environmental gradient of the shelf.

Background on Hypotheses Examined

The first hypothesis tested during my thesis research was the relationship between foraminiferal host species and microbiome compositions. I hypothesized that foraminiferal microbiome compositions would be strongly influenced by host species, and consequently, more closely related host species will demonstrate a more similar microbiome composition than more distant phylogenetic relatives. In corals and sponges, the host species is recognized as the strongest driver influencing bacterial composition, even allowing for variations with geographic location, time of year, or other environmental perturbations (Webster & Bourne, 2006; Littman et al., 2009; Webster & Taylor, 2011; Reveillaud et al., 2014; Chu & Vollmer, 2016). Moreover, more closely related species of coral have been observed to demonstrate higher similarity in their microbiome compositions (Littman et al., 2009; Sunagawa et al., 2010). Although this hypothesis has been well explored in corals and sponges, host-specificity of microbial communities is a topic not yet addressed in foraminifers.

The second hypothesis was that foraminifers with similar algal symbionts would have a more similar microbiome composition than those with different algal symbionts. Studies of coral microbiomes have identified algal symbionts to be a factor that may contribute to an organism's core microbiome (Littman et al., 2009). The algal symbionts themselves likely have their own unique relationship with microbes, thus contributing to the diversity and composition of the holobiont (Bourne et al., 2013; Ainsworth et al., 2015). In a study of marine organisms including corals, foraminifers, bryozoans, sponges, ascidians, and bivalves, the composition of the organisms' microbiomes differed both between taxa and between members of the same group with and without symbionts (Bourne et al., 2013). This may indicate that the presence or absence of an algal symbiont, or different types of symbionts, can influence the distributions of microbes that make up an organism's microbiome. Because different lineages of foraminifers have symbiotic relationships with different types of algae, including diatoms, dinoflagellates, chlorophytes and rhodophytes (Lee, 2006), study of their microbiomes provides an opportunity to investigate the role of algal symbionts in structuring core microbiome assemblages.

My third hypothesis was that bleached foraminiferal specimens would have an altered composition of microbes compared specimens exhibiting normal color. Samples of corals taken before, during, and after bleaching events showed a tight correlation between microbiome disruption and bleaching (Bourne et al., 2007). Corals that were visibly bleached showed a higher diversity of microbes than before bleaching, the bacterial diversity then decreased if the coral recovered from the bleaching event (Bourne et al., 2007). Interestingly, during periods of higher temperatures, the shift in microbial diversity could be detected before the visible characteristics of the bleaching were observed leading some researchers to view microbial shifts as an indicator for large scale bleaching events (Bourne et al., 2007). Moreover, *Amphistegina*

spp. of Foraminifera have been observed to consistently bleach weeks prior to coral bleaching events, making them a potential indicator species or "early warning system" for predicting such events (e.g., Spezzaferri et al., 2018).

The mechanisms for the observed shift in microbiota before and during a bleaching event likely include the effects of photoinhibition, temperature and pH on the holobiont. The combined effects of increased ocean temperature, and decreased pH have been shown to alter microbial compositions in a variety of calcifying invertebrates (Webster et al., 2016). Sharp & Ritchie (2012) hypothesized that lower pH impacts the host metabolism, shifting the availability of nutrients and carbon to the microbiota, causing a perturbation in the core microbiome. Much like the effects observed with increasing temperature, lowered pH caused the microbes associated with corals to exhibit lowered antimicrobial activity (Sharp & Ritchie, 2012). The microbiome response to both lowered pH and increased temperature offers a mechanism by which these conditions can lower host defenses and lead to bleaching or disease.

Symbiont loss (partial to extensive bleaching) has been recognized in *Amphistegina* spp. for more than three decades (Hallock et al., 1986, 1995). Based on studies of the microbiomes in corals in response to bleaching, I hypothesized that bleached *Am. gibbosa* specimens would exhibit an altered composition of microbes as compared to their visibly healthy counterparts.

CHAPTER 2. MICROBIAL ASSOCIATIONS OF FOUR SPECIES OF ALGAL SYMBIONT-BEARING FORAMINIFERA FROM THE FLORIDA REEF TRACT, USA

Note: This chapter has been prepared as a manuscript to be submitted for publication. The abstract of the thesis will serve as the abstract of the manuscript.

Introduction

As genomic research has become more affordable and thus more widespread, the scientific community now recognizes that most organisms are holobionts, hosting diverse assemblages of bacteria, archaea, fungi, viruses, and in some cases, symbiotic algae (McFall-Ngai et al., 2013). The unique assemblage of microbes hosted within and on the surface of an organism is known as a microbiome. Studies of invertebrate microbiomes have shown that the diversity and composition of microbes is specific to each organism and may play a vital role in maintaining the health of the host (Rosenberg et al., 2007; McFall-Ngai et al., 2013).

In corals and sponges, the host species is recognized as the strongest driver influencing microbiome composition, even allowing for variations with geographic location, time of year, and environmental perturbations (Webster & Bourne, 2007; Littman et al., 2009; Reveillaud et al., 2014; Chu & Vollmer, 2016). Moreover, more closely related species of coral have been observed to demonstrate higher similarity in their microbiome compositions (Littman et al., 2009; Sunagawa et al., 2010). Although the relationship between host and microbiome structure

has been extensively explored in corals and sponges, host-specificity of microbial communities is just beginning to be addressed in the Foraminifera.

Despite being the most abundant shelled organisms in the oceans, only a handful of studies have included foraminifers in microbiome research (Bourne et al., 2013; Webster et al., 2016; Bird et al., 2017; Prazeres et al., 2017). My study examined the microbial associations of four common Western Atlantic and Caribbean species of foraminifers that host algal symbionts (e.g., Hallock, 1999, and references therein). *Archaias angulatus* (Fichtel & Moll) and *Cyclorbiculina compressa* (d'Orbigny) are classified in the Order Miliolida, Family Soritidae, Subfamily Archaiasinae, and both host chlorophyte symbionts. *Sorites orbiculus* (Forskål), Order Miliolida, Family Soritidae, Subfamily Soritinae, hosts dinoflagellate symbionts. *Amphistegina gibbosa* d'Orbigny, Order Rotaliida, Family Amphisteginidae, hosts diatom endosymbionts. Subsequently in this paper, the genus name *Amphistegina* will be abbreviated *Am*. to distinguish it from *Archaias*, which will be abbreviated *Ar*.

In a study of marine organisms including corals, foraminifers, bryozoans, sponges, ascidians, and bivalves, the composition of the organisms' microbiomes differed both between taxa and between members of the same taxon with and without symbionts (Bourne et al., 2013). This may indicate that the presence or absence of an algal symbiont, or different types of symbionts, can influence the distributions of bacteria that make up an organism's microbiome. Because different lineages of foraminifers have symbiotic relationships with different types of algae, including diatoms, dinoflagellates, rhodophytes and chlorophytes (Lee, 2006, and references therein), study of their microbiomes provides an opportunity to investigate the role of algal symbionts in structuring core microbial assemblages.

Another aspect that can influence microbiome composition in marine species is bleaching, or the loss of the algal endosymbiont from the holobiont (Bourne et al., 2007). Bleaching is rapidly becoming one of the most prevalent afflictions of tropical reef-building corals; over the past several decades, bleaching events have increased in frequency and scale (Hughs et al., 2003; Baker et al., 2008). Samples of corals taken before, during, and after bleaching events have shown a tight correlation between microbiome disruption and bleaching (Bourne et al., 2007; Littman et al., 2011; Lins-de-Barros et al., 2012; Pootakham et al., 2018). During periods of higher temperatures, shifts in microbial diversity could be detected before visible signs of bleaching were observed (Bourne et al., 2007). *Amphistegina* spp. of foraminifers have been observed to exhibit bleaching several weeks prior to coral bleaching events, making them a potential indicator species or "early warning system" for predicting such events (e.g., Spezzaferri et al., 2018). Symbiont loss (partial to extensive bleaching) has been recognized in *Amphistegina* spp. for more than three decades (Hallock et al., 1986, 1995), yet the potential influence of bleaching on foraminiferal microbiomes has not been investigated.

This study had two major objectives. The first was to describe microbial assemblages of four common foraminiferal species that host algal endosymbionts. The second objective was to use the data from the core microbial assemblages to examine three hypotheses. The first hypothesis was that the microbiome composition is strongly influenced by host species, and thus more closely related host species will have more similar microbiome compositions. The second hypothesis was that foraminifers with similar algal symbionts will have a more similar microbiome composition than those with different algal symbionts. The last hypothesis was that visibly partly-bleached specimens of *Am. gibbosa* have an altered composition of microbes compared to specimens exhibiting normal coloration.

Methods

Sample Collection

Foraminiferal specimens were collected from two sites in the vicinity of Long Key, Florida Keys, USA, on 16 May 2016 (Table 1). The first site was at 6 m depth in the immediate vicinity of the Tennessee Reef lighthouse (24.7453°, –80.7818°), where specimens of *C. compressa* and *Am. gibbosa* were collected from coral-rubble substrate. The second sampling site was the shallow, protected inlet on the Florida Bay side of the Keys Marine Laboratory (24.8252°, –80.8125°), where specimens of *S. orbiculus* and *Ar. angulatus* were collected from a mixture of sand and algal substrate.

Table 1. Site information for the foraminiferal specimens collected and successfully sequenced.

Sample IDs	Species	Symbiont Type	Collection Site	Depth	Temp (°C)	Notes
SK1, SK3, SK4, SK5	***Sorites orbiculus***	**Dinoflagellate**	Keys Marine Laboratory	1.5 m	30	Healthy
RK2, RK6, RK8	***Archaias angulatus***	**Chlorophyte**	Keys Marine Laboratory	1.5 m	30	Healthy
C61, C62, C64, C66, C67, C68	***Cyclorbiculina compressa***	**Chlorophyte**	Tennessee Reef	6 m	29	Healthy
M61, M62, M64	***Amphistegina gibbosa***	**Diatom**	Tennessee Reef	6 m	29	Healthy
M6B1, M6B2, M6B3, M6B7, M6B8	***Amphistegina gibbosa***	**Diatom**	Tennessee Reef	6 m	29	Partly Bleached
6E1, 6E2	-	-	Tennessee Reef	6 m	29	Substrate Sample
KE1, KE2	-	-	Keys Marine Laboratory	2 m	30	Substrate Sample

To minimize contamination, divers carried new, unopened plastic bags, which were opened underwater immediately before the sample was placed inside. The bags were turned inside out without touching the inside. Using the inside of the bag, the samples were "grabbed"

and the bag was closed. At the 6 m site, the sample bags were placed in a dark-colored mesh bag, and carried to the support boat, where the sealed sample bags were placed in a covered container of seawater to protect the samples from sunlight during transport to the field laboratory. The samples from the second site were taken into the laboratory within a few minutes of collection.

Sterile Picking and Rinsing

The pieces of algae and rubble were removed from the sample bags and placed in sterile petri dishes under a stereomicroscope to facilitate identification of foraminiferal specimens. The specimens were individually picked from the algae or rubble using flame-sterilized forceps, then placed in a separate sterile petri dish containing 0.22 μm filtered seawater. The foraminifers were cleaned of visible debris using a sterilized brush, then placed into a third sterile petri dish with filtered seawater. This rinsing process was completed a total of three times, after which the specimens were picked with sterile forceps into sterile cryovials. Each cryovial contained only one specimen and each vial was labeled with species and collection location information.

For each soritid species, 5−8 individual specimens were selected. For *Am. gibbosa*, seven normal-appearing and nine partly bleached specimens were isolated. For each location, two additional samples of the original substratum (algae or rubble from inside the collection bags) were placed into individual cryovials for use as substrate controls (Table 1). All cryovials were flash frozen in a liquid nitrogen dewar at the field laboratory for transport to the University of South Florida College of Marine Science in St. Petersburg, FL, where they were placed into a −80°C freezer.

Microbial DNA Extraction

Microbial DNA was extracted from each specimen using a Qiagen DNeasy Powersoil Kit. To evaluate potential kit contamination, one "Kit Blank" sample was processed using all the same methods, but without adding any sample material. Extracted DNA was sent to the University of Minnesota Genomics Center (UMGC) for amplification and sequencing. Amplification was done using UMGC's dual-indexing approach (Gohl et al., 2016). The V4 region of the 16S rRNA gene was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 860R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) on an Illumina MiSeq using V2 chemistry to generate 2x250 bp paired-end reads. Sequences are available from NCBI SRA under accession number PRJNA471153.

Each sample was checked for sequence quality using FastQC (version 0.11.5). Samples that did not pass the quality check were removed from the data set; these included one *S. orbiculus* specimen, five *Ar. angulatus* specimens, two *C. compressa* specimens, three normal-appearing *Am. gibbosa* specimens, and three partly bleached *Am. gibbosa* specimens. Data from the remaining 21 foraminiferal specimens and four substrate samples were used in further analysis (Table 1).

Data Analyses

The results from the bacterial DNA sequencing were analyzed using QIIME (Quantitative Insights Into Microbial Ecology, Caporaso et al., 2010). This bioinformatic platform allows microbiome analyses from raw sequencing data, including identifying Operational Taxonomic Units (OTUs), assigning taxonomy, reconstructing phylogeny, and analysis of microbial diversity. The QIIME 1.9.1 AMI (derived from the Starcluster Ubuntu

12.04 AMI) was used on Amazon EC2 along with specific python scripts detailed in a full workflow (Appendix 1).

From the original sequences, a shell script, Merged_Reads_Script.sh written by Jackson Sorrenson, was used to merge reads (https://github.com/edamame-course/Amplicon_Analysis/blob/master/resources/Merged_Reads_Script.sh). Prior to analyses, OTUs were picked using pick_open_reference_otus.py script (Rideout et al., 2014), singletons were removed, alignment was performed with pyNAST (Caporaso et al., 2009), taxonomy was assigned with uclust (Edgar, 2010), and no prefiltering was performed, as recommended by Rideout et al. (2014). Failed alignments of paired-end reads, chloroplasts, and mitochondrial sequences were removed from the data. One *Am. gibbosa* foraminiferal sample (M66) with comparatively low sequence reads (9,260) was removed from the data to rarify the data to the second lowest number of sequence reads (20,647). After rarefaction, diversity metrics were calculated using alpha_diversity.py and beta_diversity.py.

The term "core microbiome" is used to describe bacterial taxa that appear in all of the individuals of the defined group (e.g., "*S. orbiculus* core microbiome" refers to bacterial taxa found in all *S. orbiculus* specimens sequenced). Core microbiomes for each species were calculated from the un-rarefied OTU table, to better represent the core microbiome of species with higher numbers of sequences, using the compute_core_microbiomes.py script requiring presence in 100% of the specimens being compared.

Comparisons of microbiome dissimilarity between foraminiferal hosts were calculated using PERMANOVA+, a non-parametric multivariate statistical test designed for the analysis of ecological data (Anderson et al., 2008). PERMANOVA was run using the weighted UniFrac distance matrix with the host genus names (*Sorites, Archaias, Cyclorbiculina, Amphistegina*) as

factors. The test design was based on dissimilarity, using partial sum of squares type III, with 9,999 permutations. Pairwise dissimilarity was also performed to compare hosts using Monte Carlo permutations to account for the small numbers of specimens.

Results

Forty-five specimens from four foraminiferal species, along with four substrate samples, were collected from two locations along the middle Florida Keys reef tract. Of these, 21 specimens were successfully sequenced for prokaryotic DNA to investigate their microbial associations (Table 1). Four substrate samples and one control kit blank were also sequenced.

The total number of sequence reads across all samples was >10 million. After removing failed alignments of paired-end reads, and removing chloroplasts and mitochondrial sequences, the total was 8.3×10^6. The specimens of each species had relatively similar numbers of sequence reads and OTUs (Table 2, Appendix 2). A rarefaction curve of observed OTUs plotted against sequences per sample revealed that *Am. gibbosa, Ar. angulatus* and *C. compressa* OTUs saturated at >2,000 sequences per sample. For *S. orbiculus* and the substrate samples, the OTUs continued to increase out to 20,000 sequence reads, though the rate of increase for the substrate OTUs was at least four times faster than for *S. orbiculus* OTUs (Fig. 1). The *Am. gibbosa* specimens consistently had lower numbers of sequence reads and OTUs than the other species. However, the Shannon diversity index revealed comparable values for the microbial assemblages of *Ar. angulatus* (6.6) and *Am. gibbosa* (6.5) and slightly lower values for *S. orbiculus* (5.5) and *C. compressa* (5.1). On the other hand, the environmental samples yielded much higher OTUs, with the Shannon Index averaging 10.4.

Table 2. Sequencing information and Shannon diversity index*.

Species	Sample ID	Sequence Reads*	OTUs	Shannon Index
S. orbiculus	SK1	549,377	990	5.6
	SK3	662,209	1,322	3.6
	SK4	755,146	2,000	6.1
	SK5	382,586	1308	6.6
Ar. angulatus	RK2	282,469	737	7.5
	RK6	156,225	407	5.8
	RK8	205,650	481	6.5
C. compressa	C61	124,969	387	4.8
	C62	432,152	180	2.7
	C64	349,799	306	3.1
	C66	354,082	883	8.5
	C67	315,677	604	6.4
	C68	289,638	493	5.2
Am. gibbosa (healthy appearing)	M61	58,328	415	6.8
	M62	20,467	293	5.9
	M64	71,177	348	6.6
Am. gibbosa (partially bleached)	M6B1	57,814	306	5.0
	M6B2	22,059	394	5.9
	M6B3	174,051	510	7.2
	M6B7	104,624	504	7.1
	M6B8	182,342	557	7.1
KML Substrate	KE1	831,748	4,833	10.6
	KE2	584,520	3,519	10.2
Tennessee Reef Substrate	6E1	967,637	5,057	10.7
	6E2	288,620	4,034	10.0

**Samples were rarified to 20,467 sequences (lowest observed sequence read amount) before diversity metrics were calculated.*

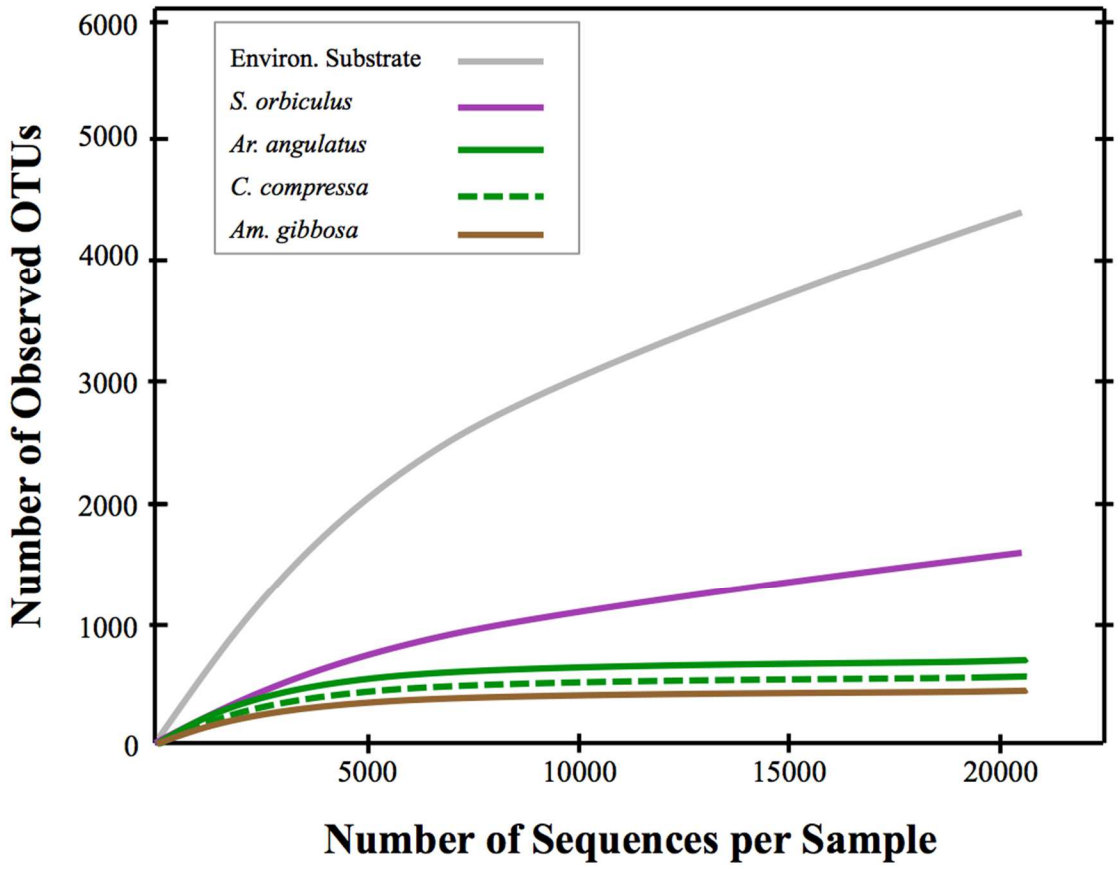

Figure 1. Number of OTUs to number of sequence reads for each foraminiferal species showing the diversity of microbial taxa found in the microbiomes of each species group.

The similarities in microbial taxa within species were observed by comparing the relative abundances of taxa recognized in the microbiomes from each specimen (Fig. 2). Relative abundances of microbial taxa were most similar among specimens of *S. orbiculus*; those for *Ar. angulatus* and *C. compressa* were also relatively similar among individuals. In contrast, substantial variability of microbial taxa was evident within and between the partly-bleached (M6B) and normal-appearing (M6) specimens of *Am. gibbosa*. Clear differences in microbial composition also were evident between foraminiferal specimens and samples from the substrate from which the specimens were collected.

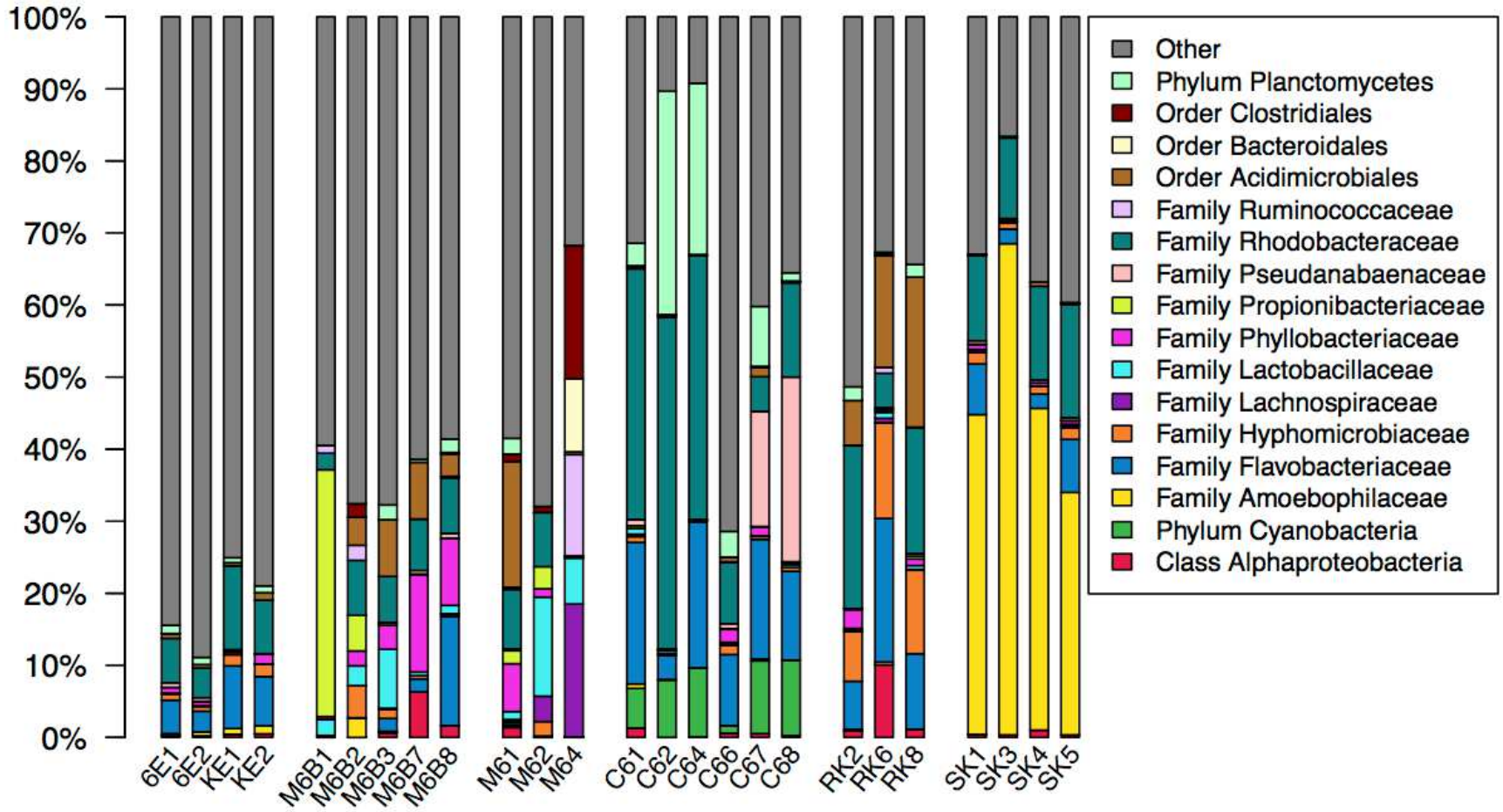

Figure 2. Relative abundances of each microbial family (or their lowest discernable taxonomic classification) identified in each foraminiferal specimen successfully sequenced. Sample identifiers are listed in Table 1.

Microbiome Comparisons

The microbial OTUs from all four taxa were dominated by Proteobacteria (Fig. 3). *Archaias angulatus* samples included high proportions of OTUs from the Class Alphaproteobacteria, Order Acidomicrobiales, and familes Flavobacteriaceae, Hyphomicrobiaceae, and Rhodobacteraceae, with varied abundances among host specimens (Fig. 2). *Cyclorbiculina compressa* samples included the phyla Cyanobacteria and Planctomycetes, and families Flavobacteriaceae, Rhodobacteraceae, and Pseudanabaenaceae; the latter were particularly abundant in two of the *C. compressa* specimens (C67, C68) (Fig. 2). The microbial OTUs from *S. orbiculus* specimens also were dominated by Proteobacteria, most notably by Amoebophilaceae, a family poorly represented in the OTUs identified from the other species. The second largest proportion of the *S. orbiculus* microbiome consisted of Rhodobacteraceae and Flavobacteriaceae. The healthy-appearing and partly-bleached *Am. gibbosa* microbiomes varied greatly in terms of composition and abundance.

Based on their microbiome compositions, the samples clustered together by species and photosynthetic endosymbiont type, as seen in a weighted UniFrac principal coordinate analysis (PCoA) plot (Fig. 4). The microbiome compositions of the two species that host chlorophyte symbionts, *C. compressa* and *Ar.angulatus*, grouped most closely to each other and to the substrate samples. The microbiome compositions of specimens of the dinoflagellate-bearing *S. orbiculus* were the most distinct. The *Am. gibbosa* specimens again showed the greatest variability. Because the weighted UniFrac plot describes the highest percentage of variability in the data, abundances (not just presence/absence) and phylogenetic relatedness of the microbial taxa each played a role in structuring the groupings observed.

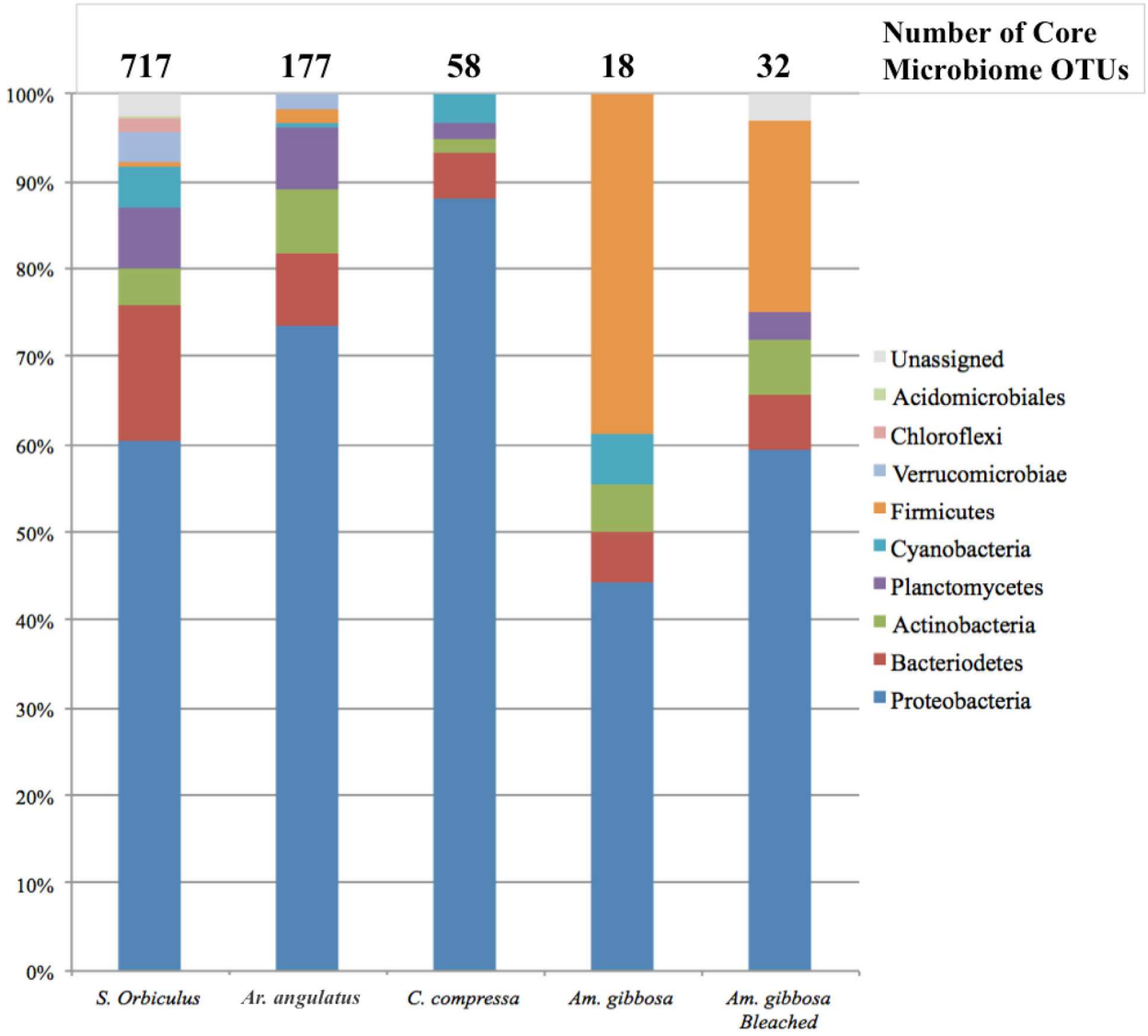

Figure 3. The bacterial phyla represented in the core microbiome of each foraminiferal species and their relative abundances.

The PERMANOVA analysis of the microbiome composition by host species tested the null hypothesis of no difference among the host microbiomes. The P-value (0.0001) and the pseudo-F statistic value (6.04) indicated that microbiome composition was significantly different among the host species. The Pairwise PERMANOVA tests comparing microbiomes of host pairs, using Monte Carlo permutations to account for the small sample sizes, revealed that all host microbiomes differed significantly, with P(MC) values all falling below the 0.05 threshold.

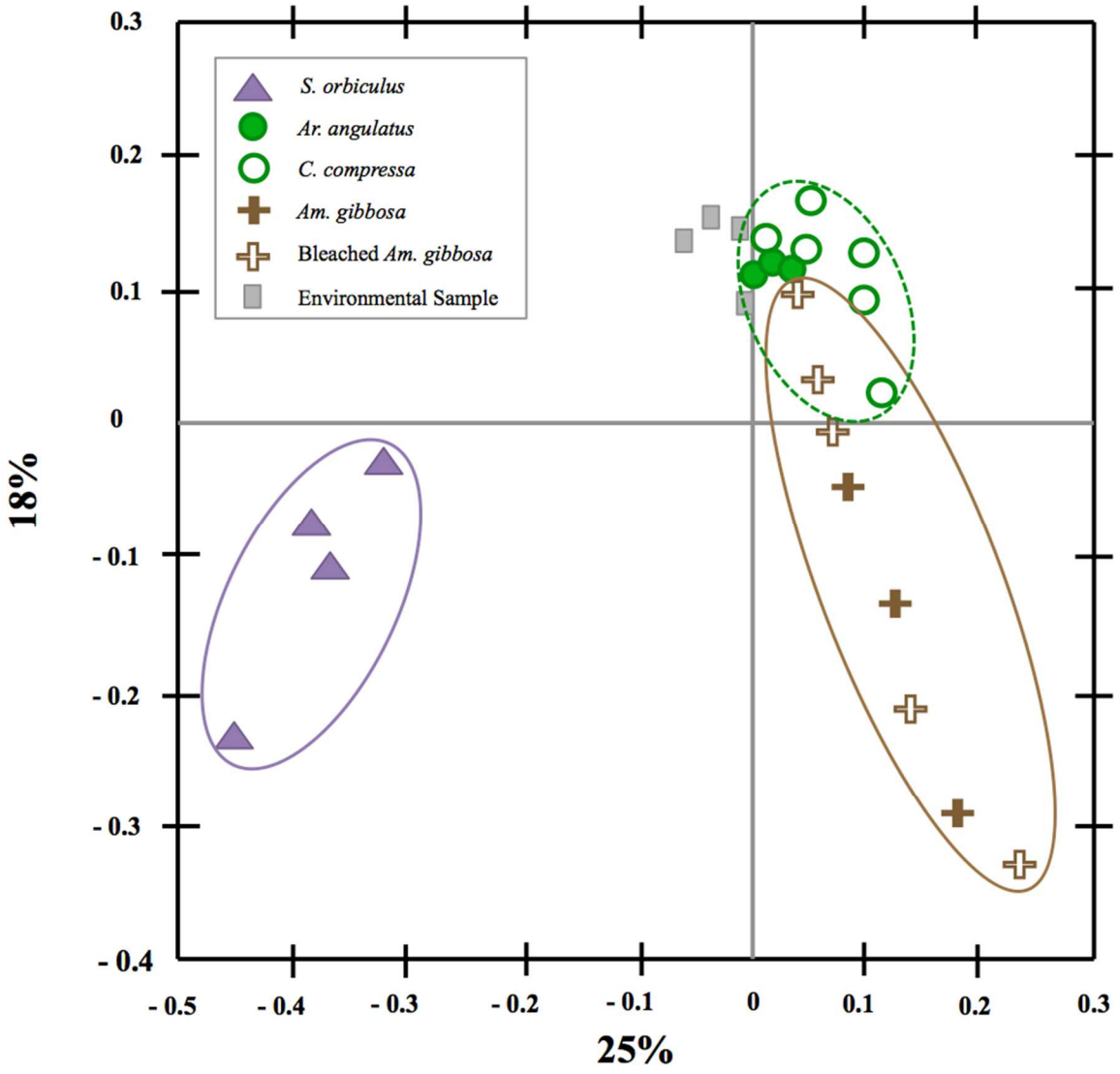

Figure 4. Clustering of foraminiferal samples based on weighted UniFrac dissimilarity matrix of microbial OTU abundance. Axes represent the percent of variability explained.

Core Microbiomes

Four taxa appeared in every foraminiferal specimen across all species (Table 3). These four OTU sequences were compared against the Kit Blank (KB) to determine if their presence could be due to contamination from the DNA extraction kit reagents or during DNA amplification. The *Propionibacterium* OTU was completely absent from the KB. However, hundreds of sequence reads of the two *Ralstonia* OTUs were detected in the KB. The

Rhodobacteraceae OTU was present in the KB, but with only six sequences. The *Propionibacterium* and *Ralstonia* OTUs were also present in the substrate samples, but at much lower abundances than in the foraminiferal samples; the substrate samples consistently had sequences of these OTUs under one hundred, while the foraminiferal samples had hundreds to thousands of sequence reads. The Rhodobacteraceae OTU was present in the substrate samples at similar numbers of sequence reads to those observed in the foraminiferal specimens.

***Archaias angulatus* and *Cyclorbiculina compressa*.** Although only three specimens of *Ar. angulatus* were successfully sequenced, they yielded a core microbiome of 177 OTUs. The percentage of the total *Ar. angulatus* microbiome represented by core taxa averaged 70% (range: 61–77%). The six *C. compressa* specimens shared 58 OTUs and the percentage of the total *C. compressa* microbiome represented by core taxa averaged 65% (range: 37–96%).

Archaias angulatus and *C. compressa* share the same endosymbiont type, a chlorophyte. The microbial taxa shared between them are collectively referred to as the "archaiasine core microbiome", consisting of 31 OTUs in three distinct microbial phyla (Table 4). The majority of the archaiasine core microbiome was comprised of the microbial families Rhodobacteraceae and Flavobacteriaceae, with 14 Rhodobacteraceae OTUs making up to 46% of the microbiome in one specimen of *C. compressa*. Overall, *Ar. angulatas* had somewhat lower relative abundances (38%) of the shared microbial OTUs compared to *C. compressa* (48%). For the nine specimens representing the two archaiasine species, the percentage of the total microbiome that represented core taxa averaged 43% (range: 32–67%).

Table 3. Core microbiome OTUs shared by all four foraminiferal species.

Phylum	Family	Genus	OTU ID	Sequence
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	1088265	GTGCCAGCAGCCGCGGTGATACGTA GGGTGCGAGCGTTGTCCGGATTTATT GGGCGTAAAGGGCTCGTAGGTGGTT GATCGCGTCGGAAGTGTAATCTTGGG GCTTAACCCTGAGCGTGCTTTCGATA CGGGTTGACTTGAGGAAGGTAGGGG AGAATGGAATTCCTGGTGGAGCGGT GGAATGCGCAGATATCAGGAGGAAC ACCAGTGGCGAAGGCGGTTCTCTGGG CCTTTCCTGACGCTGAGGAGCGAAAG CGTGGGGAGCGAACAGGCTTAGATA CCCCGGTAGTCC
Proteobacteria	Oxalobacteraceae	*Ralstonia*	437105	GTGCCAGCAGCCGCGGTAATACGTA GGGTCCAAGCGTTAATCGGAATTACT GGGCGTAAAGCGTGCGCAGGCGGTT GTGCAAGACCGATGTGAAATCCCCG GGCTTAACCTGGGAATTGCATTGGTG ACTGCACGGCTAGAGTGTGTCAGAG GGGGGTAGAATTCCACGTGTAGCAGT GAAATGCGTAGAGATGTGGAGGAAT ACCGATGGCGAAGGCAGCCCCCTGG GATAACACTGACGCTCATGCACGAA AGCGTGGGGAGCAAACAGGATTAGA TACCCCGGTAGTCC
Proteobacteria	Oxalobacteraceae	*Ralstonia*	287547	GTGCCAGCAGCCGCGGTAATACGTA GGGTCCAAGCGTTAATCGGAATTACT GGGCGTAAAGCGTGCGCAGGCGGTT GTGCAAGACCGATGTGAAATCCCCG AGCTTAACTTGGGAATTGCATTGGTG ACTGCACGGCTAGAGTGTGTCAGAG GGGGGTAGAATTCCACGTGTAGCAGT GAAATGCGTAGAGATGTGGAGGAAT ACCGATGGCGAAGGCAGCCCCCTGG GATAACACTGACGCTCATGCACGAA AGCGTGGGGAGCAAACAGGATTAGA TACCCCGGTAGTCC
Proteobacteria	Rhodobacteraceae		1107606	GTGCCAGCAGCCGCGGTAATACGGA GGGGGTTAGCGTTGTTCGGAATTACT GGGCGTAAAGCGCACGTAGGCGGAT CGGAAAGTTGGGGGTGAAATCCCGG GGCTCAACCCCGGAACTGCCTCCAAA ACTATCGGTCTAGAGTTCGAGAGAGG TGAGTGGAATTCCGAGTGTAGAGGTG AAATTCGTAGATATTCGGAGGAACAC CAGTGGCGAAGGCGGCTCACTGGCTC GATACTGACGCTGAGGTGCGAAAGT GTGGGGAGCAAACAGGATTAGATAC CCCGGTAGTCC

Sorites orbiculus. The four specimens of *S. orbiculus* shared 717 OTUs (Figure 3), demonstrating the largest and most conserved microbiome of the species examined. The percentage of the total *S. orbiculus* microbiome made up of core taxa averaged 91% (range: 82–95%). Proteobacteria and Bacteroidetes were the dominant phyla represented. Proteobacteria of the Family Rhodobacteraceae represented 28% (204) of the 717 core OTUs, but averaged only 10% relative abundance. Only 31 OTUs were identified as representing the Phylum Bacteroidetes, Family Amoebophilaceae, but comprised 48% of the total sequence reads.

Interestingly, *S. orbiculus* shared 26 OTUs with the other two species from the Family Soritidae (Table 5). These 26 OTUs represent 84% of the 31 OTUs shared by *Ar. angulatus* and *C. compressa.*

Table 4. Sub-family Archaiasinae core taxa: Core microbial taxa shared by *Ar. angulatus* and *C. compressa*, both of which host chlorophyte endosymbionts and belong to the same sub-family.

Phylum	Family	Genus	# OTUs	*Ar. angulatus*			*C. compressa*					
				RK2	RK6	RK8	C61	C62	C64	C66	C67	C68
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	1	0.2	0.1	0.4	0.4	0.2	0.01	0.1	0.1	0.1
Bacteroidetes	Flavobacteriaceae		2	6.2	19.1	8.8	19.6	3.3	20.2	9.1	13.2	12.3
Proteobacteria	Hyphomicrobiaceae	*Roseibium*	1	4.6	12.2	9	0.06	0	0.01	0.3	0.1	0
Proteobacteria	Hyphomicrobiaceae		1	2.1	0.9	2.4	0.4	0.06	0.1	1	0.3	0.5
Proteobacteria	Phyllobacteriaceae		1	2.5	0.3	0.4	0.01	0	0.01	1.6	1.2	0.2
Proteobacteria	Rhodobacteraceae		14	18.1	4.1	12	34.7	45.9	36.5	6.8	4.4	12.6
Proteobacteria	Oxalobacteraceae	*Ralstonia*	4	0.3	0.1	0.9	1.1	1.5	0.3	0.2	1.2	0.4
Proteobacteria	OM60	*Congregibacter*	2	0.2	0.9	0.6	0.5	0	3.7	0.4	0.2	8.6
Proteobacteria	Enterobacteriaceae		2	0.2	0.6	0.2	1.8	0.1	0.09	0.5	0.2	0.4
Proteobacteria	HTCC2089		1	1.4	0.5	1	7.2	0	0.3	5	0.9	0.2
Proteobacteria	Piscirickettsiaceae		2	1.5	1.9	0.6	1.3	3.6	0.4	7.1	9.9	7.5
		Total	**31**	**37.3**	**40.7**	**36.3**	**67.1**	**54.7**	**61.6**	**32.1**	**31.7**	**42.8**
			Avg.	**38**			**48**					
			Over-all Avg.	**43**								

Table 5. Core microbial taxa shared by all three foraminiferal species belonging to the Soritidae family.

Phylum	Family	Genus	# OTUs	*Ar. angulatus*			*C. compressa*						*S. orbiculus*			
				RK2	RK6	RK8	C61	C62	C64	C66	C67	C68	SK1	SK3	SK4	SK5
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	1	0.2	0.1	0.4	0.4	0.2	0.01	0.1	0.1	0.1	0.1	0.03	0.01	0.04
Bacteroidetes	Flavobacteriaceae		2	6.2	19.1	8.8	19.6	3.3	20.2	9.1	13.2	12.3	6	1.8	1.7	6.4
Proteobacteria	Hyphomicrobiaceae	*Roseibium*	1	4.6	12.2	9	0.06	0	0.01	0.3	0.1	0	0.1	0.09	0.04	0.02
Proteobacteria	Hyphomicrobiaceae		1	2.1	0.9	2.4	0.4	0.06	0.1	1	0.3	0.5	1.4	0.7	1	1.6
Proteobacteria	Phyllobacteriaceae		1	2.5	0.3	0.4	0.01	0	0.01	1.6	1.2	0.2	0.4	0.1	0.3	0.3
Proteobacteria	Rhodobacteraceae		13	18.1	4.1	12	34.7	45.9	36.5	6.8	4.4	12.6	8.7	9.7	10.8	11.5
Proteobacteria	Oxalobacteraceae	*Ralstonia*	3	0.3	0.1	0.9	1.1	1.5	0.3	0.2	1.2	0.4	0.2	0.03	0.03	0.1
Proteobacteria	OM60	*Congregibacter*	2	0.2	0.9	0.6	0.5	0	3.7	0.4	0.2	8.6	0.3	0.08	0.3	0.7
Proteobacteria	HTCC2089		1	1.4	0.5	1	7.2	0	0.3	5	0.9	0.2	0.3	0.5	0.9	0.7
Proteobacteria	Piscirickettsiaceae		1	1.5	1.9	0.6	1.3	3.6	0.4	7.1	9.9	7.5	0.9	0.7	1.7	1.5
		Total	**26**	**37.1**	**40.1**	**36.1**	**65.3**	**54.6**	**61.5**	**31.6**	**31.5**	**42.4**	**18.4**	**13.7**	**16.8**	**22.9**
			Avg.	**38**			**48**						**18**			

Amphistegina gibbosa. The three normal-appearing *Am. gibbosa* specimens had the fewest conserved core OTUs (18), making up an average of 25% (range: 8–49%) of the microbiome (Table 6A). Of those 18 shared OTUs, most belonged to the phyla Proteobacteria or Firmicutes, with smaller, roughly equal percentages of Bacteroidetes, Actinobacteria, and Cyanobacteria (Fig. 3).

The five partly bleached *Am. gibbosa* specimens shared 32 OTUs, averaging 32% (range: 23–50%) of the microbiome (Table 6B). These OTUs were from five distinct phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Planctomycetes, and one unassigned OTU. The Family Rhodobacteraceae (Phylum Proteobacteria) contributed ten OTUs, with relative abundances averaging 5%. Two OTUs with the highest average relative abundances belonged to the genus *Propionibacterium* (8%). Other notable constituents were the genus *Streptococcus* (Phylum Firmicutes) with seven OTUs averaging 4%, and the genus *Ralstonia* (Phylum Proteobacteria) with four OTUs averaging 3%. However, as previously noted, two of these *Ralstonia* OTUs may be due to contamination.

All eight *Am. gibbosa* specimens (normal-appearing plus partly-bleached specimens) shared six core OTUs, comprising an average of only 13% (range: 1–39%) of the microbiome (Table 6C). These six core taxa represented three phyla: Proteobacteria, Bacteroidetes, and Actinobacteria. The genus *Ralstonia* (Phylum Proteobacteria) contributed three OTUs with relative abundances averaging 3%, but again two of these OTUs may represent contamination. Other notable constituents were the genus *Propionibacterium* (Phylum Actinobacteria) with only one OTU, but higher average relative abundance (6%). These six core OTUs varied widely in relative abundances among samples; for example, the relative abundance of *Propionibacterium* ranged from 0.1–34%.

Table 6. Core microbial taxa of *Am. gibbosa* and their relative percent abundances: **A** core shared by all normal-appearing specimens; **B** core shared by all partly bleached specimens; **C** core shared by all specimens.

A.

A. Phylum	Family	Genus	# OTUs	M61	M62	M64
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	1	1.8	3.1	0.2
Bacteroidetes	[Amoebophilaceae]	*Ucs1325*	1	0.07	0.2	0.02
Cyanobacteria	Synechococcaceae	*Synechococcus*	1	0.005	1	0
Firmicutes	Staphylococcaceae	*Staphylococcus*	2	1	1.2	0.5
Firmicutes	Lactobacillaceae	*Lactobacillus*	5	1.1	13.7	6.4
Proteobacteria	Rhodobacteraceae		3	6.7	7.5	0.06
Proteobacteria	Oxalobacteraceae	*Ralstonia*	3	1.5	9.8	0.5
Proteobacteria	Enterobacteriaceae		1	2.5	4.9	0.4
Proteobacteria	Piscirickettsiaceae		1	3.8	7.4	0
		Total	**18**	**18. 5**	**48.8**	**8.1**
			Avg.	**25**		

B.

Phylum	Order	Family	Genus	# OTUs	M6B 1	M6B 2	M6B 3	M6B 7	M6B 8
Actinobacteria	Actinomycetale	Propionibacteriaceae	*Propionibacterium*	2	34.2	4.9	0.3	0.1	0.08
Bacteroidetes	Cytophagales	[Amoebophilaceae]	*Ucs1325*	1	0.03	2.6	0.2	0.01	0.01
Bacteroidetes	Flavobacteriales	Flavobacteriaceae		1	0.005	0.06	1.3	1.7	4.7
Firmicutes	Lactobacillales	Streptococcaceae	*Streptococcus*	7	9.3	5.8	0.9	0.7	0.7
Planctomycetes	Pirellulales	Pirellulaceae		1	0.01	1.3	7.4	5.8	4.4
Proteobacteria	Rhizobiales	Hyphomicrobiaceae	*Roseibium*	1	0	2.2	0.4	0	0.1
Proteobacteria	Rhodobacterales	Rhodobacteraceae		10	2	6.5	5.3	5.3	7
Proteobacteria	Burkholderiales	Oxalobacteraceae	*Ralstonia*	4	2.3	8.4	1.3	1	0.5
Proteobacteria	Chromatiales			1	0.6	0.03	3.4	3.7	1.2
Proteobacteria	Enterobacteriales	Enterobacteriaceae		1	2	0.01	0.02	0.2	0.01
Proteobacteria	HTCC2188	HTCC2089		1	0.01	1.7	1	2.6	1.7
Proteobacteria	Thiotrichales	Piscirickettsiaceae		1	0.01	1.1	1.5	1.5	7.4
			Total	**32**	**50.5**	**34.6**	**23.0**	**22.6**	**27.8**
				Avg.	**32**				

C.

C. Phylum	Family	*Genus*	# OTUs	M6 1	M6 2	M6 4	M6B 1	M6B 2	M6B 3	M6B 7	M6B 8
Actinobacteria	Propionibacteriaceae	*Propionibacterium*	1	1.8	3.1	0.2	34.2	4.9	0.3	0.1	0.08
Bacteroidetes	[Amoebophilaceae]	*Ucs1325*	1	0.1	0.2	0	0.03	2.6	0.2	0.01	0.01
Proteobacteria	Rhodobacteraceae		1	6.7	7.5	0.1	2	6.5	5.3	5.3	7
Proteobacteria	Oxalobacteraceae	*Ralstonia*	3	1.5	9.8	0.5	2.3	8.4	1.3	1	0.5
		Total	**6**	**10**	**21**	**0.8**	**39**	**22**	**7.1**	**6.4**	**7.6**
			Avg.	**11**			**16**				
			Over-all Avg.	**13**							

Most of the *Am. gibbosa* specimens had relatively high portions of Firmicutes. In the normal-appearing *Am. gibbosa*, the core Firmicutes OTUs included two bacterial genera, *Staphylococcus* and *Lactobacillus*. In the partly bleached *Am. gibbosa*, the core Firmicutes OTUs were only from the genus *Streptococcus.*

Discussion

Pan-Species Core Microbiome

While the core microbial taxa varied in quantity and diversity among the four foraminiferal species, the phyla Proteobacteria, Bacteroidetes, and Actinobacteria were represented in the core microbiomes of all specimens (Fig. 3). Cyanobacteria were found in all specimens except the partly-bleached *Am. gibbosa*. Proteobacteria represented the majority of the core OTUs for all species. Of the four OTUs found in every foraminiferal specimen sampled, two represent the genus *Ralstonia*, one the genus *Propionibacterium*, and the last could only be classified to the Family Rhodobacteraceae (Table 3).

The *Propionibacterium* OTU is very similar to a sequence commonly found in coral microbiomes. When the *Propionibacterium* sequence from this study was compared to a *Propionibacterium* sequence found in a coral microbiome study by Kellogg et al. (2016), the sequences appeared very similar, with BLASTn sequence comparison showing a 99% identity match over 229 base pairs. *Propionibacterium* sequences have been reported as members of coral microbiomes by a number of studies (de Castro et al., 2010; Ainsworth et al., 2015; Kellogg et al., 2017; Yang et al., 2017). This bacterium has also been seen, via laser microdissection and fluorescence *in situ* hybridization (FISH), inside the coral's endosymbiotic dinoflagellates, leading Ainsworth et al. (2015) to hypothesize that these bacteria may have a

role in facilitating the relationship between host coral and symbiotic algae. However, *S. orbiculus*, which hosts dinoflagellate endosymbionts, did not appear to have more *Propionibacterium* sequences than specimens of other three foraminiferal species. Among the eight *Am. gibbosa* specimens, relative abundances of *Propionibacterium* sequence reads varied from <0.1% to >34%. Because this OTU was completely absent from the control Kit Blank sample, its presence in all samples likely was not due to contamination. The *Propionibacterium* OTU was also present in the substrate samples, but at sequence reads that were orders of magnitude lower than in the foraminiferal samples. The higher abundances of *Propionibacterium* in the foraminiferal samples may indicate that this bacterium plays an important role in the holobiont.

Another bacterial genus present in all samples was *Ralstonia* (Family Oxalobacteraceae). Interestingly, this genus is also commonly associated with coral core microbiomes (Ainsworth et al., 2015; Leite et al., 2017; Yang et al., 2017) and, like *Propionibacterium*, has been observed within the coral's endosymbiotic dinoflagellates (Ainsworth et al., 2015). Hernandez-Agreda et al. (2017) hypothesized that a *Ralstonia* species may play a role in carbon uptake by the host coral from the endosymbiont, based upon the localization of the bacterium in the "peri-algal space", the area within the coral that hosts the endosymbiotic algae. In our study, large quantities of two *Ralstonia* OTUs observed in all samples also were present in the Kit Blank, indicating potential contamination, rather than being true members of the core microbiomes analyzed (e.g., Salter et al., 2014). However, all species sampled had at least three distinct OTUs of *Ralstonia*; each species bearing at least one *Ralstonia* OTU in addition to the two found in the "pan-species core". Thus, we cannot rule out the possibility that this bacterial genus was common to the core microbiomes of the foraminiferal species examined. Like the *Propionibacterium* OTU, the two

Ralstonia OTUs were present in the substrate samples at much lower sequence reads than in the foraminiferal samples, indicating a potential role in the holobiont.

The Rhodobacteraceae also appear to be major contributors to foraminiferal microbiomes since OTUs identified as representing this family were present in all specimens. Furthermore, the Rhodobacteraceae consistently contributed a larger proportion than other families of the core taxa for all species. Again, Rhodobacteraceae is a family of bacteria that has been observed as an important component of the microbiomes of coral and benthic foraminifers (Bourne et al., 2013; Pantos et al., 2015). Webster et al. (2016), examining samples collected on Australia's Great Barrier Reef, recorded Rhodobacteraceae in the benthic symbiont-bearing foraminifer, *Heterostegina depressa* d'Orbigny*,* which hosts a diatom symbiont (Lee, 2006). Unlike *Ralstonia* and *Propionibacterium*, Rhodobacteraceae have also been identified in association with marine pathogens that cause algal bleaching and mortality (Pantos et al., 2015; Zozaya-Valdes et al., 2015). Like the *Ralstonia* OTUs, the Rhodobacteraceae OTU found in all samples was also observed in the Kit Blank. However, the Kit Blank contained only six sequences of this OTU, while the foraminiferal specimens had hundreds to thousands of sequences of the same OTU. Given these abundance differences, the Rhodobacteraceae OTU found in all samples may be a true member of the core microbiome of these foraminifers. Interestingly, the counts of the Rhodobacteraceae OTU were similar in both foraminifers and substrate samples, suggesting a more passive or environment-driven relationship with the holobiont.

The foraminiferal taxa examined most likely do not share a "pan-species" microbiome; three of the four OTUs found in all foraminiferal specimens could be contaminants, and the fourth, the *Propionibacterium* OTU, could be associated with algal symbiosis, given that similar OTUs are found in zooxanthellate corals. However, Bourne et al. (2013, fig. S2), based on

redundancy analysis, found stronger similarities among the microbial communities of three foraminiferal taxa (*H. depressa*, *Marginopora vertebralis* Quoy & Gaimard, and *Sorites* sp.) than with the microbial communities of photosymbiont-bearing invertebrates included in their study. Bourne et al. (fig. S3) also reported higher taxonomic richness in the three foraminiferal taxa, which they attributed to the close association of the foraminifers with reef rubble, filamentous algae and sediment.

We found by far the most OTUs and higher Shannon diversity indices in the substrate samples compared to the foraminiferal specimens (Table 2). Moreover, *S. orbiculus* not only had a very large core microbiome, but rarefaction analyses revealed that, like the substrate samples, the number of OTUs continued to increase as number of sequences per sample increased (Fig. 1) Fujita & Hallock (1999) studied *Ar. angulatus* and *S. orbiculus* populations from the same location as we collected specimens of these species for our study. Fujita & Hallock reported that *S. orbiculus* is sensitive to nutrification that promotes epiphytic growth on the algae or seagrass upon which this species often is found. In the context of the Fujita & Hallock paper, our findings are consistent with the possibility that the microbial association on *S. orbiculus* can be influenced by the substrate to which it adheres.

The minimal similarities that we observed in the core microbiome of *Am. gibbosa* compared to the core microbiomes of the three soritid species is consistent with their evolutionary histories. The soritids are classified in the Class Tubothalamea and *Amphistegina* in the Class Globothalamea (Pawlowski et al., 2013). Although all four species are Cenozoic in origin, the classes to which they are assigned have been stratigraphically distinct since the Lower Cambrian (i.e., >510 mya).

Influence of the Host

Of the three soritid species (Order Miliolida, Family Soritidae), *S. orbiculus* belongs to the Subfamily Soritinae and hosts dinoflagellate endosymbionts, while *Ar. angulatus* and *C. compressa* belong to the Archaiasinae and host chlorophyte endosymbionts (e.g., Holzmann et al., 2001, and references therein). These three species shared a core microbiome composed of 26 OTUs representing three bacterial phyla (Table 5), representing 84% of the 31 OTUs shared by *Ar. angulatus* and *C. compressa* (Fig. 5). At the family level, only the Enterobacteriaceae OTUs, shared by *Ar. angulatus* and *C. compressa*, are absent from the *S. orbiculus* core. The similarities among the core microbiomes of the three soritids indicate that host phylogenetic relatedness plays a major role in structuring the core microbiome, as has been previously noted by Sunagawa et al. (2010).

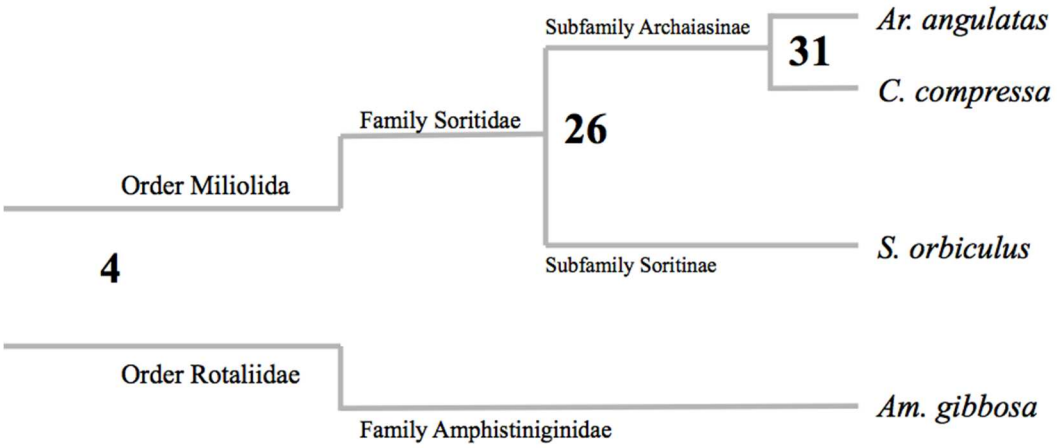

Figure 5. Numbers of core bacterial taxa shared by foraminiferal species with closer and more distant phylogenetic relationships. Branching indicates relatedness, but branch lengths are not quantitative.

Holzmann et al. (2001) reported the molecular phylogeny of the superfamily that includes the Soritidae, with a phylogenetic tree for the Soritinae for which *Sorites* spp. are near basal for two of the three branches. The Archaiasinae form a separate major branch of the Soritidae. Holzmann et al. further concluded that the Family Peneroplidae is ancestral to the Family Soritidae. Thus, future studies of the microbiomes associated with members of the Peneroplidae, which host rhodophyte symbionts, will be key to understanding the evolution of the core microbiome of the Soritidae.

The patterns of host specificity observed in this study agree with conclusions made in studies of coral microbiomes. In corals, the host is recognized as one of the strongest drivers of core microbiome composition, even considering temporal and geographic variation (Littman et al., 2009; La Rivière et al., 2015; Chu & Vollmer, 2016; McCauley et al., 2016). Studies comparing different species of corals found the microbial community to be more similar amongst closely related coral species (Sunagawa et al., 2010; La Rivière et al., 2015). Sunagawa et al. (2010) observed microbiome similarity among coral species belonging to the same genus and family, with microbiome profiles differing at higher taxonomic levels.

The trends seen in our study support the hypothesis that host phylogeny plays a major role in structuring the foraminiferal core microbiome (Fig. 5). Species in the same subfamily shared 31 core taxa, species in the same family shared 26 core taxa, and species spanning different orders shared at maximum only four core taxa. Authors of previous studies of coral or sponge microbiomes proposed a possible explanation for strong host species specificity: different species of host may offer different niches or host-derived nutrients, thereby favoring specific microbes (Littman et al., 2009; Sunagawa et al., 2010; Morrow et al., 2012; Reveillaud et al., 2014; Chu & Vollmer, 2016).

As noted above, the fourth species in this study, *Am. gibbosa*, shared at most four OTUs with the soritids. The microbial assemblages found associated with *Am. gibbosa* specimens were highly variable, with only six core taxa represented by three distinct phyla: Proteobacteria, Bacteroidetes, and Actinobacteria. Moreover, OTUs of Firmicutes contributed the largest average relative abundance (8%) in normal-appearing *Am. gibbosa*. In partly-bleached *Am. gibbosa*, Firmicutes contributed the fourth largest average relative abundance at 3.5%. The average relative abundances of 8% and 3.5% seen in healthy-appearing and partially-bleached *Am. gibbosa*, respectively, are higher than the average relative abundances observed in *S. orbiculus* and *Ar. angulatus*, where Firmicutes contributed <1% average relative abundance. Futhermore, Firmicutes was completely absent from the core microbiome of *C. compressa*. Prazeres et al. (2017) also reported that the most consistent and abundant members of the core microbiome of *Amphistegina lobifera* Larsen from the Great Barrier Reef, also belonged to the bacterial phyla Proteobacteria, Bacteroidetes, Firmicutes, and Planctomycetes. Moreover, Prazeres et al. (2017) identified Firmicutes as having the highest average abundance across all samples of *Am. lobifera.* These findings, taken together, suggest that Firmicutes is more closely associated with the genus *Amphistegina*. However, because both *Am. gibbosa* and *Am. lobifera* host diatom endosymbionts, we cannot determine if host phylogeny, endosymbiont type, or a combination of the two factors is driving the similarities observed.

Proteobacteria and Bacteroidetes were represented in the core microbiome of all four foraminiferal species; Firmicutes, and Planctomycetes were represented in three species cores. Webster et al. (2016) also reported Proteobacteria, Bacteroidetes, Firmicutes, and Planctomycetes in the foraminiferal species *Marginopora vertebralis* Quoy & Gaimard and *Heterostegina depressa*, which were observed to be dominated by Alphaproteobacteria,

Gammaproteobacteria, and Bacteroidetes. *Marginopora vertebralis* is a member of the Soritinae and is most closely related to *S. orbiculus* (Holzmann et al., 2001). *Heterostegina depressa* belongs to the same order (Rotaliida) as *Am. gibbosa* and also hosts diatom endosymbionts, but is not otherwise closely related. Additionally, we found that Alphaproteobacteria made up the largest proportion of the core microbiomes of all species, which is consistent with the findings of Webster et al. (2016) and Prazeres et al. (2017).

Influence of Algal Symbionts

As predicted, the two foraminiferal species that host chlorophyte symbionts, *Ar. angulatus* and *C. compressa*, had the most similar microbiomes, with 43% (*Ar. angulatus*) and 51% (*C. compressa*) of their microbiomes made up of shared core constituents. Moreover, the weighted UniFrac PCoA plot (Fig. 4) closely grouped the *Ar. angulatus* and *C. compressa* samples, and substantially separated that group from the *S. orbiculus* samples. These observations are consistent with the hypothesis posed by Bourne et al. (2013) that the microbiome can be heavily influenced by the photosynthetic symbiont.

The difference in location of origin did not appear to impact similarity between these two species; the *C. compressa* samples were from the six meter deep Tennessee Reef site, located in the Atlantic Ocean, whereas the *Ar. angulatus* were collected from the Keys Marine Laboratory site, located six miles away on the Gulf of Mexico. However, that distance may not be meaningful because Tennessee Reef can be influenced by tidal outflow from Florida Bay (Ogden et al., 1994). Because *Ar. angulatus* and *C. compressa* belong to the same subfamily, how much their similarity is influenced by their common endosymbiont type versus their phylogenetic proximity will require further research with additional members of the Superfamily Soritinacea,

including the ancestral Family Peneroplidae (Holzmann et al., 2001), which hosts a rhodophyte endosymbiont (Lee, 2006, and references therein).

Influence of Bleaching

Although elevated temperatures are hypothesized to be the primary factor in coral bleaching, most studies of foraminifers have shown a stronger response to photoinhibitory stress than to elevated temperature alone (e.g., Hallock et al., 1995; Talge & Hallock, 2003). The samples for our study were collected in late spring (mid May). Light intensity reaching the sea surface at the latitude of the Florida reef tract peaks in June, and time-series studies of prevalence of bleaching in *Am. gibbosa* populations have consistently shown that onset of partial bleaching occurs in spring, typically peaking in prevalence in early summer, well before maximum sea-surface temperatures (Hallock et al., 1995; Williams et al., 1997). Bleaching in foraminifers has been experimentally observed at temperatures as low as 20ºC when light intensity is at its peak (Talge & Hallock, 2003).

Recent studies specifically targeting temperature stress (Schmidt et al., 2011; Prazeres et al., 2016; Stuhr et al., 2017) have confirmed the experimental results of Talge & Hallock (2003) that elevated temperature can induce symbiont loss in foraminifers. Both Talge & Hallock (2003) and Stuhr et al. (2017) reported partial bleaching in *Am. gibbosa* after several weeks at 32ºC. Because sea-surface temperatures were indeed anomalously high in May 2016 (according to ocean data from the NOAA Station PKYF1: http://www.ndbc.noaa.gov/station_page.php?station=pkyf1, a site in the general area of the sampling locations), we cannot rule out the possibility that partial bleaching was induced by

photo-oxidative stress resulting from the combination of the approaching solar maximum combined with unusually high sea-surface temperatures.

The lack of consistent differences in the microbial OTUs found in partly-bleached versus normal-appearing specimens, combined with the overall variability of the *Am. gibbosa* individuals (Figs. 2, 4), be a consequence of sampling at a time when early stages of bleaching are common. Using cytological examination, Talge & Hallock (2003) observed that normal-appearing specimens of *Am. gibbosa* often exhibited early stages of damage to the diatom symbionts in spring and early summer. Thus, in our study, although some individuals appeared to be "normal" and others were "partly bleached", given the time of sampling and the high variability in microbial OTUs among the specimens of *Am. gibbosa* (Figs. 2, 4), all specimens likely had experienced photo-oxidative stress. For future studies, the addition of a seasonal sampling component would allow for comparisons of the possible effects on microbiome composition between "bleaching" and "non-bleaching" seasons. Similarly, studies of coral bleaching also have revealed that microbiome shifts can occur before any visual indications of bleaching are present (Bourne et al., 2007).

Besides having the most variable microbiomes, the *Am. gibbosa* specimens were the smallest individuals successfully sequenced and consistently produced the lowest numbers of sequence reads (Table 2). One specimen produced only ~9000 sequence reads and was eliminated from further analyses. In addition to size, the fundamental difference in calcification process in the Rotaliida as compared to the Miliolida (e.g., Erez, 2003; Pawlowski et al., 2013 and references therein) may contribute to lower sequence reads in *Am. gibbosa*. The Miliolida construct one chamber at a time, calcifying within the organic template where the new chamber is formed (e.g., Wetmore, 1999). As illustrated by Crevison & Hallock (2007) and Souder et al.

(2010), specimens of *Ar. angulatus* and *C. compressa* collected live from apparently thriving populations can exhibit surface pits, dissolution, microborings, microbial biofilm, and epibionts. The Rotaliida, in contrast, produce secondary lamellae over the entire test with each chamber addition (Hemleben et al., 1986, and references therein) and, as a consequence, their tests typically show much less evidence of infestation.

When symbiont loss/partial bleaching was documented in *Am. gibbosa* populations from the Florida Keys in the 1990s, anomalously high proportions of broken and epiphytized tests were observed (Hallock et al., 2006 and references therein). When imaged with Scanning Electron Microscopy, some tests of partially bleached specimens exhibited an unusual blotchy appearance and some were extensively microbored and even epiphytized (Hallock et al., 1995; Toler & Hallock, 1998). Moreover, when *Am. gibbosa* populations exhibited intermediate stress that induced partial bleaching, specimens tended to exhibit higher percentages of test damage than when stress was more acute, likely because the latter induced higher rates of mortality (Hallock et al., 2006, and references therein). Thus, stress associated with partial bleaching in *Am. gibbosa* may allow proliferation of microbes on the surface of the test as well as within the damaged chambers. Corals sampled before, during, and after bleaching events have revealed microbiome disruption (Bourne et al., 2007; Littman et al., 2011; Lins-de-Barros et al., 2012; Pootakham et al., 2018) and, as noted previously, shifts in microbial diversity have been detected before visible signs of bleaching became apparent (Bourne et al., 2007).

In spite of the overall variability, the shared core microbiome of the partly-bleached and normal-appearing *Am. gibbosa* specimens revealed similarity between the two groups. Together, they shared six core taxa representing the bacterial phyla Actinobacteria, Bacteroidetes, and Proteobacteria (Table 6C). When their core taxa are evaluated separately, the bacterial Phylum

Firmicutes was present in both groups in abundances significantly higher than in the other foraminiferal species sampled (Fig. 3). However, Firmicutes genera are not represented in the core taxa shared between the two groups because the OTUs associated with normal-appearing *Am. gibbosa* were from *Staphylococcus* and *Lactobacillus*, while those from partly-bleached *Am. gibbosa* were from *Streptococcus.* All three of these bacterial genera have been observed in coral microbiomes (Beleneva et al., 2005; de Castro et al., 2010; Godoy-Vitorino et al., 2017). However, studies of microbiome composition in healthy and diseased corals have found that *Staphylococcus* is common in healthy corals, but is not observed in diseased corals (Beleneva et al., 2005; de Castro et al., 2010). The presence of *Staphylococcus* in the healthy-appearing *Am. gibbosa*, and absence from the partly-bleached samples may indicate that *Staphylococcus* is lost from the holobiont when the host experiences stress, whether from bleaching, disease, or other potential stressors.

In a study of the influence of increased temperature on *Marginopora vertebralis* and *Heterostegina depressa,* Webster et al. (2016) found a reduction in overall OTUs when temperature was increased from 28 to 31°C. Although we found similar overall average numbers of OTUs between normal-appearing and partly-bleached specimens (353 vs. 352, Table 2), the partly-bleached specimens exhibited a higher number of core OTUs than the normal-appearing specimens (32 vs. 18, Table 6A,B). However, neither species in the Webster et al. (2016) study exhibited any visible changes such as the loss of coloration seen in partly bleached specimens.

In studies of microbiome changes during bleaching in corals, bleaching resulted in the loss or reduction of cyanobacteria from coral microbiomes (Ainsworth et al., 2008; Littman et al., 2011; Lins-de-Barros et al., 2012). In our study, cyanobacteria were represented in the core microbiome of all species except for the partly bleached *Am. gibbosa*. However, cyanobacteria

were a very small component of the normal-appearing *Am. gibbosa*, contributing only one OTU and an average relative abundance of 0.3%, so testing the hypothesis that cyanobacterial OTUs decline with bleaching will require further study.

Microbial Associations and Environmental Stress

With growing evidence for the role of microbiomes in protection from pathogens, it is not surprising that diseases in marine organisms can be correlated with a perturbation of the core microbiome. Heightened diversity of core microbiomes has been linked to White Band Disease in corals (Gignoux-Wolfsohn & Vollmer, 2015), sponge disease and die-off (Blanquer et al., 2016), unknown lesions in corals (Meyer et al., 2014), and other visible indicators of disease. There are two main hypotheses regarding the disruption of the microbiome and its contribution to disease. The first suggests that when the antimicrobial activity of the associated microbes is hindered by environmental stressors, outside pathogens are able to colonize the organism and cause disease (Rosenberg et al., 2007). The second hypothesis states that when the health of the host is compromised by outside stressors, normal constituents of the host's own microbiome may opportunistically grow beyond their normal populations, causing disease (Bourne et al., 2007).

Several intriguing pieces of evidence from our study suggest that the microbiomes of algal symbiont-bearing foraminifers should similarly reflect environmental stress. The high variability in the microbiomes associated with *Am. gibbosa* specimens experiencing photo-oxidative stress are consistent with previous reports of individual and population responses associated with bleaching (e.g., Hallock et al., 2006, and references therein). Hallock et al. (1995) actually described the variety of signs as indicating a "new disease". The observations of Fujita & Hallock (1999) that *S. orbiculus* is sensitive to nutrient pollution that results in

increased epiphytization of its preferred algal and seagrass substrates are consistent with our microbiome data that indicate the influence of the substrate on the microbiome of *S. orbiculus*. And while we selected specimens of *Ar. angulatus* and *C. compressa* that appeared undamaged, Souder et al. (2010) illustrated individuals of both species that were collected from habitats in which notable proportions of the specimens exhibited microboring, epiphytization and other features that would certainly influence the microbiomes of such specimens.

The benthic foraminifers that host algal symbionts are important carbonate sediment producers in tropical shelf and reef environments (e.g., Hallock, 1981; Yamano et al., 2000). They also are widely used as bioindicators of the potential of environments to support hypercalcification by zooxanthellate corals (e.g., Hallock, 2012, and references therein). Thus, future microbiome studies of these protists, using both field-collected specimens and laboratory experiments of their responses to environmental changes, hold great promise to advance both our understanding of foraminiferal biology and ecology, and their usefulness as bioindicators of environmental stressors.

Conclusions

This study examined microbiomes associated with specimens of four species of algal symbiont-bearing foraminifers from the Florida Reef Tract, USA.

1. The microbial phyla Proteobacteria, Bacteroidetes, and Actinobacteria were represented in the core microbiomes of all specimens, though only one *Propionibacterium* OTU was unquestionably found in all foraminiferal specimens analyzed and absent in the Kit Blank.

2. The quantity of Operational Taxonomic Units (OTUs) in core microbiomes differed widely among the species.
    a. The core microbiome of *Sorites orbiculus* comprised 717 distinct OTUs, dominated by Proteobacteria, most notably by the Family Amoebophilaceae; Rhodobacteraceae and Flavobacteriaceae were also well represented.
    b. In the core microbiome of *Archaias angulatus*, 177 OTUs were found, dominated by those from the Class Alphaproteobacteria, Order Acidomicrobiales, and families Flavobacteriaceae, Hyphomicrobiaceae, and Rhodobacteraceae.
    c. The core microbiome of *Cyclorbiculina compressa* included 58 OTUs, represented by the phyla Cyanobacteria and Planctomycetes, and families Flavobacteriaceae, Rhodobacteraceae, and Pseudanabaenaceae.
    d. The microbial assemblages found in *Amphistegina gibbosa* specimens varied widely in composition and abundance. Only 6 OTUs, representing the Proteobacteria, Bacteroidetes, and Actinobacteria, were found in all specimens.
3. Host phylogeny appeared to be the strongest driver of foraminiferal microbiome composition.
    a) Specimens of *Archaias angulatus* and *Cyclorbiculina compressa*, both hosting chlorophyte endosymbionts and both in the Family Soritidae, Subfamily Archaiasinae, shared 31 core OTUs.
    b) *Sorites orbiculus*, hosting dinoflagellate endosymbionts, from the Family Soritidae, Subfamily Soritinae, shared 26 core OTUs with specimens of both *Ar. angulatus* and *C. compressa*, which represents 84% of the core shared by the two archaiasine species.

c) The major differences between the very limited core microbiome of *Amphistegina gibbosa* compared with the diverse core microbiome shared by the three soritid species indicate that representatives of the two major classes of the Foraminifera do not share a core microbiome.

CHAPTER 3. ADDITIONAL DISCUSSION

Notes on Unsuccessful Extraction Attempts

During the planning stages of this thesis, I initially aimed to sequence three chlorophyte-bearing species to make a comparison of the microbiomes of foraminiferal species that all bear the same endosymbiont type, *Archaias angulatus*, *Cyclorbiculina compressa*, and *Androsina lucasi* Levy, 1977. The *Androsina* samples were collected during the same sampling trip and were collected from shallow mudflats on Little Torch Key. DNA extraction, PCR, and gel electrophoresis were performed on specimens of each species prior to sequencing to assess the potential for success of bacterial DNA extraction. The *Androsina* samples were the only foraminiferal species that failed to appear on the gel electrophoresis, leading me to believe that DNA extraction was unsuccessful. As the *Androsina* specimens were the smallest of the foraminiferal specimens collected, it is possible that their small size may have contributed to the difficulties in bacterial DNA extraction.

Another study question that was eliminated from the thesis was the comparison of *Ar. angulatus* from two different sampling locations. *Archaias angulatus* was collected from both the Tennessee Reef and Keys Marine Laboratory field sampling locations. However, after quality checking the sequencing results, all of the *Ar. angulatus* specimens from the Tennessee Reef field site failed basic sequence-quality metrics, eliminating them from further analysis.

Additionally, foraminiferal samples were kept in aquaria for seven days and then prepared in the same manner as the field samples in an attempt to observe any possible differences in microbiome structure between field samples and samples from aquaria. However, after submission of the extracted DNA to the sequencing facility, all of the samples that had been kept in aquaria yielded extremely low bacterial DNA content upon attempted amplification and further analysis was not attempted.

Recommendations for Future Research

Future research that would work to develop my initial hypotheses would be to expand the study to include more species of larger benthic foraminifers and the inclusion of planktonic foraminifers, both groups where algal symbiosis is prevalent. Expanding the research to include more foraminiferal species that host similar endosymbionts, such as *Laevipeneroplis* that hosts a chlorophyte similar to *C. compressa* and *Ar. angulatus,* or *Heterostigina depressa* that hosts a diatom symbiont similar to *Am. gibbosa,* as well as additional species of *Amphistegina*, could provide useful comparisons for evaluating the potential role of endosymbiont type in microbiome structure. Additionally, to continue testing the connection between foraminiferal phylogenetic relatedness and microbiome similarity, inclusion of the family Peneroplidae would be appropriate for future study. This family is ancestral to the family Soritidae and thus could be key to understanding the evolution of the core microbiome of the Soritidae. Futhermore, the family Peneroplidae hosts rhodophyte symbionts, which would add to the comparison of algal symbiont taxa on microbiomes.

Future studies focusing on the effects of bleaching on foraminiferal microbiomes should include seasonal sampling components to allow for comparisons between “bleaching” and “non

bleaching" seasons. Additionally, study of the impact of bleaching-induced test structure changes on microbiome structure is a topic of interest. Study of this topic could be achieved by collecting a subset of foraminiferal samples to be imaged using a scanning electron microscope (SEM) or other similar imaging technology. This topic could be expanded to include multiple species of both healthy and bleached-appearing specimens to study any connection between test structure and microbiome diversity.

Furthermore, additional study of the role of habitat on foraminiferal microbiomes is a topic that could be addressed with future study, following the lead of Prazeres et al. (2016) who examined differences in *Am. lobifera* microbiomes with habitat across the Great Barrier Reef, Australia. Roder et al. (2015) found that, where a coral species is abundant, its microbial community has a higher degree of structure and less variability. In marginal/fringe habitats, away from the coral's preferred geographic, light, depth, substrate, and/or nutrient conditions, Roder et al. observed a shift in the species' microbial assemblage. The further away from a species' "ideal" habitat, the less structured and more diverse their microbiome (Roder et al., 2015). This observation suggests that availability of an organism's preferred habitat or environmental conditions could be an important factor in the host's ability to maintain the composition of its core microbiome.

In this study, *Ar. angulatus* collected from the Keys Marine Laboratory field site at 1.5 meters were successfully sequenced, but the *Ar. angulatus* specimens from the Tennessee Reef field site at 6 meters failed sequence quality analysis. Larger miliolid foraminifers, like *Ar. angulatus*, are more commonly found in shallower sites and are more restricted by depth than rotaliids (Hallock, 2003). The difficulty in sequencing the *Ar. angulatus* from the deeper site could possibly be due to the fact that these specimens were in a fringe environment. Future

studies could address this topic by incorporating individuals from multiple locations and environmental conditions including fringe or marginal habitats.

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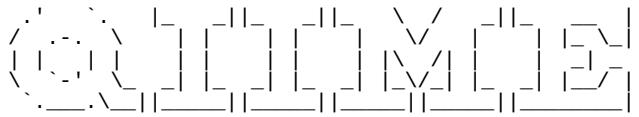
APPENDICES

Appendix 1: Full QIIME workflow.



```
QIIME 1.9.1 AMI (derived from the StarCluster Ubuntu 12.04 AMI)
www.qiime.org

Getting help: help.qiime.org
QIIME script index: scripts.qiime.org
QIIME workshops: workshops.qiime.org
QIIME help videos: videos.qiime.org
StarCluster (building AWS-based clusters): star.mit.edu/cluster
IPython, and the IPython Notebook: ipython.org
Software Carpentry (educational resources for Linux and scientific computing):
    software-carpentry.org

QIIME is powered by scikit-bio: scikit-bio.org
Qiita, QIIME-powered microbiome data storage and analysis: qiita.microbio.me
biocore, collaboratively developed bioinformatics software: github.com/biocore

To print configuration and version info for QIIME and its dependencies, run:
    print_qiime_config.py

Current System Stats:

  System load:  0.0               Processes:           119
  Usage of /:   28.2% of 63.00GB  Users logged in:     1
  Memory usage: 0%                IP address for eth0: 172.31.58.64
  Swap usage:   0%

ubuntu@ip-172-31-58-64:~$ print_qiime_config.py

System information
==================
         Platform:    linux2
   Python version:    2.7.3 (default, Aug  1 2012, 05:14:39)  [GCC 4.6.3]
Python executable:    /usr/bin/python

QIIME default reference information
===================================
For details on what files are used as QIIME's default references, see here:
 https://github.com/biocore/qiime-default-reference/releases/tag/0.1.2

Dependency versions
===================
          QIIME library version:     1.9.1
           QIIME script version:     1.9.1
qiime-default-reference version:     0.1.2
                 NumPy version:     1.9.2
                 SciPy version:     0.15.1
                pandas version:     0.16.1
            matplotlib version:     1.4.3
            biom-format version:     2.1.4
                   h5py version:     2.5.0 (HDF5 version: 1.8.4)
                   qcli version:     0.1.1
                   pyqi version:     0.3.2
             scikit-bio version:     0.2.3
                 PyNAST version:     1.2.2
                Emperor version:     0.9.51
                burrito version:     0.9.1
       burrito-fillings version:     0.1.1
              sortmerna version:     SortMeRNA version 2.0, 29/11/2014
              sumaclust version:     SUMACLUST Version 1.0.00
                  swarm version:     Swarm 1.2.19 [May 26 2015 15:28:37]
                          gdata:     Installed.

QIIME config values
===================
For definitions of these settings and to learn how to configure QIIME, see here:
 http://qiime.org/install/qiime_config.html
 http://qiime.org/tutorials/parallel_qiime.html

                      blastmat_dir:    /qiime_software/blast-2.2.22-release/data
      pick_otus_reference_seqs_fp:    /usr/local/lib/python2.7/dist-
packages/qiime_default_reference/gg_13_8_otus/rep_set/97_otus.fasta
```

```
                        sc_queue:    all.q
      topiaryexplorer_project_dir:    None
     pynast_template_alignment_fp:    /usr/local/lib/python2.7/dist-
packages/qiime_default_reference/gg_13_8_otus/rep_set_aligned/85_otus.pynast.fasta
                  cluster_jobs_fp:    start_parallel_jobs.py
pynast_template_alignment_blastdb:    None
assign_taxonomy_reference_seqs_fp:    /usr/local/lib/python2.7/dist-
packages/qiime_default_reference/gg_13_8_otus/rep_set/97_otus.fasta
                    torque_queue:    friendlyq
                   jobs_to_start:    1
                      slurm_time:    None
            denoiser_min_per_core:    50
assign_taxonomy_id_to_taxonomy_fp:    /usr/local/lib/python2.7/dist-
packages/qiime_default_reference/gg_13_8_otus/taxonomy/97_otu_taxonomy.txt
                        temp_dir:    /home/ubuntu/temp/
                    slurm_memory:    None
                     slurm_queue:    None
                     blastall_fp:    /qiime_software/blast-2.2.22-release/bin/blastall
                seconds_to_sleep:    1

#Use shell script from 2016 edamame tutorial:
for file in $(<list.txt)
do
    join_paired_ends.py -f ${file}L001_R1_001.fastq -r ${file}L001_R2_001.fastq -o ${file}/
    mv ${file}/fastqjoin.join.fastq Merged_Reads/${file}_merged.fastq
    convert_fastaqual_fastq.py -c fastq_to_fastaqual -f Merged_Reads/${file}_merged.fastq -o
Merged_Reads/${file}
    mv Merged_Reads/${file}/${file}_merged.fna Merged_Reads/${file}_merged.fasta
    rm -r Merged_Reads/${file}
    rm Merged_Reads/${file}_merged.fastq
    rm -r ${file}/
done

#Renamed above script Merged_Reads_Script.sh
#changed permissions with:
> chmod 755 Merged_Reads_Script.sh
#Above script from: https://github.com/edamame-
course/Amplicon_Analysis/blob/master/resources/Merged_Reads_Script.sh
#Script written by Jackson Sorrenson

#Set up tmux session named 'Success'
#Started 9:30, ended 14:30. Time elapsed: 5 hours
#Output: combined_seqs.fna

> count_seqs.py -i combined_seqs.fna
#Counted 10,383,845 total sequences

#Using mothur to show summary statistics
> mothur
> summary.seqs(fasta=combined_seqs.fna)
              Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:      1       251     251     0       3       1
2.5%-tile:    1       291     291     0       4       259597
25%-tile:     1       292     292     0       4       2595962
Median:       1       292     292     0       4       5191923
75%-tile:     1       292     292     0       4       7787884
97.5%-tile:   1       293     293     0       6       10124249
Maximum:      1       496     496     0       217     10383845
Mean:   1     272.451 272.451 0       4.16645
# of Seqs:    10,383,845

Output File Name:
combined_seqs.fna.summary

> quit()

#Start new tmux session "OTUP":
> tmux new -s OTUP

> pick_open_reference_otus.py -i combined_seqs.fna -o uclust_openref/ -f
#Had to move combined_seqs.fna file from Merged_Reads folder to main directory
#Started at 9:45, ended at 12:15 . Total time elapsed: 2.5 hours

#In the above script:
#We tell QIIME to look for the input file -i, "combined_seqs.fna".
#We specify that output files should go in a new folder, uclust_openref/
#We tell the program to overwrite already-existing files in the folder if we are running this
program more than once (-f)
#Other default parameters of interest:
#Singletons are removed from the OTU table (default flag --min_otu_size)
#Alignment is performed with PyNAST
```

```
#Taxonomy is assigned with uclust
#We do not perform prefiltering, as per the recommendations of Rideout et al.


#Navigate to uclust_openref/pynast_aligned_seqs
#Check how many failed alignments there were:
> count_seqs.py -i rep_set_failures.fasta
2362  : rep_set_failures.fasta (Sequence lengths (mean +/- std): 342.8544 +/- 66.4463)
2362  : Total

#Check how many successful alignments
> count_seqs.py -i rep_set_aligned.fasta
44993  : rep_set_aligned.fasta (Sequence lengths (mean +/- std): 7682.0000 +/- 0.0000)
44993  : Total

#Used blast to look at 10 different sequence from rep_set_failures.fasta. First few were good
matches, but others were complete trash.
#So, use: otu_table_mc2_w_tax_no_pynast_failures.biom (does not include failures)

Looking at assigne
> cd uclust_assigned_taxonomy
> wc -l rep_set_tax_assignments.txt
# Results in 47,355 (# of lines in the document = # of assignments)
> grep -c k__Bact rep_set_tax_assignments.txt
# find and count number of times it sees kingdom bacteria = found 41,766
> grep -c Unassigned rep_set_tax_assignments.txt
# find and count unassigned = found 5,379
> grep -c c__Chloroplast rep_set_tax_assignments.txt
# find and count Chloroplasts = found 1,858
> grep -c f__mitochondria rep_set_tax_assignments.txt
# find and count mitochondria = found 341
# Chloroplasts + mitochondria = 2,199

> grep -c k__Arch rep_set_tax_assignments.txt
# find and count number of kingdom archaea = found 210
# Bacteria + Unassigned = 47,145
# Bacteria + Archaea + Unassigned = 47,355 (same as total)

#Move back to uclust_openref/
> filter_taxa_from_otu_table.py -i otu_table_mc2_w_tax_no_pynast_failures.biom -o
otu_table_final.biom -n c__Chloroplast,f__mitochondria
# Removed chloroplasts and mitochondrial sequences from data
> biom convert -i  otu_table_final.biom -o otu_table_final.txt --to-tsv
# creating text file to view that removal was done correctly
> biom convert -i  otu_table_mc2_w_tax_no_pynast_failures.biom -o
otu_table_mc2_w_tax_no_pynast_failures.txt --to-tsv

> wc -l otu_table_mc2_w_tax_no_pynast_failures.txt
# Result: 44,995
> wc -l otu_table_final.txt
#Result: 42,871
# input-output = 2,124
# removal of chloroplasts and mitochondria should have removed 2,199
# 75 extra lines in output file unaccounted for...
# Jackson Sorrenson (who wrote shell script) said that likely the 75 lines were removed as
"failures" because they did not align. Numbers not adding up would be a problem if MORE than our
2,199 were removed, but only 2,124 were removed, so the 75 difference were likely removed prior.
# Deciding to continue on with data analysis and assume above explanation is correct as we do not
have the time to write a script to "sanity check"

> nano otu_table_mc2_w_tax_no_pynast_failures.txt


#Summarize OTU table
> biom summarize_table -i otu_table_final.biom -o summary_otu_table_final.txt

> more summary_otu_table_final.txt

# Num observations: 42869
# Total count: 8322283
# Table density (fraction of non-zero values): 0.079

# Counts/sample summary:
 # Min: 9260.0
 # Max: 967637.0
 # Median: 282469.000
 # Mean: 308232.704
 # Std. dev.: 259651.391
 # Sample Metadata Categories: None provided
 # Observation Metadata Categories: taxonomy
```

```
# Counts/sample detail:
#  M66: 9260.0
#  M62: 20467.0
#  M6B2: 22059.0
#  M6B1: 57814.0
#  M61: 58328.0
#  M64: 71177.0
#  KB: 89657.0
#  M6B7: 104624.0
#  C61: 124969.0
#  RK6: 156225.0
#  M6B3: 174051.0
#  M6B8: 182342.0
#  RK8: 205650.0
#  RK2: 282469.0
#  6E2: 288620.0
#  C68: 289638.0
#  C67: 315677.0
#  C64: 349799.0
#  C66: 354082.0
#  SK5: 382586.0
#  C62: 432152.0
#  SK1: 549377.0
#  KE2: 584520.0
#  SK3: 662209.0
#  SK4: 755146.0
#  KE1: 831748.0
#  6E1: 967637.0


# Still have three amphistegina with higher than 20,000 counts, so we will remove the first M66
because it only have 9,260 and thus would make us give up a lot of counts

# Created file ids.tt listing the one file that we will be removing: listing sample with counts
below 20,000 -- M66
# Script will discard samples listed in this file

> filter_samples_from_otu_table.py -i otu_table_final.biom -o filtered_otu_table_final.biom --
sample_id_fp ids.txt --negate_sample_id_fp
> biom summarize_table -i filtered_otu_table_final.biom -o summary_filtered_otu_table_final.txt
> more summary_filtered_otu_table_final.txt

Num samples: 26
Num observations: 42869
Total count: 8313023
Table density (fraction of non-zero values): 0.082

Counts/sample summary:
 Min: 20467.0
 Max: 967637.0
 Median: 285544.500
 Mean: 319731.654
 Std. dev.: 257763.029
 Sample Metadata Categories: None provided
 Observation Metadata Categories: taxonomy

Counts/sample detail:
 M62: 20467.0
 M6B2: 22059.0
 M6B1: 57814.0
 M61: 58328.0
 M64: 71177.0
 KB: 89657.0
 M6B7: 104624.0
 C61: 124969.0
 RK6: 156225.0
 M6B3: 174051.0
 M6B8: 182342.0
 RK8: 205650.0
 RK2: 282469.0
 6E2: 288620.0
 C68: 289638.0
 C67: 315677.0
 C64: 349799.0
 C66: 354082.0
 SK5: 382586.0
 C62: 432152.0
 SK1: 549377.0
 KE2: 584520.0
 SK3: 662209.0
 SK4: 755146.0
```

```
 KE1: 831748.0
 6E1: 967637.0

#Successfully removed M66

> single_rarefaction.py -i filtered_otu_table_final.biom -o otu_table_final_rarified20467.biom -d
20467

> biom summarize_table -i otu_table_final_rarified20467.biom -o
summary_otu_table_rarified20467.txt
> more summary_otu_table_rarified20467.txt

Num samples: 26
Num observations: 15076
Total count: 532142
Table density (fraction of non-zero values): 0.079

Counts/sample summary:
 Min: 20467.0
 Max: 20467.0
 Median: 20467.000
 Mean: 20467.000
 Std. dev.: 0.000
 Sample Metadata Categories: None provided
 Observation Metadata Categories: taxonomy

Counts/sample detail:
 M61: 20467.0
 RK6: 20467.0
 M6B1: 20467.0
 M64: 20467.0
 KE2: 20467.0
 KE1: 20467.0
 C68: 20467.0
 KB: 20467.0
 M6B7: 20467.0
 M62: 20467.0
 6E2: 20467.0
 RK2: 20467.0
 6E1: 20467.0
 C66: 20467.0
 C67: 20467.0
 C64: 20467.0
 RK8: 20467.0
 C61: 20467.0
 C62: 20467.0
 M6B3: 20467.0
 SK5: 20467.0
 SK4: 20467.0
 SK1: 20467.0
 M6B2: 20467.0
 SK3: 20467.0
 M6B8: 20467.0

# "Clean" dataset


# Make output directory in ucluct_openref/ for next steps
> mkdir Alpha_Diversity

> alpha_diversity.py -i otu_table_final_rarified20467.biom -m
observed_otus,ace,chao1,simpson_reciprocal,shannon,simpson_e -o
Alpha_Diversity/Alpha_Diversity.txt -t rep_set.tre
> more Alpha_Diversity.txt

#       observed_otus  ace     chao1   simpson_reciprocal     shannon simpson_e
#6E1    5057.0  10172.4638819  9639.41134752  446.877538567  10.6683772827  0.0883681112453
#M6B8   557.0   842.938489444  883.071428571  47.712819795   7.1260255757   0.0856603586983
#KE1    4833.0  8757.9304507   8361.37485582  457.912709787  10.6453760771  0.0947470949279
#6E2    4034.0  6457.79939016  6207.15206186  192.578140236  10.0447883518  0.047738755636
#SK1    990.0   1187.07857729  1387.75 5.14615166897  5.64933308579  0.00519813299896
#SK3    1322.0  2335.02452655  2215.97540984  2.15452025942  3.6089047877   0.00162974301015
#KE2    3519.0  5146.42235366  5049.7654321   383.946305016  10.1699578528  0.109106651042
#SK5    1308.0  1558.00250237  1662.08227848  8.63884398335  6.61940660538  0.00660462078238
#C66    883.0   1147.32915946  1301.5  198.814364724  8.49167131787  0.225157830944
#C64    306.0   445.377825631  484.578947368  4.6556897139   3.08542474987  0.0152146722677
#M62    293.0   484.822552648  443.666666667  37.5696419585  5.94500821754  0.128224033988
#SK4    2000.0  3310.07729929  3237.95 5.00348291299  6.1344455818   0.0025017414565
#RK6    407.0   731.688565801  720.870967742  16.519812488   5.76724779017  0.0405892198723
#RK8    481.0   756.843236464  815.121212121  24.8423381438  6.49884281223  0.0516472726482
#RK2    737.0   911.576860895  1020.92 59.8143773915  7.4761184871   0.0811592637605
```

```
#M6B7   504.0   940.131452322  866.068181818  61.1483012913  7.06173622938  0.121325994626
#C67    604.0   769.039841392  910.277777778  21.3387744825  6.44992674456  0.0353290968253
#M64    348.0   464.634718428  444.184210526  46.1884629095  6.55196710971  0.132725468131
#C68    493.0   773.563628124  755.857142857  10.9077207303  5.24640664445  0.0221251941792
#M61    415.0   735.461660629  659.170731707  46.2643788832  6.77379801793  0.111480431044
#KB     173.0   347.330577009  273.1   2.92061240076  3.00305123957  0.0168821526056
#C62    180.0   397.84252667   467.272727273  3.45783636339  2.73553803942  0.0192102020188
#M6B2   394.0   1222.77402923  1125.52777778  33.6559132761  5.90766434626  0.0854210996856
#C61    387.0   752.885539223  797.322580645  7.7930649777   4.761748738    0.0201371188054
#M6B3   510.0   878.08589231   993.724137931  67.4277102916  7.20341580968  0.13221119665
#M6B1   306.0   588.286834232  553.028571429  8.13617594762  4.95267637811  0.0265888102863


# Started new tmux session: "Success2"
# QIIME workflow script that calculates summaries of OTUs at different taxonomic levels
> summarize_taxa_through_plots.py -o Alpha_Diversity/taxa_summary20467/ -i
otu_table_final_rarified20467.biom

# Generating rarefaction curves to let us know if we sequenced enough
> alpha_rarefaction.py -i otu_table_final_rarified20467.biom -o Rarefaction/ -t rep_set.tre -m
NewMappingFile3.txt -e 20467

# Generate beta diversity: To compare weighted (who is there and how much of them)/unweighted
(who is there) and phylogenetic(Relation of species)/taxonomic (OTUs) metrics, we will ask QIIME
to create four resemblance matrices of all of these different flavors.
> beta_diversity.py -i otu_table_final_rarified20467.biom -m
unweighted_unifrac,weighted_unifrac,binary_sorensen_dice,bray_curtis -o compar_div_rare20467/ -t
rep_set.tre

# Checking is samples are significantly different from one another
# Unweighted, so only presence/absence, no abundance
# Default 100 monte carlo randomizations
> beta_significance.py -i otu_table_final_rarified20467.biom -t rep_set.tre -s unweighted_unifrac
-o unw_sig.txt
# Output in unw_sig.txt

# Checking is samples are significantly different from one another
# Weighted, so presence/absence plus abundance
> beta_significance.py -i otu_table_final_rarified20467.biom -t rep_set.tre -s weighted_unifrac -
o w_sig.txt
# Output in w_sig.txt


> principal_coordinates.py -i compar_div_rare20467/ -o compar_div_rare20467_PCoA/

> make_2d_plots.py -i
compar_div_rare20467_PCoA/pcoa_weighted_unifrac_otu_table_final_rarified20467.txt -m
NewMappingFile3.txt -o PCoA_2D_plot_WU/

> make_2d_plots.py -i
compar_div_rare20467_PCoA/pcoa_unweighted_unifrac_otu_table_final_rarified20467.txt -m
NewMappingFile3.txt -o PCoA_2D_plot_UU/

> make_2d_plots.py -i
compar_div_rare20467_PCoA/pcoa_binary_sorensen_dice_otu_table_final_rarified20467.txt -m
NewMappingFile3.txt -o PCoA_2D_plot_BSD/

> make_2d_plots.py -i
compar_div_rare20467_PCoA/pcoa_bray_curtis_otu_table_final_rarified20467.txt -m
NewMappingFile3.txt -o PCoA_2D_plot_BC/


#NOTE: Need to compute core microbiome (messed around to pick and choose which samples we want to
use this script with -- see scripts that hopefully work below)
#Using unrareified table - complete list of OTUs: helps us not miss rare sequences

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_cyclorbiculina --
mapping_fp NewMappingFile3.txt --valid_states "ForamSpecies:Cyclorbiculina"
       #Looking at core of Cyclorbiculina samples only

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_amphistegina --
mapping_fp NewMappingFile3.txt --valid_states "ForamSpecies:Amphistegina"
       #Looking at core of Amphistegina samples only; note this includes both healthy and
bleached samples

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_archaias --mapping_fp
NewMappingFile3.txt --valid_states "ForamSpecies:Archaias"
       #Looking at core of Archaias samples only

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_sorites --mapping_fp
NewMappingFile3.txt --valid_states "ForamSpecies:Sorites"
```

```
        #Looking at core of Sorites samples only

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_chlorophytes --
mapping_fp NewMappingFile3.txt --valid_states "SymbiontType:Chlorophyte"
        #Looking at core of samples that have Chlorophyte symbionts (i.e., Cyclorbiculina and
Archaias)

> compute_core_microbiome.py -i filtered_otu_table_final.biom -o otu_core_amphi_bleached --
mapping_fp NewMappingFile3.txt --valid_states "Treatment:Bleached"
        #Looking at core of bleached Amphistegina samples only; compare against core of all
Amphistegina
```

Appendix 2: Additional diversity metrics calculated during data analysis

Species	Sample ID	Sequence Reads*	OTUs	Chao1 Richness	Simpson Reciprocal	Shannon Index	Simpson Evenness
S. orbiculus	SK1	549,377	990	1,388	5.1	5.6	0.005
	SK3	662,209	1,322	2,216	2.2	3.6	0.002
	SK4	755,146	2,000	3,238	5.0	6.1	0.003
	SK5	382,586	1308	1,662	8.6	6.6	0.007
Ar. angulatas	RK2	282,469	737	1,020	60	7.5	0.08
	RK6	156,225	407	720	16	5.8	0.04
	RK8	205,650	481	815	25	6.5	0.05
C. compressa	C61	124,969	387	797	7.8	4.8	0.02
	C62	432,152	180	467	3.4	2.7	0.02
	C64	349,799	306	485	4.7	3.1	0.02
	C66	354,082	883	1,302	199	8.5	0.2
	C67	315,677	604	910	21	6.4	0.04
	C68	289,638	493	756	11	5.2	0.02
Am. gibbosa (healthy appearing)	M61	58,328	415	659	46	6.8	0.1
	M62	20,467	293	444	38	5.9	0.1
	M64	71,177	348	444	46	6.6	0.1
Am. gibbosa (partially bleached)	M6B1	57,814	306	553	8.1	5.0	0.03
	M6B2	22,059	394	1,123	34	5.9	0.09
	M6B3	174,051	510	994	67	7.2	0.1
	M6B7	104,624	504	866	61	7.1	0.1
	M6B8	182,342	557	883	48	7.1	0.09
KML Substrate	KE1	831,748	4,833	8,361	458	10.6	0.1
	KE2	584,520	3,519	5,050	384	10.2	0.1
Tennessee Substrate	6E1	967,637	5,057	9,639	447	10.7	0.09
	6E2	288,620	4,034	6,207	193	10.0	0.05

**Samples were rarified to 20,467 (lowest observed sequence read amount) before diversity metrics were calculated.*