6-28-2016

Diversity and Distribution of Diatom Endosymbionts in *Amphistegina* spp. (Foraminifera) Based on Molecular and Morphological Techniques

Kwasi H. Barnes
University of South Florida, kwasi.barnes@gmail.com

Diversity and Distribution of Diatom Endosymbionts in *Amphistegina* spp. (Foraminifera) Based on Molecular and Morphological Techniques

by

Kwasi H. Barnes

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
with a concentration in Biological Oceanography
College of Marine Science
University of South Florida

Major Professor: Pamela Hallock Muller, Ph.D.
Mya Breitbart, Ph.D.
James Garey, Ph.D.
Dale Griffin, Ph.D.
John Paul, Ph.D

Date of Approval:
June 3, 2016

Keywords: DNA Extraction, Sequencing, Denaturing Gradient Gel Electrophoresis, Symbiosis, Phylogenetics, Bioinformatics

DEDICATION

I dedicate my dissertation to the "girls" of my life whose love and support got me to this point: my wife Seraphine, daughters Nykiia and Melody, and my "Granny" singing and dancing with the angels. Thank you all for your love, support, and inspiration. To my daughters: The most important ingredients to success are hard work and perseverance. However, sometimes you need the love, kindness, or help of others to achieve your goals. Always work your hardest and never give up, but also be kind, respectful, and gracious to others along the way—because burnt bridges are difficult, and sometimes even impossible, to cross! My "Granny" taught me that, my wife exemplifies it, and we hope to pass that life lesson on to you.

ACKNOWLEDGMENTS

I am eternally grateful to my major professor Dr. Pamela Hallock Muller for the immeasurable effort, dedication, and time she has invested in my academic and professional growth. Dr. Hallock Muller was largely responsible or intricately involved in every process that allowed me to complete my dissertation. There has never been a question, problem, or request that she wasn't able to help me with directly or guide me in the right direction. I can honestly say that I have never ended a conversation with her not feeling better or wiser than when I started. Dr. Hallock Muller is a great scientist, excellent mentor, and a truly kind and wonderful person.

I am tremendously appreciative of my committee members' advice, assistance, and the opportunity to complete my dissertation. I am truly grateful to Dr. Breitbart for stepping in and agreeing to serve on my committee, and for her truly insightful, thought provoking, and helpful edits, suggestions, and questions. I want to thank Dr. Dale Griffin immensely for his advice and availability to discuss all things regarding my molecular data and improving its presentation. I am very grateful to Dr. James Garey for his advice, suggestions, and the use of his labs. I want to thank Dr. John Paul for his advice, suggestions, and for his assistance securing funding for me to conduct my molecular work. I am also grateful to Dr. Gabriel Vargo for his advice and suggestions, particularly about culturing diatoms when I was having very low success rates.

I want to acknowledge and thank Prof. John J. Lee, City University of New York, for providing the baseline research for which this entire dissertation extended from. I am also very

thankful for the diatom cultures he provided for me to use as references and his suggestions regarding culture techniques.

I am extremely thankful for the academic and research funding from the following sources: National Sea Grant, Environmental Marine Biotechnology Award No. NA86RG0052, Am.7.1 (Cheryl Woodley, PI); NOAA-NURC-UNCW subcontract 2004-19B (Pamela Hallock, PI); US National Science Foundation Biocomplexity Awards No. CHE-0221834 (T. Pichler, PI) and OCE-0221763 (J. Paul, PI); US Geological Survey/University of South Florida Cooperative Agreement 99HQAG0004; Field support by the Florida Institute of Oceanography. Additional financial support was provided by the University of South Florida, Office of Graduate Studies and the College of Marine Science. Thanks to Dr. Edward VanVleet, Dr. David Naar, Brittany Sheehy, Dr. Jacqueline Dixon, Dr. Teresa Greely (GK-12 Fellowship), Rod Hale, and many others at USF for their assistance with enrollment, fellowships, grants, and tuition assistance.

I would like to thank the following people for graciously providing samples: Lore Ayoub (Cayman Islands), Elizabeth Carnahan (Florida Keys), Kate Ciembronowicz (Pulley Ridge), Elizabeth Fisher (Florida Keys, Bahamas, and Jamaica), Pamela Hallock Muller (Florida Keys and Hawaii), John Lee (Diatom Cultures), Matt Lybolt (Florida Middle Grounds), Bryan McCloskey (PNG), and Dana Williams (Navassa and Grenadines). I also thank Katie Rathmell, Jen Dupont, Jim Ivey, Roy Price and KML staff for their assistance with collecting samples from the Keys in Sept 2004, and May and June 2005. Collections in Biscayne National Park were permitted by BISC-2001-SCI-0022, BISC-2002-SCI-0012 and BISC-2003-SCI-0019 from the National Park Service and FKNMS—2001-008, 2003-002, and 2005-002 from the Florida Keys National Marine Sanctuary.

I would like to thank the following people for advice, assistance, and general support: Tony Greco, Boris Wawrik, Stefi Depovic, Steve Massey, Bina Nayak (Valerie Harwood Lab) with DGGE and Todd LaJeunesse with DGGE, Michael Martinez, Natasha Mendez, and so many others along the way!

Last, but certainly not least, I would like to thank my wife, Dr. Seraphine Pitt Barnes for her love, patience, and support beyond my merit!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Diatoms associated with foraminifers of the genus *Amphistegina* were assessed using a combination of morphological and molecular techniques. These included: 1) microscopic identification of diatoms cultured from the host, 2) sequencing of portions of the small subunit of the ribosomal RNA gene (*18S*) and the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase [i.e., RubisCO] gene (*rbcL*) from DNA extracted directly from the *Amphistegina* hosts and also from diatoms cultured from these hosts, and 3) denaturing gradient gel electrophoresis (DGGE) profiles of *rbcL* and internal transcribed spacer 1 (*ITS1*) PCR amplicons from DNA extracted directly from hosts and from cultures.

Consistent with previous culture studies, multiple species of pennate diatoms of the genera *Nitzschia*, *Fragilaria* (including *Nanofrustulum*), *Amphora*, and *Navicula*, were cultured from >900 host specimens collected from >20 sites in the western Atlantic and four sites in the Pacific. Diatoms of the genus *Nitzschia* grew in about half of all successful cultures. The genetic identities of selected cultures were consistent with those based on morphological taxonomy.

Diatom sequences from DNA extracted directly from the cytoplasm of the *Amphistegina* hosts were species specific and distinct from sequences obtained from cultured diatoms and from sequences in GenBank of diatom taxa previously reported as endosymbionts. Multiple phylogenetic analyses revealed that the *18S* and *rbcL* diatom sequences from specimens of *A. gibbosa* collected from the Atlantic sites and of *Amphistegina* spp. from Hawai'i were most similar to the *18S* and *rbcL* sequences of an unnamed Fragilariaceae diatom in GenBank

(Accession # JX413542.1 for *18S* and JX413559.1 for *rbcL*) and other closely related diatoms in that family.

Of diatom taxa previously reported as endosymbionts of larger foraminifers, *Nanofrustulum shiloi* was the most similar, but not identical, to the sequences from hosts collected from the Atlantic and Hawai'i. The *18S* and *rbcL* diatom sequences from the Atlantic host species, *A. gibbosa*, were all nearly identical, but small intra-species differences (subclades) were observed from specimens collected from the deepest (75 m) site in the Florida Keys and also from the eastern-most site, Young Island near St. Vincent. The *18S* and *rbcL* diatom sequences from the two host species from Hawai'i, *A. lobifera* and *A. lessonii*, were more variable but still within the family Fragilariaceae.

The diatom sequences from *A. radiata* collected from two sites in Papua New Guinea (PNG) were most similar to diatoms of the family Plagiogrammaceae and therefore distinct from sequences obtained from other *Amphistegina* species in this study, as well as from all diatoms previously reported as endosymbionts. A small difference was observed between the diatom sequences from host specimens collected from a Pacific site as compared to a Bismarck Sea site.

The *ITS1* DGGE profiles of DNA extracted directly from *A. gibbosa* specimens at different depths, locations, and seasons in the western Atlantic were nearly identical. Differences were seen between *rbcL* DGGE profiles of DNA extracted directly from the different *Amphistegina* host species. The *rbcL* DGGE profiles directly from all hosts were clearly different from those extracted from diatoms cultured from the same host specimens, as well as from *Nitzschia laevis*, a commonly reported diatom endosymbiont in past culture-based studies.

My findings are consistent with ultrastructural studies of endosymbionts of *Amphistegina* published in the early 1980s and congruent with recent molecular studies of endosymbionts in

other diatom-bearing foraminifers, all of which indicate specificity. Nevertheless, the consistency with which several diatom taxa have been reported in culture studies from all oceans indicates the possibility of some relationship with *Amphistegina* spp., either as important food items, epiphytes, or minor opportunistic symbionts that can thrive in culture media.

PREFACE

Algal symbiont-bearing larger foraminifers are very common and ubiquitous among tropical and sub-tropical reef environments. Their small size, high numbers, and symbiotic relationships with microalgae analogous to reef-building corals and their zooxanthellae, have led to their use as proxies in experimental studies and as bioindicators of reef health. Foraminifers in the genus *Amphistegina* are the most abundant among these reef-dwelling, algal symbiont-bearing foraminifers worldwide. In the late 1970s and early 1980s, *in situ* ultrastructural cytological studies utilizing Transmission Electron Microscopy (TEM) confirmed that the algal endosymbionts of *Amphistegina* were frustule-less diatoms.

Without frustules, the primary characteristic used for specific identification at that time, researchers attempted to grow the diatom symbionts in culture with hopes that they would regain their frustules outside the host. The foraminiferal hosts were meticulously cleaned, crushed, and placed in enriched media, where diatoms (with frustules) grew and were presumed to be endosymbionts of the foraminifers. For over 30 years, researchers grew more than two dozen different species of diatoms in culture from thousands of specimens of *Amphistegina* and other diatom-bearing foraminifers, which suggested a non-specific relationship between hosts and symbionts. Experiments and hypotheses were tested, expressed, and expanded upon in over 50 publications from the 1970s to present, which were primarily rooted on the findings of culture isolation studies.

However, two early 1980s TEM studies indicated a specific relationship between hosts and symbionts. Those TEM studies compared the cytological ultrastructure of the endosymbionts within *Amphistegina* and other diatom-bearing foraminifers to the cytological ultrastructure of presumed diatom symbionts obtained in cultures and found that the diatoms within the hosts exhibited specific ultrastructural characteristics, and most were very different from the cultured diatoms that were supposedly symbionts. More recent molecular studies of diatom endosymbionts in other species of Foraminifera have also directly conflicted with the long-standing hypothesis based on culture isolations that the endosymbionts were a "loose fit" within the hosts.

The primary objective of my dissertation research was to determine if algal symbionts in populations of *Amphistegina gibbosa* from the Florida reef tract differed taxonomically with season, depth at specific reefs, or spatially across the reef tract. I originally proposed to combine culture-isolation techniques and molecular-genetic analyses, specifically gene sequencing from both cultures and directly from individual host specimens, to determine variability in the symbiont assemblages. The organization of this dissertation, in part, reflects that original research design. The chapters of the dissertation are as follows:

In Chapter 1 (Introduction, objectives, significance, and literature review), I provide the objectives and significance of my study as it relates to the further development of algal symbiont-bearing foraminifers as bioindicators of reef health and their use as experimental proxies for coral-zooxanthellae studies. I give an overview of algal symbiosis in Foraminifera and breadth and depth of symbionts isolated in culture in past studies of *Amphistegina* and other algal-bearing foraminifers, and introduce the few ultrastructure and molecular studies that directly contradicted the findings of those numerous culture isolation studies.

In Chapter 2 (Culture isolations), I report the findings of diatoms isolated in culture from *Amphistegina gibbosa* specimens that were collected across different sites, depths, and seasons from the Florida reef tract, as well as from serendipitous collections of *Amphistegina* spp. specimens from several sites in the western Atlantic, Caribbean, and Pacific to explore possible symbiont variations based on regional, seasonal, depth, or host-species differences. These data are compared to the culture isolation data from past studies.

In Chapter 3 (Analysis of symbiont DNA sequences obtained directly from *Amphistegina gibbosa* hosts collected from the Florida reef tract), I report the findings from DNA extracted and sequenced directly from *Amphistegina gibbosa* host specimens collected across different sites, depths, and seasons on the Florida reef tract. I sequenced portions of two genes, the small subunit of the ribosomal RNA gene (*18S*) and the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (i.e., RubisCO) gene (*rbcL*) to determine the molecular identity of the algal endosymbionts and to determine whether they varied with location, depth, or seasons. These data are compared to the culture isolation data from previous studies.

In Chapter 4 (Comparison of diatom cultures to sequences from DNA extracted from the hosts and the cultured diatoms from Atlantic and Pacific *Amphistegina* spp. host specimens), the results of the culture and morphological identification presented in Chapter 2 are compared with the results from the molecular-genetic approach presented in Chapter 3, augmented by results from a substantial number of specimens of *Amphistegina* spp. collected from locations around the Caribbean and three locations in the Pacific Ocean. I report a broader geographic and host-species comparison of the endosymbionts of *Amphistegina* spp. I compared culture isolations to the *rbcL* and *18S* DNA sequences from both hosts and cultures from several species of

Amphistegina to 1) assess the validity of each method for identifying endosymbionts, and 2) determine whether the endosymbionts of *Amphistegina* vary with location or host species.

In Chapter 5 (Comparison of diatom cultures to DNA extracted directly from the host using DGGE), I report results utilizing denaturing gradient gel electrophoresis (DGGE) as an alternative molecular tool to assess and compare the identity and diversity of symbiont taxa within large numbers of foraminiferal hosts specimens from different locations, depths, and seasons and to compare them to presumed algal endosymbionts isolated in culture from their respective foraminiferal hosts.

In Chapter 6 (Summary, discussion, and future implications), I present a summary of all the findings and compared and contrasted them with past studies. I also discuss connections, implications and suggestions for future studies. I provide suggestions to improve the speed and efficiency, and reduce the costs of exploring algal symbionts in foraminifers in future studies. I end with the significance of this study to future reef bioindicators research and to experimental studies of the environmental effects on symbiosis.

CHAPTER 1: INTRODUCTION, OBJECTIVES, SIGNIFICANCE OF STUDY, AND LITERATURE REVIEW

Objectives of this Study

The original objective of my dissertation research was to explore the spatial and temporal distributions of diatom endosymbionts in the reef-dwelling foraminifers of the genus *Amphistegina* in order to strengthen their use as bioindicators of reef heath and proxies for experimental studies related to coral-algal relationships. As the study progressed, an ancillary objective of comparing the results of morphological and molecular identifications of the symbionts morphed into the co-primary objective as I worked to resolve the conflicting results obtained from the two approaches.

These were the specific questions that I addressed in my dissertation research:

1) What symbiont taxa were present in specimens of *Amphistegina* that were available for my study?

2) Do culture (morphological) techniques and genetic (molecular) analyses yield the same symbiont assemblages?

3) Do molecular-genetic analyses of the rbcL loci (which encode for ribulose-1,5-bisphosphate carboxylase/ oxygenase, i.e., RuBisCO) and the 18S (small subunit rDNA gene sequence) from individual host specimens yield the same symbiont assemblages?

4) Do symbionts in *Amphistegina gibbosa* populations differ taxonomically with depth, seasons, or location along the Florida reef tract?

5) Do symbionts in *Amphistegina* spp. populations differ taxonomically within host specimens collected from different geographic regions?

6) Do symbionts in *Amphistegina* populations differ taxonomically between host species?

7) Can DGGE fingerprinting be used to compare symbiont assemblages?

Brief Description and Importance of Foraminifera

The Foraminifera are an extremely abundant group of unicellular eukaryotes, most of which produce a shell (test) that may include agglutinated particles or precipitated calcium carbonate (Goldstein, 1999). The foraminiferal cytoplasm consists of endoplasm, in which the nucleus or multiple nuclei are found, along with other organelles, and ectoplasm, which is rich in microtubules, mitochondria and lysosomes (Talge and Hallock, 2003). The shells have one or many openings (apertures) from which characteristic extensions of the ectoplasm known as granuloreticulopodia emerge to capture food, for locomotion, for attachment, and for other functions including chamber formation in multi-chambered forms.

The Foraminifera are estimated to have arisen roughly 1,000 million years ago and have a fossil record of more than 500 million years (Sen Gupta, 1999).Their abundance, preservation, and quantifiable evolution and distribution in fossil records have made them very useful tools for a variety of paleo-research applications (Martin, 2000), including studies of past environments and climates, determining the age of rocks, and for hydrocarbon exploration. There are approximately 10,000 species of extant Foraminifera known (Vickerman, 1992).

Of those extant species, only about 1% are known to host algal symbionts (Lee and Anderson, 1991). However, these symbiont bearers are the most prolific producers of calcium

carbonate of all foraminifers and the planktic foraminifers are second only to coccolithophorids as major producers of carbonate in the world's oceans (Langer et al., 1997; Hallock, 1999).

Lineages of benthic foraminifers that evolved symbiotic relationships with algae arose multiple times over the past 350 million years, often becoming relatively large and so prolific that they have been major producers of carbonate sediments in shallow-water environments in warm-temperate to tropical seas in certain intervals of Earth's history (Hallock, 1999; Pomar and Hallock, 2008; many others). As a consequence, the term "larger benthic foraminifer" (LBF) is used commonly to refer to benthic foraminifers that host algal endosymbionts, even though not all such foraminifers grow to exceptionally large sizes.

In modern tropical shelf environments, LBFs tend to be associated with coral reefs, where they are typically important producers of sand-sized sediments (e.g., Hallock, 1981a; Langer et al., 1997). The synergistic relationship between carbonate production and photosynthesis in reef-dwelling, larger foraminifers and their algal endosymbionts is considered largely analogous to that of ecologically and economically important reef-building corals and their zooxanthellae (Hallock, 1999, 2000a, b). These symbiotic and synergistic relationships (within both corals and symbiont-bearing foraminifers) are highly sensitive to chemical, physical, and environmental stresses. Because coral reefs are in decline worldwide, the abundant and easily manipulated symbiont-bearing LBFs have been proposed, explored, and utilized as experimental proxies and as bioindicators of environmental health (e.g., Lee, 1995; Hallock, 2000a, 2011, 2012; Hallock et al., 2003, Schmidt et al., 2011).

Symbiosis in Foraminifera

Studies of zooxanthellae in corals are revealing that populations are neither static nor host specific, and may play a significant role in the ability of coral to adapt to environmental stress and changes (e.g., Buddemeier and Fautin, 1993; Rowan, 1998; Baker, 2001). Among the foraminifers, species of diatoms, dinoflagellates, chlorophytes, rhodophytes, and other algae have been identified as symbionts (e.g., Lee and Anderson, 1991; Lee, 2006, 2011a; Lee et al., 2010). There are approximately 15 extant families of Foraminifera described as hosts of algal symbionts (Lee and Anderson, 1991; Hallock, 1999). The planktic foraminifers with algal symbionts are primarily hosts to dinoflagellates, but also to chlorophytes, prymnesiophytes, and chrysophytes (Lee and Anderson, 1991; Gast and Caron, 2001). The LBFs are reported to be hosts to a wide variety of endosymbionts, including diatoms, dinoflagellates, chlorophytes, rhodophytes, cyanobacteria, and haptophytes (e.g., Lee, 2006, 2011a; Lee et al., 2010).

For the past 40 years, most of the published studies (over 90 publications), of symbiosis in LBFs have been conducted by John J. Lee of the City University of New York and colleagues (Lee, 2011a). Most of these studies (over 50 journal articles, chapters, reviews, summaries, etc.) focused on identifying, studying, reviewing, and discussing the purported endosymbionts of diatom-bearing, benthic foraminifers, particularly of the genus *Amphistegina*, that were grown in culture media (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1). However, since the diatom endosymbionts lacked their distinctive frustules within the foraminiferal hosts (e.g., Leutenegger, 1977; Berthold, 1978), almost all of the studies by Lee and colleagues (see Table 1.1) relied primarily on culturing techniques, in which the diatom "endosymbionts" were isolated and cultured in enriched media, where they produced frustules, which were then used to identify them. These culture isolation techniques were used to

"discover" over two dozen species of "endosymbiotic" diatoms from LBFs in the families Alveolinidae, Amphisteginidae, Calcarinidae, and Nummulitidae.

These cultured diatoms where mostly identified as belonging to the genera *Nitzschia*, *Navicula*, *Fragilaria* (including a new genus *Nanofrustulum*), and *Amphora*, though several less common genera were also identified in cultures from various host specimens, for a total of ~25 different species (Lee, 2011a, b). Often two and sometimes three species of diatoms were cultured from one foraminifer (e.g., Lee et al. 1979a, 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1). Eight different species (five genera) of diatoms were isolated in culture by Lee et al. (1995a) from 50 Florida reef-tract specimens of *Amphistegina gibbosa*, the species and general location of the majority of the samples used in my study.

The few studies of diatom endosymbionts in foraminifers carried out utilizing either *in situ* Transmission Electron Microscopy (TEM) for cytological ultrastructure examinations (Leutenegger, 1983, 1984) or molecular techniques (Holzmann et al., 2006; Schmidt et al., 2015) have all found specific host-symbiont relationships, which directly contradicted the diverse and non-specific relationships concluded based on the culture isolation studies (see Table 1.2). In the early 1980s, two studies, based on *in situ* cytological ultrastructural examinations of diatom endosymbionts of 16 different species of diatom-bearing larger foraminifers reported that the endosymbionts were highly specific and did not vary with locations, depth, or seasons (Leutenegger, 1983, 1984). Furthermore, the endosymbionts of only three of the 16 different hosts were ultrastructurally similar to only a single species (Leutenegger, 1983, 1984) of all the more than two dozen species of diatoms purportedly isolated in culture from the foraminiferal hosts (Table 1.2).

More recent molecular studies of diatom endosymbionts in foraminifers (Holzmann et al., 2006; Schmidt et al., 2015) have also indicated specific host-symbiont relationships. Holzmann and colleagues (2006) sequenced and analyzed portions of a small subunit ribosomal gene from diatom DNA extracted directly from multiple specimens of nine species of diatom-bearing foraminifers in the Nummulitidae family collected from various locations and spanning different years, their data showed that all of the diatom endosymbionts within these foraminifers were closely related to diatoms of the genus *Thalassionema*, a genus never identified among the more than two dozen cultured diatom "symbionts." Most recently, Schmidt et al. (2015) cultured several species of diatoms identical to those in previous culture isolation studies from the diatom-bearing foraminifer, *Pararotalia calcariformata*, but when the DNA was extracted directly from the hosts, amplified by PCR, and the PCR products directly sequenced, only a single species of diatom, *Minutocellus polymorphus*, was evident in those sequences. *Minutocellus polymorphus* was never reported among any of the previously isolated diatoms "endosymbiont" in the numerous culture isolation studies listed in Table 1.1.

The Decline of Reefs and the Growing Need for Bioindicators of Reef Health

Coral-reef communities are regarded as one of the most "valuable" of marine ecosystems (Costanza et al., 1997; Carr and Mendelsohn, 2003). Worldwide, these communities support millions of tons of food, as well as tourism and recreation industries that were valued at ~$30 billion dollars per year (Cesar et al., 2003). About 30 to 40% of all species of fish are linked to coral reefs (Ehrlich, 1975). Millions of other species are found in, on, or near coral reefs, and many of these organisms are a source of food or other products. With such biologically rich ecosystems, the prospects that cures for diseases and new products can be found among these

many organisms is not only possible, but also likely. Additionally, coral reefs attract many visitors simply because of their natural splendor, which generates revenue for the human communities adjacent to the reefs. Moreover, because those human communities live near their coastlines, the value of coral reefs for coastal protection is immense. Suffice it to say, coral-reef communities are precious ecosystems—ecologically, economically, and aesthetically.

Despite the "value" of coral-reefs communities to humans, these ecosystems have been disappearing progressively since the dawn of the Industrial Revolution (Jackson, 1997). Coral reefs worldwide are threatened by many anthropogenic and anthropogenically-linked factors, and their future seems bleak (e.g., Jackson, 1997; Hoegh-Guldberg, 1999; Lough, 2000; Pandolfi et al. 2005). Many scientists believe that these ecosystems will disappear if intervention is not undertaken promptly.

To effectively protect coral-reef communities, scientists must understand the physiology and ecology of the hermatypic corals that build the reef structure. However, the ecological, economical, and aesthetic "value" placed on coral-reef communities, coupled with their threatened status, hinder researchers' ability to study hermatypic corals. There are many local, national, and international laws that limit sampling or manipulating of hermatypic corals, which are often necessary to effectively study the corals that scientists are ultimately trying to protect. Hence, as coral-reef communities decline and regulations that limit study stiffen, the need increases for effective, practical, yet minimally intrusive ways to study coral reefs and their environments. An innovative way to accomplish this task is by using non-coral, biological proxies (i.e., bioindicators), such as LBFs, to monitor environments to determine if they are conducive for coral-reef accretion or to conduct experiments to gauge the effects of

environmental changes (Hallock, 2000a; Hallock et al., 2003; Schueth and Frank, 2008; Uthicke et al., 2010).

Coral reefs exist because of the relationship between corals and their symbiotic algae. This mutualism between hermatypic corals and their algal symbionts plays a key role in the survival of these organisms in oligotrophic tropical waters (e.g., Muscatine and Porter, 1977; Hallock, 1981b, 2000b; many others). Algal symbiosis in hermatypic corals plays a key role in the formation of the calcareous reef structures that provide habitat, protection, nurseries, etc., for the many reef dwellers (Gattuso et al., 1999). A variety of organisms, including sea anemones, jellyfish, and foraminifers, share similar relationships with algal symbionts. These organisms are increasingly being studied to assess their potential as proxies and bioindicators of coral-reef health.

Foraminifers of the genus *Amphistegina* are the most abundant of all LBFs in tropical and subtropical shallow-water environments (Talge and Hallock, 2003). Members of this genus exhibit similar physiological stress responses as zooxanthellate corals, including bleaching (Williams et al., 1997), and are being utilized as bioindicators of reef health (e.g., Hallock, 2012 and references therein).

The application of symbiont-bearing foraminifers as bioindicators of reef health is based on their similar environmental requirements and stress responses to those of hermatypic corals and their abundance in reef environments (Hallock, 2000a). Of the symbiont-bearing foraminifers, *Amphistegina* spp. are the most useful because they are nearly circumtropical in distribution, are readily maintained in culture, and are typically abundant to readily study in the field or to collect for laboratory experiments (Hallock, 2000a). As a consequence of their meiofaunal size (0.1–>2 mm), they are easy to identify and manipulate individually, while being

small enough to carry out experiments on statistically significant sample sizes (10–25 individuals/trial) within petri dishes in the laboratory or in small containers in the field.

Amphistegina have numerous commonalities with stony corals that enhance their potential as bioindicators. These foraminifers are dependent upon their diatom endosymbionts for growth and calcification in a relationship that appears to be physiologically analogous to that of corals and their zooxanthellae (Lee and Anderson, 1991). Corals on the Florida reef tract and worldwide are in decline from bleaching (Glynn, 1996; Brown, 1997; and many others), diseases (Richardson et al., 1998; Santavy and Peters, 1997; and many others), and many other stresses. Similarly, since 1991 stress symptoms have been documented in *Amphistegina* populations, including bleaching, reproductive dysfunction that has caused populations to decline, interference with normal calcification, increased susceptibility to predation, bioerosion, and cyanobacterial infestation, and overgrowth by algae and encrusting animals (Hallock et al., 1995; Talge et al., 1997; Toler and Hallock, 1998; Hallock, 2000b).

The original focus of my research was to determine if taxa of algal symbionts in populations of *Amphistegina* differ between reefs, depths, or seasons, to aid in the continued development of these foraminifers as bioindicators and experimental tools. As my study progressed, I discovered conflicting results based on different identification methods, and I shifted focus to resolving those conflicts. Nevertheless, this research provides a critical "next step" in the continued development and application of *Amphistegina* spp. as bioindicators. Understanding the potential variability of the endosymbiont taxa, and the role of those symbionts in the responses of the host, is essential to interpreting field studies and laboratory data of responses to specific stressors. This study provides an essential base for new lines of experiments

and may ultimately lead to the development of *Amphistegina* / symbiont lineages that are best suited for experimental bioindicator studies—developing "white rats" for reef studies.

Table 1.1: A chronological list of publications by John J. Lee and colleagues pertaining to *Amphistegina* and diatoms isolated from them.

Chronological list of publications by John J. Lee and colleagues pertaining to *Amphistegina*
1. Lee, J. J., Müller-Merz, E., Hagen, J., McEnery, M., Mastropa. C, & Garrison, J. R. (1974). Symbiosis in Benthic Foraminifera. *Journal of Protozoology, 21*(3), 437-437.
2. Lee, J. J., McEnery, M. E., Kahn, E., & Shuster, F. (1977). Symbiosis and Evolution of Larger Foraminifera. *Journal of Paleontology, 51*(2), 17-17.
3. Lee, J. J., McEnery, M., Rottger, R., & Reimer, C. (1979a). Isolation, Culture, and Identification of Endosymbiotic Diatoms from *Heterostegina depressa* and *Amphistegina lessonii* (Larger Foraminifera) from an Hawaiian Tide Pool. *Journal of Phycology, 15*, 18-18.
4. Lee, J. J., McEnery, M. E., Shilo, M., & Reiss, Z. (1979b). Isolation and Cultivation of Diatom Symbionts from Larger Foraminifera (Protozoa). *Nature, 280*(5717), 57-58.
5. Lee, J. J., McEnery, M. E., Kahn, E. G., & Schuster, F. L. (1979c). Symbiosis and the Evolution of Larger Foraminifera. *Micropaleontology, 25*(2), 118.
6. Lee, J. J., Reimer, C. W., & McEnery, M. E. (1980a). Identification of Diatoms Isolated as Endosymbionts from Larger Foraminifera from the Gulf-of-Eilat (Red-Sea) and the Description of 2 New Species, *Fragilaria shiloi* sp. nov. and *Navicula reissii* sp. nov. *Botanica Marina, 23*(1), 41-48.
7. Lee, J. J., McEnery, M. E., Rottger, R., & Reimer, C. W. (1980b). The Isolation, Culture and Identification of Endosymbiotic Diatoms from *Heterostegina depressa* d'Orbigny and *Amphistegina lessonii* d'Orbigny (Larger Foraminifera) from Hawaii. *Botanica Marina, 23*(5), 297-302.
8. Lee, J. J., McEnery, M. E., Lee, M. J., Reidy, J. J., Garrison, J. R., & Röttger, R. (1980c). Algal Symbionts in Larger Foraminifera. In W. Schwemmler & H Schenk (Eds.) *Endocytobiology: Endosymbiosis and Cell Biology*. (pp. 113-124) Berlin, Germany: Walter de Gruyter.
9. Lee, J. J., McEnery, M. E., & Garrison, J. R. (1980d). Experimental Studies of Larger Foraminifera and their Symbionts from the Gulf of Elat on the Red Sea. *Journal of Foraminiferal Research, 10*(1), 31-47.
10. McEnery, M. E., & Lee, J. J. (1981). Cytological and Fine Structural Studies of Three Species of Symbiont-Bearing Larger Foraminifera from the Red Sea. *Micropaleontology, 27*(1), 71-83.
11. Lee, J. J., & Ellis, R. (1982). A Comparative Study of Photo Adaptation in 4 Diatoms Isolated as Endosymbionts from Larger Foraminifera. *Marine Biology (Berlin), 68*(2), 193-198.
12. Lee, M. J., Ellis, R., & Lee, J. J. (1982). A Comparative-Study of Photo-Adaptation in 4 Diatoms Isolated as Endosymbionts from Larger Foraminifera. *Marine Biology, 68*(2), 193-197.
13. Lee, J. J., McEnery, M. E., Koestler, R., & Lee, M. J. (1982). Experimental Studies of Symbiont Preference in Diatom-Bearing Species of Larger Foraminifera from the Red-Sea. *Journal of Protozoology, 29*(3), 493-493.
14. Lee, J. J., & Reimer, C. W. (1982). Isolation and Identification of Endosymbiotic Diatoms From Larger Foraminifera of the Great Barrier Reef, Australia, Makapuu Tide Pool, Oahu, Hawaii, and the Gulf of Elat, Israel, with a Description of Three New Species *Amphora roettgerii*, *Navicula hanseniana* and *Nitzschia frustulum* var. symbiotica. In D. G. Mann (Ed.), *Proceedings 7th International Diatom Symposium* (pp. 327-343). Koenigstein, Germany: Otto Koeltz.
15. Lee, J. J. (1983). Perspective on Algal Endosymbionts in Larger Foraminifera. *International Review of Cytology, Supplement 14*, 49-77.
16. Lee, J. J., & McEnery, M. E. (1983). Symbiosis in Foraminifera. In L.J. Goff (Ed.), *Algal Symbiosis: A Continuum of Interaction Strategies*, (pp. 37-66). New York, NY: Cambridge University Press.
17. Lee, J. J., Saks, N. M., Kapiotou, F., Wilen, S. H., & Shilo, M. (1984). Effects of Host-Cell Extracts on Cultures of Endosymbiotic Diatoms from Larger Foraminifera. *Marine Biology, 82*(2), 113-120.
18. Koestler, R. J., Lee, J. J., Reidy, J., Sheryll, R. P., & Xenophontos, X. (1985). Cytological Investigation of Digestion and Reestablishment of Symbiosis in the Larger Benthic Foraminifera *Amphistegina lessonii*. *Endocytobiosis and Cell Research, 2*(1), 21-54.
19. Lee, J. J., & Corliss, J. O. (1985). Symposium on "Symbiosis in Protozoa": Introductory Remarks. *Journal of protozoology, 32*(3), 371-372.

Table 1.1 (Continued)

Chronological list of publications by John J. Lee and colleagues pertaining to *Amphistegina*
20. Lee, J. J., Erez, J., McEnery, M. E., Lagziel, A., & Xenophontos, X. (1986). Experiments on Persistence of Endosymbiotic Diatoms in the Larger Foraminifer *Amphistegina lessonii*. *Symbiosis, 1*(3), 211-226.
21. Lee, J. J., & Hallock, P. (1987). Algal Symbiosis as the Driving Force in the Evolution of Larger Foraminifera. *Annals of the New York Academy of Sciences, 503*, 330-347.
22. Lee, J. J., Chan, Y., & Lagziel, A. (1988a). An Immunofluorescence Approach Toward the Identification of Endosymbiotic Diatoms in Several Species of Larger Foraminifera. In C.M. Yentsch, F.C. Mague, & P.K. Horan, (Eds.) *Immunochemical Approaches to Coastal, Estuarine and Oceanographic Questions* (pp. 230-241). New York, NY: Springer US.
23. Lee, J. J., Erez, J., Ter Kuile, B., Lagziel, A., & Burgos, S. (1988b). Feeding Rates of Two Species of Larger Foraminifera *Amphistegina lobifera* and *Amphisorus hemprichii*, from the Gulf of Eilat (red Sea). *Symbiosis, 5*(1-2), 61-102.
24. Lee, J. J., McEnery, M. E., Kuile, B. T., Erez, J., Röttger, R., Rockwell, R. F., ... & Lagziel, A. (1989). Identification and Distribution of Endosymbiotic Diatoms in Larger Foraminifera. *Micropaleontology, 35*(4), 353-366.
25. Lee, J. J., & Reimer, C. W. (1990). Unusual Endosymbiotic Diato-ms Isolated from Larger Foraminifera From Semitropical Pacific Habitats. *Journal of Phycology, 26*(2 SUPPL), 6-6.
26. Lee, J. J., & Anderson, O. R. (1991). Symbiosis in Foraminifera. In J.J. Lee & O.R. Anderson (Eds.), *Biology of Foraminifera.* (pp. 157-220) San Diego, CA: Academic Press.
27. Lee, J. J., Faber, W. W., Nathason, B., & Rottger, R. (1991a). Endosymbiotic Diatoms From Larger Foraminifera Collected in Pacific Habitats. *Journal of Phycology, 27*(3 SUPPL), 43-43.
28. Lee, J. J., Sang, K., Terkuile, B., Strauss, E., Lee, P. J., & Faber, W. W. (1991b). Nutritional and Related Experiments on Laboratory Maintenance of 3 Species of Symbiont-Bearing, Large Foraminifera. *Marine Biology, 109*(3), 417-425.
29. Lee, J. J., Faber, W. W., & Lee, R. E. (1991c). Antigranulocytes Reticulopodal Digestion—A Possible Preadaptation to Benthic Foraminiferal Symbiosis. *Symbiosis, 10*(1-3), 47-61.
30. Lee, J. J. (1992). Isolation of Endosymbiotic Algae from Larger Foraminifera. *Protocols in Protozoology*, A5.1-A5.4.
31. Lee, J. J. (1993). On a Piece of Chalk—Updated. *Journal of Eukaryotic Microbiology, 40*(4), 395-410.
32. Lee, J. J., Faber, W. W., Jr., Nathanson, B., Rottger, R., Nishihira, M., & Kruger, R. (1993). Endosymbiotic Diatoms From Larger Foraminifera Collected in Pacific Habitats. *Symbiosis, 14*(1-3), 265-281.
33. Lee, J. J. (1995). Living Sands—The Symbiosis of Protists and Algae can Provide Good Models for the Study of Host Symbiont Interactions. *Bioscience, 45*(4), 252-261.
34. Lee, J. J., Morales, J., Symons, A., & Hallock, P. (1995a). Diatom Symbionts in Larger Foraminifera from Caribbean Hosts. *Marine Micropaleontology, 26*(1-4), 99-105.
35. Lee, J. J., Wray, C. G., & Lawrence, C. (1995b). Could Foraminiferal Zooxanthellae be Derived from Environmental Pools Contributed to by Different Coelenterate Hosts. *Acta Protozoologica, 34*(2), 75-85.
36. Lee, J. J. (1998). "Living Sands"—Larger Foraminifera and their Endosymbiotic Algae. *Symbiosis, 25*(1-3), 71-100.
37. Chai, J. Y., & Lee, J. J. (1999). Initial Recognition of Endosymbiotic Diatom Surface Antigens by the Larger Foraminifer *Amphistegina lobifera*. *Symbiosis, 26*(1), 39-53.
38. Chai, J. Y., & Lee, J. J. (2000). Recognition, Establishment and Maintenance of Diatom Endosymbiosis in Foraminifera. *Micropaleontology, 46*, 182-195.
39. Lee, J. J., Reimer, C. W., Correia, M., & Morales, J. (2000). A Revised Description of the *Nitzschia frustulum* var. symbiotica Lee and Reimer Emend. Complex, the Most Common of the Endosymbiotic Diatoms in Larger Foraminifera. *Micropaleontology, 46*, 170-181.
40. Lee, J. J. (2002). Living Sands: Symbiosis Between Foraminifera and Algae. In J. Seckbach (Ed.), *Symbiosis: Mechanisms and Model Systems* (pp. 489-506). Dordrecht, Netherlands: Springer Netherlands.
41. Lee, J. J., & Correia, M. (2005). Endosymbiotic Diatoms from Previously Unsampled Habitats. *Symbiosis, 38*(3), 251-260.
42. Lee, J. J. (2006). Algal Symbiosis in Larger Foraminifera. *Symbiosis, 42*(2), 63-75.
43. Lee, J. J., Cervasco, M. H., Morales, J., Billik, M., Fine, M., & Levy, O. (2010). Symbiosis Drove Cellular Evolution. *Symbiosis, 51*(1), 13-25.

Table 1.1 (Continued)

Chronological list of publications by John J. Lee and colleagues pertaining to *Amphistegina*
44. Lee, J. J. (2011a). Fueled by Symbiosis, Foraminifera have Evolved to be Giant Complex Protists. In J. Seckbach & Z. Dubinsky (Eds.), *All Flesh Is Grass: Plant-Animal Interrelationships* (pp. 427-452). Dordrecht, Netherlands: Springer Netherlands.
45. Lee, J. J. (2011b). Diatoms as Endosymbionts. In J. Seckbach & P. Kociolek (Eds.), *The Diatom World* (pp. 437-464). Dordrecht, Netherlands: Springer Netherlands.

Table 1.2: A comparison of the diversity of diatoms (grouped by genus or family) identified as endosymbionts of diatom-bearing foraminifers utilizing different techniques. TEM cellular ultrastructure and DNA sequencing were done directly on the foramaniniferal hosts versus identification of diatoms cultured from cleaned and crushed specimens. [1]The diatoms observed by TEM in *A. bicirculta*, *A. lessonii*, *A. lobifera*, and *A. papillosa* were specific and consistent, and shared characteristics of *Nanofrustulum* (previously *Fragilaria*) *shiloi*. [2]The diatoms observed by TEM in the nummulitid and alveolinid Foraminifera were specific and consistent but not identified, but they were placed in the specific and consistent diatom groups observed in those foraminifers in subsequent molecular studies. The unidentified diatoms observed by TEM in [3]*A. radiata* and the [4]calcarinid Foraminifera were specific and consistent, but were not similar in cell structure to any diatoms cultured from them.

Diatoms Indentified Directly from Foraminiferal Hosts											
Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatoms (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
DNA sequences directly from 9 species of nummulitids (Holzmann et al., 2006)	30		100%								
DNA Sequences directly from *P. calcariformata* (Schmidt et al. 2015)	2			100%							
TEM *in hospite* cellular ultrastructural examination in *A. bicirculta*, *A. lessonii*, *A. lobifera*, and *A. papillosa* (Leutenegger, 1983, 1984)	25	100% [1]									
TEM *in hospite* cellular ultrastructural examination in *A. radiata* (Leutenegger, 1983, 1984)	4				100% [3]						
TEM *in hospite* cellular ultrastructural examination in 5 species of nummulitids (Leutenegger, 1983, 1984)	20		100% [2]								

Table 1.2 (Continued)

Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatoms (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
TEM *in hospite* cellular ultrastructural examination in 2 species of alveolinids (Leutenegger, 1983, 1984)	4		100% [2]								
TEM *in hospite* cellular ultrastructural examination in 3 species of calcarinids (Leutenegger, 1983, 1984)	6				100% [4]						
Diatoms Identified from Cultures											
Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
Diatoms isolated in culture from *Amphistegina* spp. in previous studies (Lee et al., 1992, 1995a; Lee and Correia, 2005)	1856	13.4%				63.7%	10.8%	8.2%	2.0%	0.4%	4.1%
Diatoms isolated in culture from 3 species of nummulitids (Lee et al., 1992, 1995a; Lee and Correia, 2005)	493	4.1%				51.1%	4.5%	23.7%	7.3%	0.8%	13.4%

Table 1.2 (Continued)

Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
Diatoms isolated in culture from 5 species of calcarinids (Lee et al., 1992; Lee and Correia, 2005)	485	7.4%				44.1%	5.6%	42.5%	9.7%		1.9%
Diatoms isolated in culture from *Neorotalia calcar* (Lee et al., 1992)	105	1.9%				57.1%	7.8%	25.7%	3.8%		
Diatoms isolated in culture from *Alveolinella quoyi* (Lee and Correia, 2005)	14					60%	40%	60%	40%		
Diatoms isolated in culture from *Parasorites orbitolitoides* (Lee and Correia, 2005)	10					50%	30%	40%	10%		
Diatoms isolated in culture from *P. calcariformata* (Schmidt et al. 2015)	5			80%			60%	40%			

CHAPTER 2: SPATIAL AND TEMPORAL DISTRIBUTION OF ALGAL TAXA CULTURED FROM FORAMINIFERS OF THE GENUS *AMPHISTEGINA*

Abstract

Amphistegina spp. are widely recognized as physiologically dependent upon algal endosymbionts. Previous studies isolating algae in culture from *Amphistegina* spp. have reported a variety of taxa of small pennate diatoms. The primary goal of this study was to isolate and culture the presumed symbionts of *Amphistegina gibbosa* from the Florida reef tract to determine if the isolated diatom taxa vary across depth or season. In addition, specimens of *Amphistegina* spp. from several locations in the western Atlantic, Caribbean and the Pacific provided the opportunity to explore possible regional differences. Four genera of pennate diatoms: *Nitzschia*, *Navicula*, *Fragilaria* (including *Nanofrustulum*), and *Amphora* were the most frequently isolated genera from more than 900 host specimens, consistent with previous culture isolation studies. *Nitzschia* was most commonly isolated at all seasons, depths, and geographic locations. Diversity of algal associates varied with location and depth, but no seasonal trends were evident at the generic level at eight reefs sites in the Florida Keys, where samples were collected quarterly between March 2001 and September 2004. *Nanofrustulum shiloi* and species of *Fragilaria* were very common at depths <25m, but absent or sparse at greater depths. *Amphora*, *Navicula*, *Achnanthes*, and other less common diatom genera were isolated more frequently from hosts collected at depths >20m. Diatoms were more difficult to culture from *Amphistegina* specimens collected >25m (and to a lesser extent <5m in the Atlantic) using the methods employed. Non-

diatom microorganisms, including bacteria, fungi, and dinoflagellates were observed in 17% of isolation attempts. Non-diatom microbes were isolated more than three times more frequently from bleached or otherwise abnormal-appearing host foraminifers.

Introduction

Benthic foraminifers of the genus *Amphistegina* have been reported to host a diverse array of diatom endosymbionts (e.g., Lee et al., 1979a, 1989, 1992, 1995a; Lee and Correa, 2005; see Chapter 1, see Table 1.1), which appear to be necessary for the foraminifers' survival (Lee and Hallock, 1987). This symbiosis is considered analogous to the relationship between scleractinian corals and their zooxanthellae (dinoflagellate symbionts). *Amphistegina* inhabit tropical and subtropical reef environments worldwide and have a broad depth distribution from shallow tide pools (<1m) to deep reef slopes (>100m). Hallock (1999) postulated that a possible reason these foraminifers are capable of inhabiting such a broad range of depths (and consequently light regimes) is their ability to host multiple species and strains of diatom symbionts—in some cases simultaneously (e.g., Lee et al., 1995b).

Over the past four decades, researcher have used culturing techniques to isolate algal endosymbionts from thousands of specimens of foraminifers representing numerous species (e.g., Lee et al., 1979a, 1989, 1992, 1995a; Lee and Correa, 2005; see Table 1.1). Those studies have indicated a fluid relationship between foraminifers and several groups of microalgae. More than a dozen species of diatoms have been isolated and identified as symbionts of *Amphistegina*. The vast majority of these studies examined host foraminifers collected from the Red Sea or Pacific, though diatoms were isolated from 50 specimens of the Atlantic species, *A. gibbosa*

d'Orbigny, from the Florida reef tract, reporting *Nitzschia frustulum* to be the most commonly isolated taxon (Lee et al., 1995a)

Despite the many publications on diatom taxa isolated from *Amphistegina* spp., a systematic study of the distribution of symbionts in space and time has never been done for *A. gibbosa*, the most common Atlantic species. Therefore, the primary goal of this study was to determine if taxa of algal symbionts in populations of *A. gibbosa* differ between reefs, depths, or seasons. This research is a critical "next step" in the continued development and application of *Amphistegina* as bioindicators of reef condition (e.g., Hallock et al., 2003). Understanding the potential variability of the endosymbiont taxa and the role of those symbionts in the responses of the host is essential to interpreting field studies and laboratory experiments of responses to specific stressors.

Materials and Methods

Samples of *Amphistegina* spp. available for this project came from other studies and serendipitous sampling opportunities. From March 2001 to August 2005, specimens of *Amphistegina gibbosa* were collected quarterly from eight reefs sites in the upper Florida reef tract (Figure 2.1, Table 2.1). Sites 1 through 4 are located along a 3 to 18 m depth transect off Key Largo, Florida, and Sites 2 and 5–8 represent a south to north transect parallel to the shoreline along the 6m isobath (Table 2.2). Sampling procedures were similar to those described by Hallock et al. (1995) and Williams et al. (1997). The basic sampling procedure consisted of divers collecting reef rubble into plastic bags. Aboard the vessel or onshore, the rubble was kept submerged in seawater while being scrubbed with a plastic brush to remove sediment and associated meio/microbiota. The slurry of sediment and detached biota, which usually contained

dozens to hundreds of *A. gibbosa* specimens, was rinsed several times with seawater until the water remained relatively clear. The samples were then transferred to 500 ml screw cap jars and transported to the Reef Indicators Laboratory at the University of South Florida, St. Petersburg, FL, for further processing.

Other samples of *A. gibbosa* came from reefs sites in the Florida Keys (Table 2.2), the western Atlantic, Gulf of Mexico, and Caribbean (Table 2.3). Specimens of *A. lobifera* Larsen, *A. lessonii* d'Orbigny and *A. radiata* d'Orbigny were also collected from four sites in the Pacific. The approximate locations of all collection sites in this study are provided in Table 2.1 and Figure 2.2. These samples were collected opportunistically by wading, snorkeling, scuba diving, dredge buckets, or remote or manned submersibles from depths of 1 m to 100 m. Such samples were transported live in insulated containers to the Reef Indicators Laboratory for further processing.

Since the endosymbiotic diatoms within a host lack their characteristic frustules used for taxonomic identification (Lee et al., 1979a, b; Lee and Reimer, 1982), they were extracted, cultured, and identified using methods adapted from those previously reported (e.g., Lee et al., 1979a, b, 1980a, 1995a; Lee and Reimer, 1982). During this procedure, up to 25 *Amphistegina* specimens from each site were individually: 1) examined under a dissecting microscope at a standard magnification (60X) to record size and any obvious abnormalities or stress symptoms described previously (e.g., Hallock et al., 1995; Toler and Hallock, 1998; Talge and Hallock, 2003; Williams and Hallock, 2004), which included visible symbiont loss based on color (i.e., bleaching), broken, etched or otherwise damaged tests, and discolorations such as black, green, or red spots; 2) placed in the first well of a sterile nine-hole spot plate with each well filled with 1 ml of sterile seawater (0.2 µm filtered and autoclaved) containing an antibiotic solution (Fisher

#: BW17-745E) at 1 ml/100 ml; 3) cleaned by agitating with two alcohol-sterilized, fine artist brushes; 4) cleaned repeatedly through the other eight wells by moving the foraminifer each time with flamed sterilized forceps and using new sets of brushes for cleaning; 5) removed from the last well with sterile forceps, brushed while being held with forceps and rinsed with a stream of sterile seawater (containing antibiotic) from a wash bottle; 6) examined under a stereomicroscope at highest (~100X) magnification to check its exterior surface for contamination (selected individuals were dried, mounted on an scanning electron microscope (SEM) stub, sputter coated with Au/Pd mixture, and examined further for contamination by external microbes and to assess the effectiveness of the cleaning method); 7) if free of obvious external contamination, were placed inside a sterile microtube and crushed with a flame-sterilized forceps or metal rod; and 8) ~1/3rd of each crushed sample was added to an Erdschreiber (soil extract) enriched sterile seawater solution (Hallock et al., 1986) containing an antibiotic solution (Fisher #: BW17-745E) at 1ml/100ml and incubated with 12 hours of light per day at 25ºC for two weeks. An aliquot of each culture was filtered from the media solution onto a 0.2 µm filter. The filter was dried and mounted on an SEM stub, sputter coated with Au/Pd mixture, and examined using an SEM to identify all organisms that grew in culture, with special attention on diatoms. Then SEM photographs were taken of new or uncommon observations.

The basic isolation procedures described above were refined over the course of the multi-year project. Beginning in June 2002, the cleaning of the foraminifers was preceded by an incubation of three days in sterile seawater containing antibiotics to allow digestion of all microorganisms consumed by the foraminifer, as well as to prevent further feeding that could contaminate the isolation process. In September 2004, the cleaning procedure was modified to more efficiently process specimens. Instead of brushing each foraminifer clean, single

foraminifers were placed inside a bleach-cleaned and alcohol- sterilized syringe-filter holder (Fisher # 09-753-10A) without a filter inside and pressure washed by forcing 20 ml of sterile seawater containing antibiotics 10 times through the filter-holder unit. This cleaning method was verified by SEM to be as effective as the brushing method at removing external microbes from the test of the foraminifers. In November 2004, the growth media was changed from the soil-extract enriched-seawater solution with antibiotics to a commercially available diatom growth media (NuSalts II, Argent Chemical Laboratories Redmond, WA, USA). This change was made because some soil extracts appeared to inhibit diatom growth. Sterilization of the growth media by filtration and autoclaving, and the addition of antibiotics continued as described above.

The taxonomic identity of all the diatoms isolated from individual *Amphistegina* specimens were classified to species level when possible. However, the data are reported to genus level because species-level identification was often not possible because of the highly variable morphology of some species in culture (see also Lee, 1995a; Lee et al., 2000) and the variable morphology of diatoms in general. Species of the genus *Fragilaria* were combined with those of the genus *Nanofrustulum* (Round et al. 1999), whose species were formerly classified as *Fragilaria*. Images and descriptions from Lee et al. (e.g., 1989, 1992, 1995a, 2000) and other diatom-taxonomic references (Hustedt, 1955; Round et al., 1990; Hasle and Syvertsen, 1997) were used for the identification of the diatoms isolated. Non-diatom species were noted when observed and classified into taxonomic groups whenever possible.

The frequency of each genus of diatom symbiont isolated from all hosts from particular sites, depths, and sample dates were calculated. Chi-Squared tests were used for statistical comparisons, and some of the less common taxa were combined in some analyses because of small sample sizes.

To examine the temporal distributions from Florida sites, three taxonomic categories were compared: *Nitzschia, Nanofrustulum,* and other genera. Sites 1-8 were grouped by date from March/April 2001 to September 2004 and the combined frequency distributions for each collection date were compared to an expected frequency distribution calculated from the entire set of data. Additionally, sites that were not statistically different were grouped by dates from August 2001 to September 2004 and compared to the group frequency distribution.

To examine spatial distributions of the symbionts, data were grouped by general locations: Florida Keys (Upper, Middle, Lower Keys), Andros Island, Juno Beach, Grenadines, Navassa, Little Cayman, Jamaica, Hawaii, and Papua New Guinea, and by depths (shallow ≤20m and deep >20 m). For the regional comparisons, four taxonomic categories were used: *Nitzschia*, *Nanofrustulum, Amphora,* and other. For depth comparisons, six categories were used: *Nitzschia*, *Nanofrustulum, Amphora, Navicula, Achnanthes*, and other. Data were then compared to an expected frequency distribution calculated from the entire data set. The frequency distributions of diatoms isolated were also compared to distributions of diatom symbionts isolated from *A. gibbosa*, *A. lessonii*, and *A. lobifera* specimens by Lee et al. (1989, 1992, 1995a) and Lee and Correia (2005).

Results

A total of 2016 *Amphistegina* specimens, collected between March 2001 and August 2005, were used in this study. Of these foraminifers, 1733 were normal golden-brown in color and tests were intact at the start of processing. The other 283 specimens exhibited at least one abnormal characteristic, as defined above. Diatoms were isolated and identifiable by SEM from 47% (953 of 2016) of the specimens (Table 2.3). The success rate of diatom isolation from

particular sites ranged from 0% to 100% and showed a general increase over the course of the study (Tables 2.2 and 2.3). At least 10 genera and 16 different species of diatoms (Figure 2.3-2.6) were isolated and identified. More than one genus of diatom was isolated in 29% of the successful culture attempts. Of the samples with positive diatom identifications, one species of diatom was identified from 677 host specimens (71%), 2 species from 208 host specimens (22%), 3 species from 57 host specimens (6.0%), and 4 species from 11 hosts (1%).

Four genera of pennate diatoms: *Nitzschia* (Figure 2.3, 2.4H, 2.5A), *Nanofrustulum* (Figure 2.4), *Amphora* (Figure 2.3E, 2.4G, 2.5A, 2.5B), and *Navicula* (Figure 2.5C) were consistently isolated from hosts specimens collected from both the Atlantic and Pacific. Of the 953 successful isolations, 55% contained species of *Nitzschia*, 36% contained species of *Nanofrustulum*, 21% contained species of *Amphora*, 17% contained species of *Navicula*, and 6% contained species of *Achnanthes* (Table 2.5). Four other pennate diatom genera, *Diploneis* (Figure 2.6C), *Cocconeis* (Figure 2.6E), *Entomoneis* (Figure 2.6D), and *Protokeelia* (Figure 2.5F) were also isolated, but each from fewer than 1% of the *Amphistegina* specimens. A species of *Cyclotella* (Figure 2.3F) was the only centric diatom observed in the isolations.

Some of the diatoms were unidentifiable because of aberrant morphologies, which prevented reliable classification even to the genus or family level. One additional distinct diatom morphology, which was isolated from a single individual from 21 m depth at the Pear Tree site in Jamaica (Figure 2.5F), remains unidentified.

Diatoms were extracted and identifiable from 53% (924 of 1733) of normal-appearing *Amphistegina* as compared to only 10% (29 of 283) of foraminifers with visible anomalies (i.e., partial bleaching, shell damage, etc.). Of the remaining isolations where diatoms were not identifiable by SEM, 157 had apparent diatom growth, but identification was obstructed by

excessive organic material; 214 had only non-diatom growth, which included bacteria, fungi, dinoflagellate, and other unidentified groups of microorganisms (Figure 2.7); and 692 had no apparent microbial growth.

No diatom growth was observed in an unusually high percentage (82%) of isolations from hosts specimens collected from depths >25 m (Table 2.2 and 2.3). To a lesser extent, diatoms were also difficult to isolate from hosts from very shallow (<5 m) sites in the Atlantic. The most notable examples were from the Site 1 (3 m, 16% success) and Marquesas Shallow (<5 m, 10% success) in the Florida Keys, and Salt Whistle Bay (3 m, 5% success) from Mayreau Island in the Grenadines (Tables 2.2 and 2.3). Ten specimens of *Amphistegina radiata* from a 13 m site in Tutum Bay, Ambitle Islands, Papua New Guinea, collected in June 2005, also yielded no diatom growth, but all the individuals from this site were pale when compared to other specimens from this area (Table 2.3).

Temporal Studies

For the eight Florida Keys sites with seasonal data, *Nitzschia* was the most common genus isolated from specimens of *Amphistegina gibbosa* throughout the year, present in more than half the isolations (58%, 262 of 449). *Nanofrustulum*, *Amphora*, *Navicula*, and *Achnanthes* were isolated from 38%, 17%, 11%, and 4% of the *A. gibbosa* specimens at these sites, respectively (Table 2.4). No other genera were isolated from specimens from these sites.

The ratios of *Nitzschia*, *Nanofrustulum*, *Amphora*, and *Navicula* isolated from the eight Florida reef sites showed significant statistical differences (χ^2: d.f. = 28, $p < 0.01$). Sites 5, 6, and 7 accounted for most of the variability. The ratio of diatoms isolated from Sites 1, 2, 3, 4, and 8 were not significantly different (Sites 1–4 and 8, χ^2: d.f. = 12, $p = 0.85$). No seasonal trends were

observed in the distributions of the diatom isolates (Table 2.4). Specifically, no significant differences were observed in the abundance of *Nitzschia*, *Nanofrustulum*, or the other genera (*Amphora*, *Navicula*, and *Achnanthes*) among all 8 sites or the 5 similar sites (Site 1–4 and 8) grouped by date (all 8 Sites, χ^2: d.f. = 22, p = 0.12; Sites 1–4, and 8, χ^2: d.f. = 20, p = 0.18).

Geographic Distributions

Nitzschia was the most or second most common genus of diatom isolated from most sites and depths from both Atlantic and Pacific hosts (Table 2.2 and 2.3). This genus was not isolated from host specimens from only two sites (Killi Bob 12 m, Papua New Guinea, N = 10; and Nancy's Cup of Tea 12 m, Northern Little Cayman, N = 10), of the more than 50 sites from which diatoms were isolated and identifiable (Table 2.2 and 2.3).

Nanofrustulum was the second most common genus isolated from all samples. However, this genus was more than three times as common in isolations from hosts collected from depths ≤20 m (39%, 324 of 831) than from >20 m (12%, 15 of 122), with the exception of isolates from hosts collected from the remote 25 m site off Navassa Island. *Nanofrustulum* was isolated from 35% (7 of 20) of the hosts from Navassa.

Cyclotella (Figure 2.3 F) was the only centric diatom observed in my isolations and was isolated from hosts from Andros Island, Bahamas, and Juno Beach, Florida (Table 2.3). These were two of the northernmost sites sampled.

The frequency distributions of diatom symbionts isolated from hosts grouped by regions (Table 2.5) were statistically different (χ^2: d.f. = 24, p < 0.01). Species of *Nitzschia* were the most frequently isolated symbionts from all locations except the Grenadines and Papua New Guinea. Species of *Nanofrustulum* were isolated less frequently from samples from Little

Cayman, Jamaica, and Hawaii, and more frequently from samples from Juno Beach, FL, the Bahamas, Grenadines, and Papua New Guinea. Species of *Amphora* were isolated more frequently from samples from the Bahamas, Juno Beach, Navassa, Little Cayman, and Papua New Guinea. *Navicula* and other uncommon genera were isolated less frequently from samples from the Florida Keys and Little Cayman than from other locations.

Depth Trends

A significant difference was observed between the frequency distribution of diatoms isolated from hosts collected from shallower (≤20 m) water compared to deeper (>20 m) water (χ^2: d.f. = 5, $p < 0.01$) (Table 2.5). Species of the genera *Amphora*, *Navicula*, *Achnanthes*, and less common genera (*Diploneis*, *Cocconeis*, *Entomoneis*, and *Protokeelia*) were more abundant at depths >20 m (26%, 27%, 17%, and 11% respectively, N=122) compared to ≤20 m (20%, 16%, 4%, and 2% respectively, N = 813) (Table 2.5). Species of the genus *Achnanthes* had a wide distribution similar to *Nitzschia*, appearing at the shallowest collection depths of 1 m to the deepest at 75 m (Table 2.2 and 2.3). Furthermore, in the few successful isolations obtained from hosts from depths >20 m, a species of *Achnanthes* was among the most common (25%, 12 of 48) diatoms isolated.

Other Taxa Isolated

Non-diatom taxa, which included what appeared to be bacteria, fungi, dinoflagellates, and other unidentified groups of microorganisms (Figure 2.7), were observed in 17% (339 of the 2016) of all isolations attempted. These non-diatom taxa were observed along with diatom taxa in 125 isolations and in 214 isolations in which no identifiable diatoms were observed.

The isolations with non-diatom taxa occurred far more often from foraminifers with color or test abnormalities (43%, 122 of 283), than from foraminifers that appeared to be normal (13%, 217 of 1733). Furthermore, a higher proportion of apparently normal foraminifers where non-diatom taxa were isolated (45%, 98 of 217) yielded a combination of diatom taxa and non-diatom taxa than the obviously abnormal individuals (22%, 27 of 122).

Discussion

Based on my observations and previous culture isolation studies, species of *Nitzschia* appear to be the most common diatoms that can be isolated from *Amphistegina* spp., typically found associated in all seasons, depths and geographic locations. Species of *Nitzschia* were found in more than half of all *Amphistegina* specimens from which diatoms could be grown in culture in this study (55%), similar to results from previous studies (62%) (Lee et al., 1989, 1992, 1995a, 2000; Lee and Correia, 2005).

In my study, species of *Nitzschia* were isolated almost twice as frequently from *Amphistegina* than the second most common genus, *Nanofrustulum*. In previous studies, species of *Nitzschia* were isolated more than four times more frequently than *Nanofrustulum* (Table 2.5). These observations suggest that diatoms of the genus *Nitzschia* may be the "generalists" among associates of *Amphistegina*. However, another possible explanation for this observation could be that species of this genus are more cultivable than other species using the methods employed. Several observations support the latter hypothesis and are discussed below.

Diatoms associated with *Amphistegina* exhibited some variability with the depth and geographic location of their hosts. Diatoms of the genus *Nanofrustulum* appear to be better adapted to higher light regimes in shallow water. Species of *Nanofrustulum* were isolated more

than three times more frequently at depths ≤20 m compared to depths >20 m (Table 2.5). This observation is consistent with previous observations where *Nanofrustulum* was isolated more frequently at a 6m site compared to a 20 m site (Lee and Correia, 2005) and rarely isolated from hosts collected deeper than 25 m (Lee et al., 1989). Experimental studies also showed that *Nanofrustulum* grew best under higher light conditions (Lee et al., 1982).

Most of the *Nanofrustulum* isolated in my study were from hosts collected at shallower depths, with the exception of the samples from Navassa Island, where the collection depth was about 25 m. *Nanofrustulum* was isolated from 35% of the *Amphistegina* specimens from this site. Navassa Island is a remote, uninhabited, rock island with minimal anthropogenic impact and runoff affecting the surrounding water. The waters around this island are exceptionally clear, so zonation of photosynthetic organisms extends into deeper water (Williams, 2003). However, specimens from this site were only processed using the more advanced culture-isolation techniques, which may have biased results by being more efficient.

In contrast to *Nanofrustulum* distributions, the genera *Amphora*, *Navicula*, *Achnanthes*, and to some extent the other less common genera (*Diploneis*, *Cocconeis*, *Entomoneis*, and *Protokeelia*) were slightly more abundant at depths >20 m. These observation are generally consistent with the observations of Lee et al. (1989), who only isolated species of *Amphora*, *Achnanthes*, and *Protokeelia* from hosts collected at depths >25 m. The differences in these genera from depths above and below 20 m were not as pronounced as the opposite trend with *Nanofrustulum*, but these other genera could be better adapted to the lower light regimes in deeper water. However, these genera also were quite common at some shallower sites in Jamaica, Little Cayman, Juno Beach (Florida), and Andros Islands. As most of the contrary observations occurred after changes to the culture isolation techniques in the fall of 2004,

additional studies are needed to clarify whether the observed differences are real or an artifact of methods.

Symbionts from hosts collected at depths >25 m, and from three <5 m sites from the Atlantic were very difficult to isolate. A majority of the cultures from these samples had no observable diatom growth. Foraminifers from these sites likely contained diatom endosymbionts, because they all had the characteristic golden-brown color. I suspect that symbionts at these sites uncultivable using the original methods. Additional collections and isolations using the revised techniques will be needed to resolve questions of distributions of symbionts in the shallowest and deepest dwelling *A. gibbosa*.

No seasonal variability was observed in the diatoms isolated from the Florida reef-tract sites sampled between March 2001 and September 2004. Previous studies with diatom-bearing foraminifers, including *Amphistegina*, collected from the Indo-Pacific also found no significant seasonally variability in the diatoms isolated (Lee et al., 1989). Nevertheless, the fact that I observed no significant temporal differences in these samples does not necessarily mean none exist. Changes in associated diatom populations may occur on longer time scales than seasonal. I did find significant differences in frequencies of diatoms isolated from hosts from sites that were sampled for previous studies more than a decade ago (discussed further below). Another limitation to detecting seasonal changes was that specimen numbers from seasonally sampled sites were smaller than expected, because no diatoms were isolated in two-thirds of the isolation attempts. Therefore, to test for temporal differences in the distribution of the diatom taxa isolated, I pooled sites by date and also pooled the less common genera. To discern temporal variability in the less common taxa will require larger sample sizes.

Overall, observations of the symbionts of *Amphistegina* predominantly from the Atlantic were similar to those reported in previous studies (Lee et al., 1989, 1992, 1995a, 2000; Lee and Correia, 2005), which sampled *Amphistegina* predominantly from the Indo-Pacific (Table 2.5). As in previous studies, *Nitzschia*, *Nanofrustulum*, *Amphora*, *Navicula*, and *Achnanthes* accounted for the bulk of all isolations, and certain species of the genera *Nanofrustulum*, *Amphora*, *Achnanthes*, and *Protokeelia* exhibited similar depth trends to those previously reported (Lee et al., 1989, 1992, 1995a, 2000; Lee and Correia, 2005).

However, some significant differences were observed between my study and previous ones. A higher proportion of *Amphistegina* in my study, as well as those reported by Lee and Correia (2005), produced multiple species as compared to hosts from earlier studies (Lee et al., 1989, 1992). Over a quarter (29%) of the individual host specimens in my study hosted multiple genera of diatom symbionts. Whether these observations are a reflection of actual changes in diatom distributions or of differences in methods of isolating the associates remains to be determined.

The frequency distributions of diatoms isolated from hosts from the same sites and regions of previous studies were sometimes quite different. In a previous study of 50 *A. gibbosa* specimens from Conch Reef in the Upper Florida Keys (Lee et al., 1995a), diatoms of the genera *Nanofrustulum* and *Amphora* were isolated much less frequently from hosts (6.6% and 0%) than in my study (37% and 18%). On the other hand, diatoms of the genera *Cocconeis* and *Diploneis* were isolated much more frequently (8.0% and 16%) than in my study (0% and 0.7%) (Table 2.5). *Cocconeis* were only observed in the symbiont cultures isolated from two Molasses Reef sites about 25 and 27 m deep, though it was isolated from 28% of hosts from these sites. The

only species of *Diploneis* isolated in my study was from 3 of the 20 *Amphistegina* specimens collected from Navassa Island.

The differences in diatoms that I isolated from *A. gibbosa* from the Florida Keys and those reported by Lee et al. (1995a) were statistically significant (χ^2: d.f. = 4, $p < 0.01$). These differences could certainly be the result of slightly different isolation and culturing techniques between the two laboratories. However, the differences also could reflect real changes in the symbiont populations that occurred during the decade between the two sets of samplings.

Beginning in 1991, Florida Keys *A. gibbosa* populations were impacted by a massive bleaching event (Hallock et al., 1995); bleaching has continued to occur seasonally in the *A. gibbosa* population through at least 2015. The host population has responded to the stress in a variety of ways including by increasing the frequency of asexual reproductions as compared to sexual (Harney et al., 1998). Thus, symbiont populations likely responded as well. Just increasing the relative frequency of vertical transmission of symbionts (i.e., during asexual reproduction) as compared to horizontal transmission (i.e., acquisition of symbionts from the environment by zygotes or small juveniles) has the potential to alter symbiont proportions in a population.

Two of the most striking differences between pre-1991 isolations and my results are the increase in multiple symbionts and the increase in *Nanofrustulum*; both are consistent with the conclusions reached by Talge and Hallock (2003) and Williams and Hallock (2004) that the *A. gibbosa* population in the Florida Keys has seasonally bleached in response to photic stress. If *Nanofrustulum* spp. are indeed adapted to higher light conditions than other symbiont taxa, an increase in photic stress would logically result in the increased prevalence of *Nanofrustulum* symbionts. Furthermore, since photic stress can kill resident symbionts (Talge and Hallock,

2003), this may increase the probability that newly ingested diatoms with the potential to be symbiotic (e.g., Chai and Lee, 2000) can proliferate within a host, increasing the frequency of occurrence of multiple symbionts within individual hosts.

The frequency distributions of the diatoms isolated from *Amphistegina* collected in the Pacific in this study were also statistically different (χ^2: d.f. = 4, $p < 0.01$) from those in previous studies (Lee et al., 1989, 1992). Species of *Nitzschia* were isolated slightly less frequently in this study compared to previous studies, while species of *Navicula*, *Nanofrustulum*, and *Amphora* occurred more frequently. Species of *Cocconeis* were not isolated from any of my Pacific samples but were isolated in previous studies. These differences could easily be a consequence of the sources of samples; a third of the hosts from my Pacific samples were *Amphistegina radiata* collected from sites in Papua New Guinea, which are different from the host species and sites examined in previous studies of Pacific taxa.

Over the course of my study, I observed general increases both in success in isolating diatoms from their *Amphistegina* hosts and in the diversity of taxa isolated, particularly after changing methods in late 2004. Most of the less common genera (*Diploneis*, *Protokeelia*, *Entomoneis*, and *Cocconeis*) were only isolated after making these changes. In addition to an increase in the number of genera isolated, previously uncultured species or variants of the four most common genera (e.g., different *Nitzschia* spp. and *Fragilaria* spp.) also appeared in the cultures for the first time (Figure 2.2 E,F, 2.3A-D,H). The commercial diatom growth media was clearly nutritionally superior to the soil extract-enriched seawater solution and thus promoted growth of a wider range of diatom taxa. I also suspect that the pressure wash-method enhanced isolation success by cleaning the foraminifer of external microorganisms quickly and effectively with less abrasion and stress to the foraminifer and its symbionts.

Other factors could have contributed to differences in isolation efficiency. Early in the study I used a higher proportion (25–50%) of abnormal-appearing host specimens, which typically did not yield successful diatom cultures. Fewer of these abnormal individuals were used in later isolations (0–30%). Additionally, specimens used in the earlier part of the study, particularly from Sites 1–8, were individually assessed and recorded photographically for another project. Manipulations included moving, measuring, and photographing each specimen. I only proceeded with cleaning and isolations after these analyses were completed, typically two weeks after the samples were collected. A previous study showed no substantial effect on isolations after twelve days of starvation (Lee et al., 1989). However, the extended time between host collection and symbiont isolation in conjunction with the manipulations, notably light stress associated with the photography, could have negatively impacted isolation success.

Nevertheless, the increase in the number of genera isolated from host specimens after methods changes could be real differences, since many of the sites sampled after the changes were previously unsampled. This conclusion is supported by the fact that I did not culture any of the rarer diatom taxa from Sites 2 or 6, which were the only temporal-study sites sampled after processing changes. Additionally, I only isolated *Cyclotella* from two sites, Andros Island just before changes to methods, and Juno Beach sampled after changes. This suggests that *Cyclotella* is simply a rare associate and the isolation was independent of the culture media or technique. *Cyclotella* indeed appears to be an extremely rare isolate, reported in only one previous paper (Lee and Reimer, 1982).

As with any study where microorganisms are cultured in the laboratory, there is always the concern of "culture bias," where the most cultivatable species are selected for. The reality that no diatom growth was observed in 45% (906 of 2016) of the culture isolations and no

microbial growth was observed in 34% (692 of 2016) exacerbates this concern. Although methodological changes improved the success of isolations, the new methodology did not successfully isolate diatoms from all host specimens. There are many possible reasons why I observed no diatom growth in many of my isolation attempts. For instance, an algal associate may simply be uncultivatable using the methods employed. For many years, microbiologists have known that culturing methods do not capture many of the microorganisms that we are trying to identify or enumerate (e.g., Tanaka et al. 2014). The physical and chemical conditions in culture may be unsuitable, such as too much or too little light or of a particular nutrient. The use of antibiotics to inhibit growth of prokaryotic microorganisms may inhibit growth of some photosymbionts. Another possibility is that some soils used to make the soil extract to fortify the media contained inhibitors for diatom growth, since I observed striking differences in the success of diatom isolations based on the type of soil used.

Previous reports of culture isolations of diatoms from *Amphistegina* spp. (Lee et al., 1989, 1992, 1995a, 2000; Lee and Correia, 2005) did not quantitatively report the proportion of successful isolations to the total number of isolation attempts. In one of the earliest reports, Lee and Reimer (1982) noted that some diatoms grew better in some types of media than in other types and have a larger number of isolations in particular media types, but they did not report the efficiency of isolation directly from the host foraminifer. Therefore, previously reported distributions also reflect diatoms that were cultivable and should be interpreted with that in mind.

Non-diatom microorganisms, both heterotrophic and autotrophic taxa, grew in cultures from 17% of my host specimens. Most were unidentifiable, but isolates included what appeared to be bacteria, fungi, cyanobacteria, and dinoflagellates (Figure 2.7). Identification of these organisms by SEM is more difficult than for diatoms because most lack external characteristics.

However, non-diatom taxa were isolated more frequently from *Amphistegina* with obvious abnormalities and bleaching-stress symptoms. Often abnormal individuals had broken, etched or pitted tests, which were more difficult to clean. Such epibionts may have been isolated in culture. However, I also isolated non-diatom taxa from abnormal individuals with smooth tests and also from some normal-appearing individuals. Some of these non-diatom taxa are likely either external or internal pathogens or parasites associated with the causes or results of the visible stress symptoms in *Amphistegina*, as has been reported in corals (Kushmaro et al., 1996; Richardson et al., 1998). Some of these non-diatom taxa, particularly the autotrophic ones, also may be mutualistic symbionts of *Amphistegina*, as they were also isolated from apparently healthy individuals. Lee et al. (1980b) reported unidentified chlorophytes as symbionts of *Amphistegina*. Exploring these hypotheses likely will require more advanced molecular approaches.

Morphological taxonomy can be difficult and somewhat subjective, especially when subtle microscopic features distinguish between species, genera, and higher orders of classification. This is the case with diatom taxonomy. The number of diatom species has been estimated to be as low as 10,000 (Round and Crawford, 1989) to as high as 200,000 (Mann, 1999). Species such as *Nitzschia frustulum* and *Nanofrustulum shiloi*, two of the most commonly isolated from *Amphistegina*, can have widely variable morphologies, particularly in culture (Lee et al., 2000). The species concept for these diatoms and many others are debatable (Mann, 1999). For these reasons these diatoms were only identified to the genus level for this report.

Advances in image analysis and molecular taxonomy can help researchers more objectively decipher the difficult process of distinguishing species, but such methods also are far from perfect. The Automatic Diatom Identification and Classification (ADIAC) project was a

mammoth undertaking and has made many significant advancements in the field of diatom taxonomy, but more research needs to be done for this technique to be practical for routine use (du Buf and Bayer, 2002; Jalba et al., 2005). Molecular taxonomy is being utilized by many researchers (Zechman et al., 1994; Beszteri et al., 2001; Pawlowski et al., 2001a, b). Nevertheless, based on personal experiences and communications with other molecular taxonomists, molecular techniques are plagued with technical problems that remain largely unreported in the scientific literature. Therefore, future studies of the diatom endosymbionts in foraminifers should be based on a combination of morphological and molecular taxonomic approaches.

Table 2.1: Approximate latitudes and longitudes of the collection sites for host specimens.

Amphistegina gibossa from Caribbean and Western Atlantic Sites		
Site Name	Latitudes	Longitudes
Site 1: Rodriguez Key	25.0408	-80.4240
Site 2: SW 3 Sisters	25.0182	-80.3974
Site 3: Between Molasses and Pickels	25.0024	-80.3938
Site 4: SW Molasses	25.0034	-80.3837
Site 5: White Banks Dry Rocks	25.0372	-80.3749
Site 6: Algae Reef	25.1467	-80.2930
Site 7: Alina's Reef	25.3864	-80.1629
Site 8: Biscayne National Park	25.4867	-80.1577
Andros Island	24.7607	-77.7927
Carysfort Reef	25.2194	-80.1993
Conch Reef	24.9616	-80.4534
Coral Cities Reef	19.6837	-80.0236
Dancing Lady	18.4728	-77.4119
East Rio Bueno	18.4790	-77.4491
Florida Middle Grounds	28.4109	-84.2264
Grundy's Gardens	19.6570	-80.0894
Juno Beach	26.8705	-80.0196
Looe Key	24.5450	-81.4083
Marquesas Keys	24.5152	-82.1325
Molasses Reef	25.0152	-80.3785
Navassa Island	18.4421	-74.0143
Nancy's Cup of Tea	19.6992	-80.0122
Pear Tree Reef	18.4646	-77.3553
Pulley Ridge	24.7937	-83.6578
Sailfin Reef	19.7069	-80.0122
Salt Whistle Bay	12.6473	-61.3902
Tennessee Reef	24.7667	-80.7500
Union Island	12.5923	-61.4346
West Rio Bueno	18.4832	-77.4764
Young Island Reef	13.1298	-61.2036
Amphistegina lobifera and *Amphistegina lessonii* from Makapu'u Tidepools of O'ahu, Hawai'i		
Makapu'u Tide Pools	21.3041	-157.6491
Amphistegina radiata from New Britain and Ambitle Island, Papua New Guinea		
Killi Bob Reef	-5.02493	150.9559
Tutum Bay	-4.0697	153.5789

Table 2.2: The frequency of isolation of diatoms from *Amphistegina gibbosa* specimens collected from sites along the Florida reef tract between March 2001 and July 2005. Data reported as the percentage of successful isolations from each site that contained diatoms of each genus. Abbreviations for genera are: *Nitz = Nitzschia*, *Nano* (*Frag*) = *Nanofrustulum* and *Fragilaria*, *Amph = Amphora*, *Navi = Navicula*, *Achn = Achnanthes*, *Dipl = Diploneis*, *Cycl = Cyclotella*, *Ento = Entomoneis*, and *Prot = Protokeelia, and Cocc = Cocconeis.* Other abbreviations are: UKeys = Upper Keys, MKeys = Middle Keys, LKeys = Lower Keys, BNP = Biscayne Bay National Park, a = March/April 2001, b = June 2001, c = August 2001, d = October 2001, e = March 2002, f = June 2002, g = August 2002, h = November 2002, i = February 2003, j = May 2004, k = July 2004, l = September 2004, m = May 2005, n = June 2005, o = July 2005, and ND = No Data.

Site Name	**Location**	**Dates of Collection***	**Depth (meters)**	**#Isolations Attempted**	**%Successful Isolations**	**%*Nitz***	**%*Nano* (*Frag*)**	**%*Amph***	**%*Navi***	**%*Achn***	**%*Dipl***	**%*Cycl***	**%*Prot***	**%*Ento***	**%*Cocc***
Site 1	UKeys	d, e, f, g, h, i, j, k, l	3	105	16.2	58.8	23.5	17.6	5.9	0	0	0	0	0	0
Site 2	UKeys	a, c, d, e, f, g, h, i, j, k, l, m	6	190	36.3	69.6	26.1	20.3	5.8	8.7	0	0	0	0	0
Site 3	UKeys	a, b, c, d, e, f, g, h, i, k, l	9	180	31.1	57.1	33.9	14.3	14.3	0	0	0	0	0	0
Site 4	UKeys	b, c, d, e, f, g, h, i, k, l	18	170	46.5	54.4	26.6	15.2	10.1	8.9	0	0	0	0	0
Site 5	UKeys	a, b, c, d, e, f, g, h, i, k, l	6	170	47.6	60.5	71.6	18.5	2.5	8.6	0	0	0	0	0
Site 6	UKeys	a, b, c, d, e, f, g, h, i, j, k, l, m, o	6	220	33.2	53.4	20.5	19.2	19.2	0	0	0	0	0	0
Site 7	BNP	c, d, e, f, g, h, i	6	110	39.1	51.2	62.8	16.3	14.0	0	0	0	0	0	0
Site 8	BNP	e, f, g, h, i, j	6	95	32.6	61.3	32.3	16.1	16.1	0	0	0	0	0	0
Carysfort Reef	UKeys	l, m, o	10	40	92.5	43.2	51.4	10.8	24.3	2.7	0	0			
Carysfort Reef	UKeys	l, m	25	30	70.0	52.4	14.3	28.6	33.3	14.3	0	0	0	0	0
Carysfort Reef	UKeys	l, m	50	34	8.8	66.7	0	33.3	33.3	100.0	0	0	0	0	0
Carysfort Reef	UKeys	l, m	75	14	7.1	100.0	0	0	0	100.0	0	0	0	0	0
Carysfort Reef	UKeys	l	100	5	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Conch Reef	UKeys	k	10	20	70.0	78.6	71.4	14.3	14.3	0	0	0	0	0	0
Conch Reef	UKeys	k	20	20	85.0	58.8	29.4	17.6	23.5	0	0	0	0	0	0
Conch Reef	UKeys	k	30	20	15.0	66.7	0	33.3	33.3	33.3	0	0	0	0	0
Looe Key Shallow	LKeys	j	<5	20	80.0	56.3	68.8	31.3	12.5	0	0	0	0	0	0

Table 2.2 (Continued)

Site Name	**Location**	**Dates of Collection***	**Depth (meters)**	**#Isolations Attempted**	**%Successful Isolations**	**%*Nitz***	**%*Nano* (*Frag*)**	**%*Amph***	**%*Navi***	**%*Achn***	**%*Dipl***	**%*Cycl***	**%*Prot***	**%*Ento***	**%*Cocc***
Looe Key 6m	LKeys	m	6	10	80.0	75.0	50.0	0	0	0	0	0	0	0	0
Looe Key 10m	LKeys	m	10	10	100.0	50.0	60.0	0	0	0	0	0	0	0	0
Looe Key	LKeys	n	14	10	100.0	40.0	0	50.0	0	20.0	0	0	0	0	0
Looe Key	LKeys	m	25	10	70.0	42.9	28.6	14.3	14.3	0	0	0	0	0	0
Marquesas Shallow	LKeys	j	<5	10	10.0	100.0	0	0	0	0	0	0	0	0	0
Marquesas	LKeys	j	6	10	40.0	75.0	100.0	0	0	0	0	0	0	0	0
Molasses Reef	UKeys	m	10	10	100.0	40.0	50.0	10.0	20.0	0	0	0	0	0	0
Molasses Reef	UKeys	o	12	10	100.0	30.0	0	20.0	30.0	10.0	0	0	0	40.0	0
Molasses Reef	UKeys	m	25	10	80.0	37.5	0	25.0	12.5	25.0	0	0	0	0	12.5
Molasses Reef	UKeys	o	27	10	100.0	30.0	0	20.0	30.0	0	0	0	0	0	40.0
Molasses Reef	UKeys	m	50	12	25.0	66.7	0	0	0	66.7	0	0	0	0	0
Tennessee Reef	MKeys	m	6	10	70.0	42.9	42.9	0	28.6	0	0	0	0	0	0
Tennessee Reef	MKeys	k, m, o	10	40	85.0	38.2	38.2	20.6	29.4	0	0	0	0	0	0
Tennessee Reef	MKeys	m	25	10	80.0	50.0	12.5	12.5	50.0	0	0	0	0	0	0
Tennessee Reef	MKeys	o	27	10	100.0	40.0	0	30.0	30.0	0	0	0	0	0	0
Tennessee Reef	MKeys	k	30	20	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tennessee Reef	MKeys	m	50	8	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total				**1653**	**42.4**	**54.9**	**36.8**	**17.7**	**14.7**	**5.1**	**0**	**0**	**0**	**0.6**	**0.7**

Table 2.3: The frequency of isolation of diatoms from *A. gibbosa* (all Atlantic sites), *A. lessonii* and *A. lobifera* (Makapuu, Oahu, Hawaii), and *A. radiata* (Papua New Guinea). Data reported as the percentage of successful isolations from each site that contained diatoms of each genus. Abbreviations are as follows: N = North, S = South, E = East, W = West, FL = Florida, GoM = Gulf of Mexico, Is. = Island, LCI = Little Cayman Island, JA = Jamaica, HI = Hawaii, PNG = Papua New Guinea, other abbreviations as in Table 2.1.

Site Name	Location	Dates of Collection	Depth (meters)	#Isolations Attempted	%Successful Isolations	%*Nitz*	%*Nano* (*Frag*)	%*Amph*	%*Navi*	%*Achn*	%*Dipl*	%*Cycl*	%*Prot*	%*Ento*	%*Cocc*
Gulf of Mexico															
Pulley Ridge	SW FL Shelf, GoM	03/03	75	3	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FL Middle Grounds	W FL Shelf, GoM	05/03	30	30	6.7	50.0	0	0	0	100.0	0	0	0	0	0
Atlantic															
Andros Is.	Bahamas	08/04	10	20	80.0	62.5	50.0	43.8	31.3	0	0	25.0	0	0	0
Juno Beach	SE FL	04/05	18	20	100.0	70.0	85.0	65.0	10.0	15.0	0	15.0	0	0	0
Caribbean															
Union Is.	Grenadines	05/03	10	20	55.0	45.5	81.8	27.3	36.4	9.1	0	0	0	0	0
Young Is.	Grenadines	05/03	6	20	35.0	42.9	71.4	14.3	14.3	0	0	0	0	0	0
Young Is.	Grenadines	05/03	20	20	75.0	60.0	33.3	26.7	20.0	20.0	0	0	0	0	0
Salt Whistle Bay	Grenadines	05/03	3	20	5.0	100.0	100.0	0	0	0	0	0	0	0	0
Grenadines Total				80	42.5	52.9	58.8	23.5	23.5	11.8	0	0	0	0	0
Navassa Is.	SW of Haiti	11/04	25	20	100.0	50.0	35.0	40.0	45.0	0	15.0	0	15.0	0	0
Coral Cities	S LCI	06/05	11	10	100.0	70.0	40.0	0	0	0	0	0	0	0	0

Table 2.3 (Continued)

Site Name	Location	Dates of Collection	Depth (meters)	#Isolations Attempted	%Successful Isolations	%*Nitz*	%*Nano* (*Frag*)	%*Amph*	%*Navi*	%*Achn*	%*Dipl*	%*Cycl*	%*Prot*	%*Ento*	%*Cocc*
Grundy' Gardens	S LCI	06/05	9	10	100.0	80.0	0	40.0	0	0	0	0	0	0	0
Nancy's Cup of Tea	N LCI	06/05	12	10	70.0	0	0	85.7	0	0	0	0	28.6	0	0
Sailfin	N LCI	06/05	11	10	100.0	50.0	30.0	20.0	10.0	0	0	0	0	0	0
LCI Total				40	92.5	54.1	18.9	32.4	2.7	0	0	0	5.4	0	0
W Rio Bueno	N JA	08/05	6	10	100.0	70.0	0	20.0	20.0	0	0	0	0	0	0
E Rio Bueno	N JA	08/05	11	10	100.0	50.0	0	40.0	20.0	0	0	0	0	0	0
E Rio Bueno	N JA	08/05	20	10	100.0	70.0	20.0	0	20.0	0	0	0	0	0	0
Pear Tree	N JA	08/05	29	10	70.0	85.7	0	0	0	0	0	0	0	42.9	0
Pear Tree	N JA	08/05	21	10	100.0	70.0	0	0	0	40.0	0	0	0	0	0
Pear Tree	N JA	08/05	12	10	100.0	50.0	0	20.0	30.0	30.0	0	0	0	0	0
Dancing Lady	N JA	08/05	15	10	100.0	60.0	20.0	40.0	30.0	10.0	0	0	0	0	0
JA Total				70	95.7	64.2	6.0	17.9	17.9	11.9	0	0	0	4.5	0

Table 2.3 (Continued)

Site Name	Location	Dates of Collection	Depth (meters)	#Isolations Attempted	%Successful Isolations	%*Nitz*	%*Nano* (*Frag*)	%*Amph*	%*Navi*	%*Achn*	%*Dipl*	%*Cycl*	%*Prot*	%*Ento*	%*Cocc*
Pacific															
Makapuu	Hawaii	03/01	1	25	76.0	68.4	36.8	15.8	57.9	0	0	0	0	0	0
Makapuu	Hawaii	06/03	1	25	72.0	44.4	11.1	16.7	44.4	5.6	0	0	0	0	0
HI Total				50	74.0	56.8	24.3	16.2	51.4	2.7	0	0	0	0	0
Tutum Bay 120	Ambitle Is., PNG	06/05	13	10	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tutum Bay 300	Ambitle Is., PNG	06/05	28	10	90.0	66.7	22.2	77.8	33.3	33.3	0	0	0	0	0
Killi Bob	PNG	06/05	12	10	100.0	0	70.0	0	40.0	0	0	0	0	0	0
PNG Total				30	63.3	31.6	47.4	36.8	36.8	15.8	0	0	0	0	0

Table 2.4: The frequency of isolation of diatoms from *Amphistegina gibbosa* specimens collected from eight sites along the Florida Keys reef tract between March 2001 and September 2004. Data reported as the percentage of successful isolations from a particular collection date that contained diatoms of each genus. Abbreviations as in Table 2.2.

Sites	Dates of Collection	Depth (meters)	#Isolations Attempted	%Successful Isolations	%*Nitz*	%*Nano* (*Frag*)	%*Amph*	%*Navi*	%*Achn*
All Sites (Site 1-8)									
2, 3, 5, 6	3-4/01	6-9	40	37.5	20.0	46.7	40.0	6.7	0
3-6	06/01	6-18	80	32.5	76.9	38.5	15.4	3.8	3.8
2-7	08/01	6-18	120	34.2	58.5	26.8	17.1	12.2	7.3
1-7	10/01	3-18	100	40.0	32.5	47.5	15.0	15.0	7.5
1-8	03/02	3-18	115	27.0	61.3	48.4	12.9	3.2	0
1-8	06/02	3-18	120	35.8	41.9	46.5	16.3	11.6	2.3
1- 8	08/02	3-18	120	35.0	59.5	38.1	16.7	7.1	4.8
1-8	11/02	3-18	120	30.8	75.7	40.5	10.8	13.5	0
1-8	02/03	3-18	120	35.8	60.5	44.2	14.0	14.0	2.3
1, 2, 6, 8	05/04	3-18	65	30.8	40.0	40.0	15.0	15.0	5.0
1-6	07/04	3-18	110	36.4	50.0	32.5	22.5	10.0	10.0
1-6	09/04	3-18	100	47.0	78.7	31.9	17.0	10.6	6.4
Total			**1210**	**35.1**	**56.7**	**39.5**	**16.7**	**10.6**	**4.5**
Similar Sites (Site 1-4, 8)									
2-4	06/01	6-18	60	38.3	60.9	8.7	17.4	13.0	8.7
1-4	08/01	3-18	55	38.2	23.8	42.9	14.3	14.3	9.5
1-4, 8	10/01	3-18	70	22.9	62.5	37.5	6.3	0	0
1-4, 8	03/02	3-18	75	29.3	36.4	36.4	18.2	9.1	4.5
1-4, 8	06/02	3-18	75	29.3	63.6	22.7	13.6	9.1	4.5
1-4, 8	08/02	3-18	75	28.0	90.5	23.8	14.3	9.5	0
1-4, 8	11/02	3-18	75	34.7	57.7	34.6	11.5	19.2	0
1, 2, 8	02/03	3-18	45	33.3	46.7	40.0	13.3	13.3	6.7
1-4	05/04	3-18	70	37.1	61.5	26.9	15.4	11.5	11.5
1-4	07/04	3-18	70	47.1	81.8	30.3	18.2	12.1	6.1
Total			**670**	**33.6**	**60.0**	**29.8**	**14.7**	**11.6**	**5.3**

Table 2.5: The frequency of isolation of diatoms from *A. gibbosa*, *A. lessonii*, *A. lobifera*, and *A. radiata* from sites in the Atlantic and Pacific compared to previous isolations from the Gulf of Aqaba, Pacific, and Atlantic (Lee et al., 1989, 1992, 1995a). Data reported as the percentage of successful isolations from each site that contained diatoms of each genus. Abbreviations as in Table 2.3.

This Study												
Location	**Host Species**	**#Successful Isolations**	**%*Nitz***	**%*Nano* (*Frag*)**	**%*Amph***	**%*Navi***	**%*Achn***	**%*Dipl***	**%*Cycl***	**%*Prot***	**%*Ento***	**%*Cocc***
BNP	*A. gibbosa*	74	55.4	50.0	16.2	14.9	0.0	0.0	0.0	0.0	0.0	0.0
UKeys	*A. gibbosa*	512	56.4	34.6	17.6	13.7	6.6	0.0	0.0	0.0	0.8	1.0
MKeys	*A. gibbosa*	59	40.7	28.8	18.6	32.2	0.0	0.0	0.0	0.0	0.0	0.0
LKeys	*A. gibbosa*	56	55.4	48.2	19.6	5.4	3.6	0.0	0.0	0.0	0.0	0.0
FL Middle Grounds	*A. gibbosa*	2	50.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
Andros Is.	*A. gibbosa*	16	62.5	50.0	43.8	31.3	0.0	0.0	25.0	0.0	0.0	0.0
Juno Beach	*A. gibbosa*	20	70.0	85.0	65.0	10.0	15.0	0.0	15.0	0.0	0.0	0.0
Grenadines	*A. gibbosa*	34	52.9	58.8	23.5	23.5	11.8	0.0	0.0	0.0	0.0	0.0
Navassa Is.	*A. gibbosa*	20	50.0	35.0	40.0	45.0	0.0	15.0	0.0	15.0	0.0	0.0
Little Cayman	*A. gibbosa*	37	54.1	18.9	32.4	2.7	0.0	0.0	0.0	5.4	0.0	0.0
Jamaica	*A. gibbosa*	67	64.2	6.0	17.9	17.9	11.9	0.0	0.0	0.0	4.5	0.0
Makapuu, HI	*A. lobifera* and *A. lessonii*	37	56.8	24.3	16.2	51.4	2.7	0.0	0.0	0.0	0.0	0.0
PNG	*A. radiata*	19	31.6	47.4	36.8	36.8	15.8	0.0	0.0	0.0	0.0	0.0
Shallow Sites ≤20m	**All *Amphistegina* Hosts**	**831**	**55.7**	**39.0**	**19.9**	**16.0**	**4.3**	**0.0**	**0.8**	**0.2**	**0.5**	**0.0**
Deep Sites >20m	**All *Amphistegina* Hosts**	**122**	**53.3**	**12.3**	**26.2**	**27.0**	**17.2**	**2.5**	**0.0**	**2.5**	**2.5**	**4.1**
All Sites	**All *Amphistegina* Hosts**	**953**	**55.4**	**35.6**	**20.7**	**17.4**	**6.0**	**0.3**	**0.7**	**0.5**	**0.7**	**0.5**
Previous Studies by J.J. Lee and Colleagues												
Pacific	*A. lobifera*	178	40.4	34.3	11.2	6.7	4.5	0.0	0.0	0.0	0.0	2.8
Pacific	*A. lessonii*	138	62.4	17.4	10.8	7.9	0.0	0.0	0.0	0.0	0.0	1.4
Gulf of Aqaba	*A. lobifera*	797	63.7	11.9	9.0	8.5	2.4	0.0	0.0	1.8	1.1	1.5
Gulf of Aqaba	*A. lessonii*	543	66.9	10.5	6.3	11.0	2.4	0.0	0.0	0.9	0.0	2.0
Conch Reef, Upper Keys	*A. gibbosa*	50	54.0	6.6	0.0	22.0	0.0	16.0	0.0	0.0	0.0	8.0
All Sites	**All *Amphistegina* Hosts**	**1706**	**61.8**	**14.1**	**8.2**	**9.5**	**2.3**	**0.5**	**0.0**	**1.1**	**0.5**	**2.0**

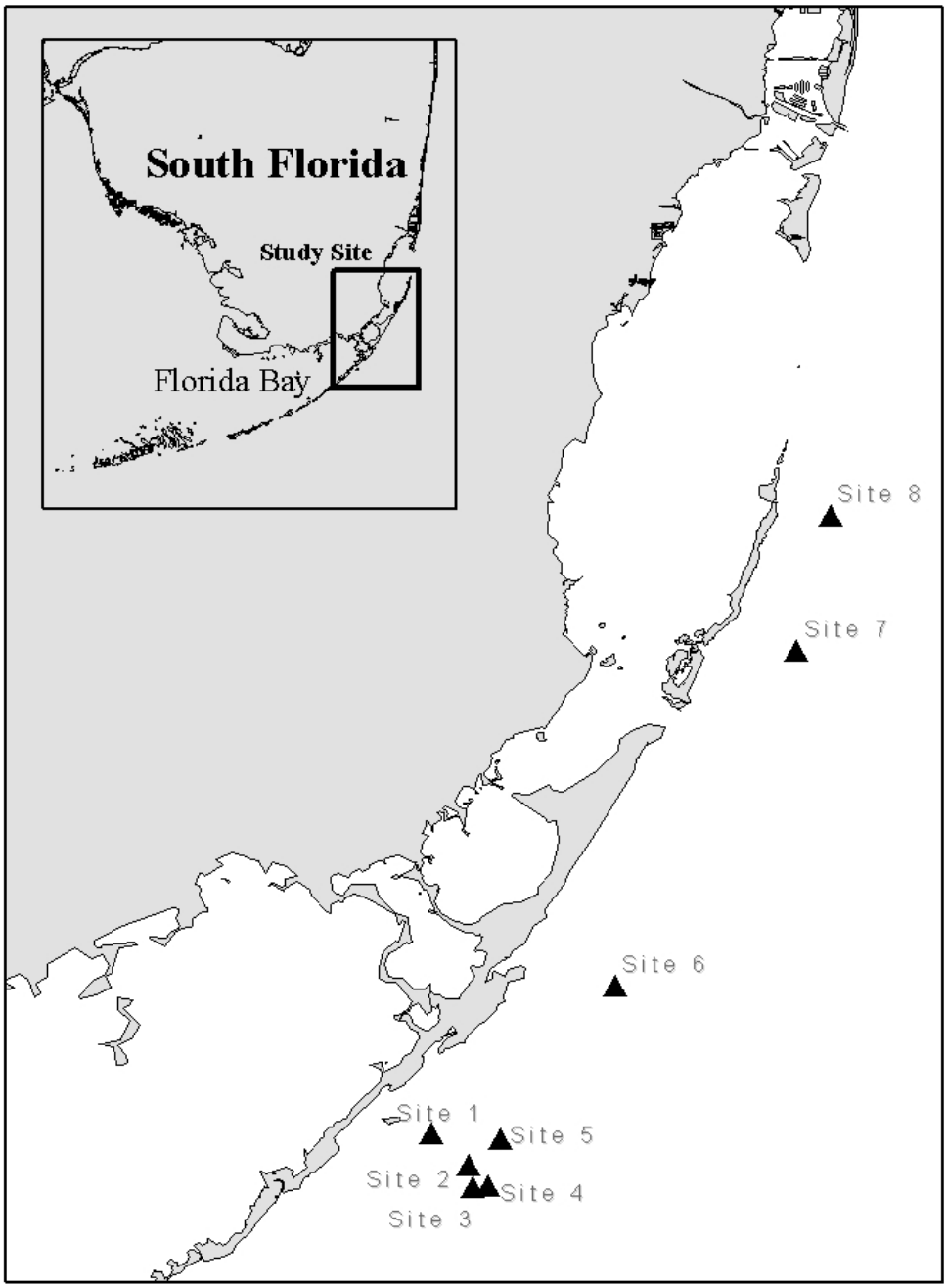


Figure 2.1: Sites 1-8 in the Upper Florida Keys and Biscayne National Park where samples were collected periodically between March 2001 and July 2005. See Table 2.1 for depths of these sites.

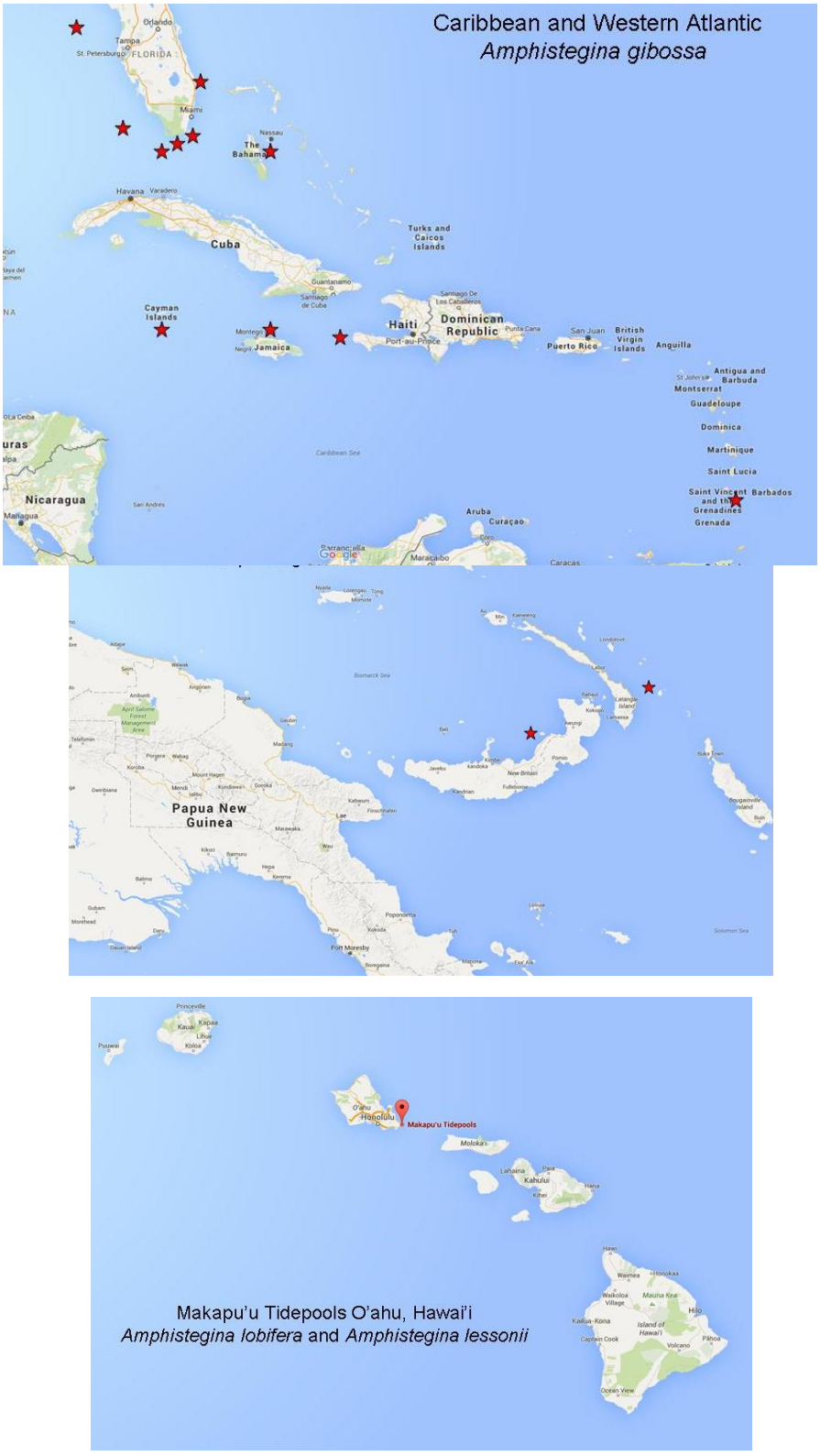


Figure 2.2: Approximate locations where host specimens were collected.

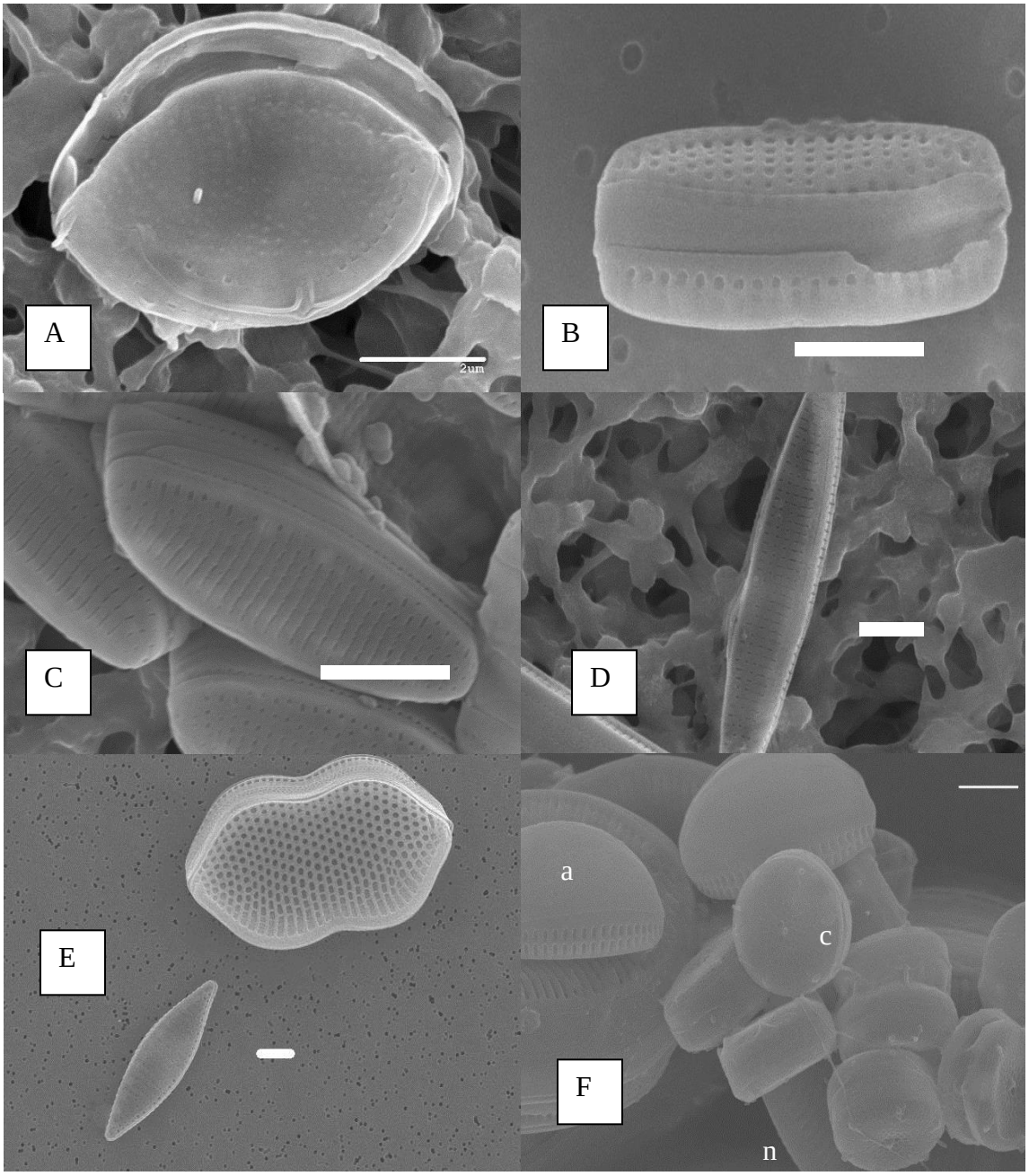


Figure 2.3: SEM micrographs of a variety of diatoms isolated in culture from *Amphistegina* specimens. *Nitzschia* sp. from Site 2, Florida Keys, March 2001 (A), *Nitzschia* sp. from Site 4, Florida Keys, July 2004 (B), *Nitzschia* sp. from Site 5, Florida Keys, February 2003 (C), *Nitzschia* sp. Union Island, May 2003 (D), and *Nitzschia* sp. (small) and *Nitzschia panduriformis* (big) isolated from Pear Tree 21m, Jamaica, August 2005 (E). *Cyclotella* sp. (c), *Amphora* sp. (a), and *Nitzschia* sp. (n), isolated from *Amphistegina* specimens from Juno Beach, FL, April 2005 (F). Scale bars equal 2 μm.

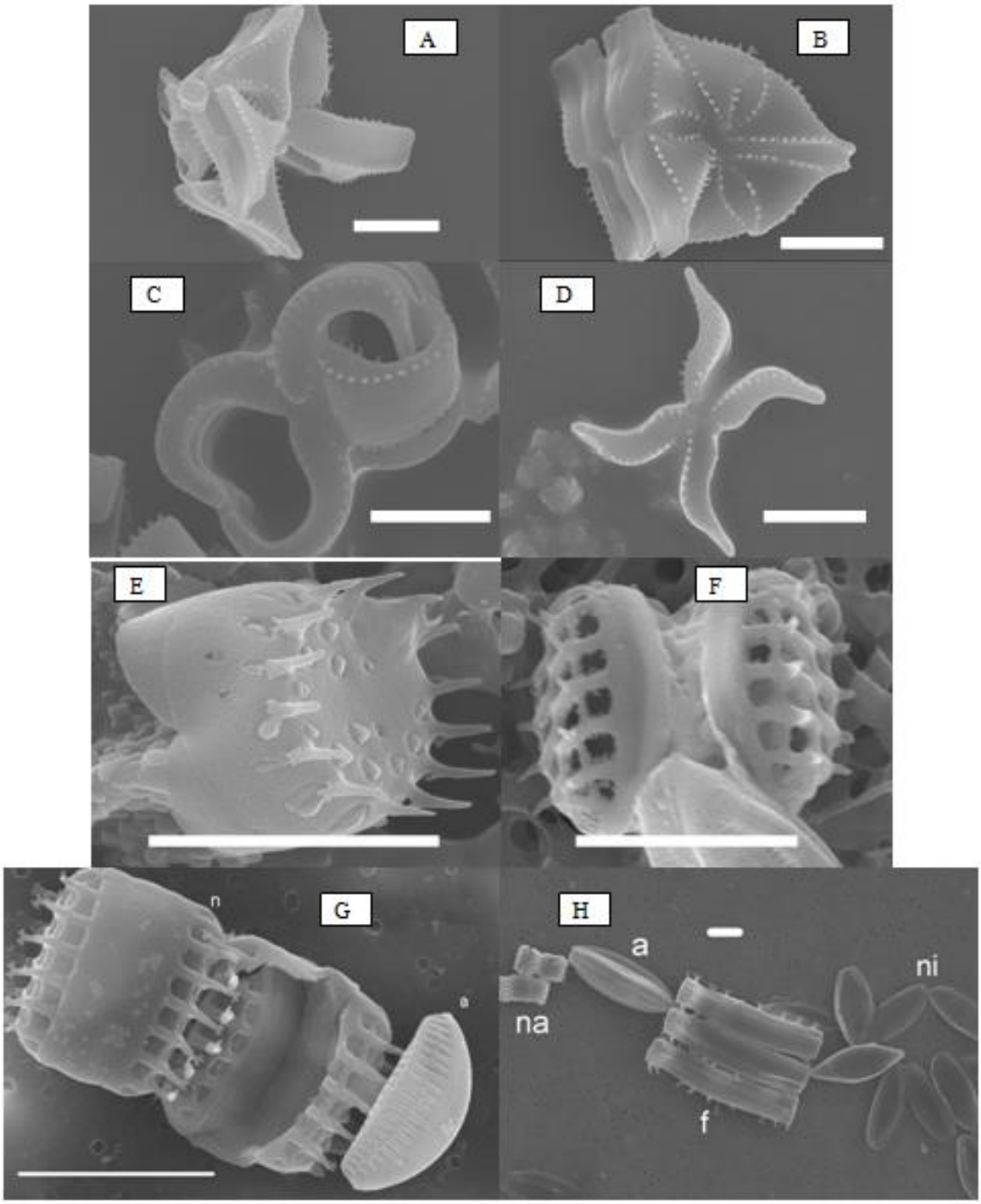


Figure 2.4: SEM micrographs of a variety of diatoms isolated in culture from *Amphistegina* specimens. Fragilariaceae sp. from Juno Beach, April 2005 (A-D), *Nanofrustulum shiloi* from Makapuu Tide Pools, March 2001 (E), Fragilariaceae sp. from Navassa Island, November 2004 (F). Fragilariaceae sp. (n) and *Amphora* sp. (a) isolated from *Amphistegina gibbosa* specimens from Site 6, Florida Keys, May 2004 (G). Four species of diatoms, *Nanofrustulum shiloi*. (na), *Amphora* sp. (a), Fragilariaceae sp. (f), and *Nitzschia* sp. (ni), isolated from a single *Amphistegina gibbosa* specimen from Sail Fin Reef, Little Cayman Island, June 2005 (H). Scale bars equal 5 μm.

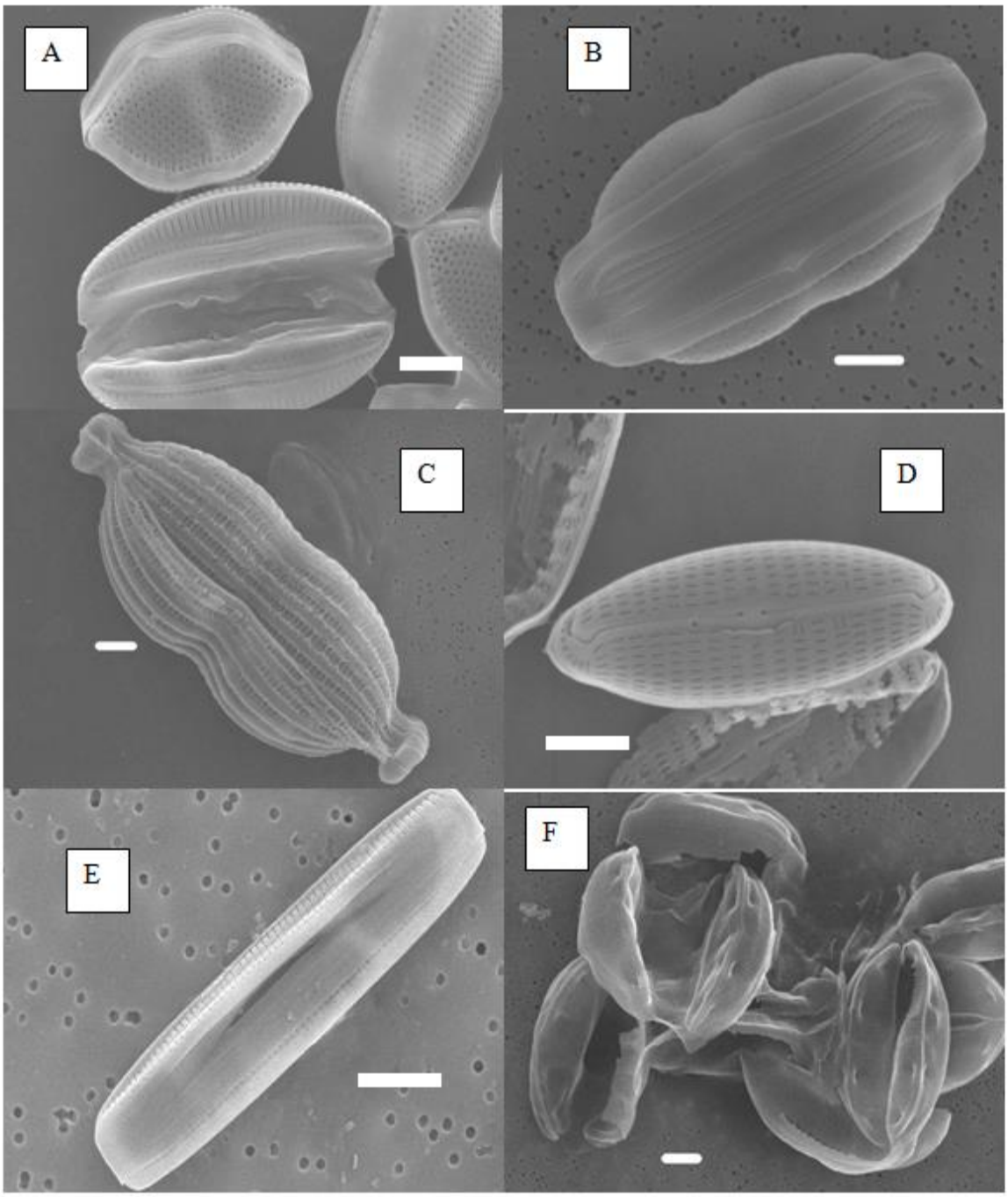


Figure 2.5: SEM micrographs of a variety of diatoms isolated in culture from *Amphistegina* specimens. *Nitzschia panduriformes* (top) and *Amphora* sp. (bottom) isolated from *A. gibbosa* specimens from Juno Beach, April 2005 (A). *Amphora* sp. isolated from *A. radiata* specimens from Tutum Bay 28 m, Papua New Guinea, June 2005 (B). *Amphora* sp. isolated from *A. gibbosa* specimens from Tennessee Reef 10 m, July 2005 (C). *Navicula* sp. isolated from *A. gibbosa* specimens from Young Island Reef 6 m, May 2003 (D). Girdle view of *Nitzschia* sp. isolated from *A. gibbosa* specimens from Looe Key Shallow, May 2004 (E). *Protokeelia* sp. isolated from *A. gibbosa* specimens from Nancy's Cup of Tea Reef, Little Cayman, June 2005(F). Scale bars equal 2 μm.

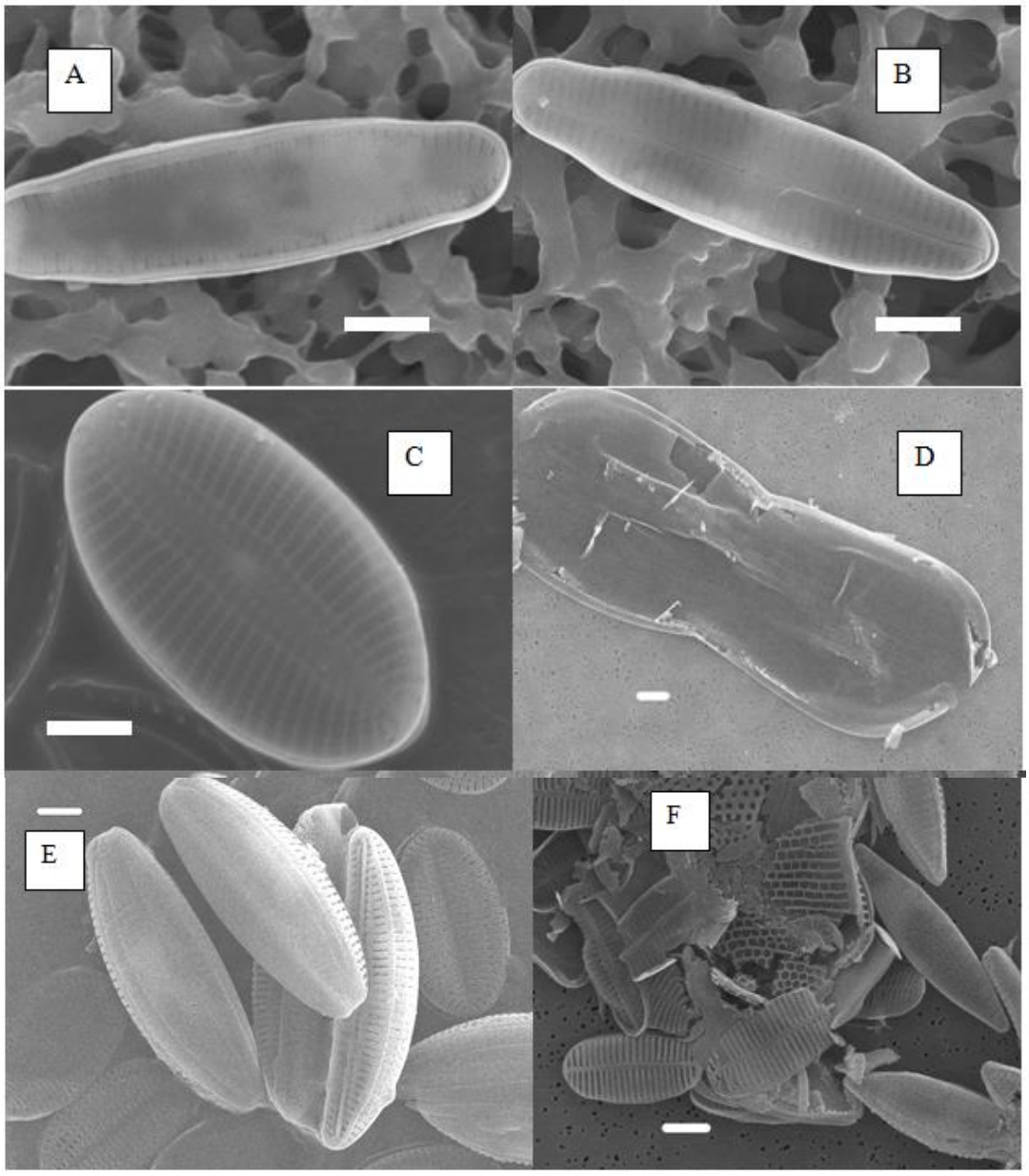


Figure 2.6: SEM micrographs of a variety of diatoms isolated in culture from *Amphistegina* specimens. *Achnanthes* sp. from *Amphistegina gibbosa* specimens from Young Island Rock 20 m, May 2003 (A: raphe-less valve, B: raphe valve). *Diploneis* sp. isolated from *A. gibbosa* specimens from Navassa Island, November 2005 (C). *Entomoneis* sp. isolated from *A. gibbosa* specimens from Pear Tree Reef 29 m, Jamaica, July 2005 (D). *Amphora* sp. (foreground) and *Cocconeis* sp. (background) isolated from *Amphistegina gibbosa* specimens from Molasses Reef 27 m, July 2005 (E). Surirellaceae sp. (s) and *Nitzschia* sp. (n) isolated from an *A. gibbosa* from Pear Tree Reef 21 m, Jamaica, July 2005 (F). Scale bars equal 2 μm.

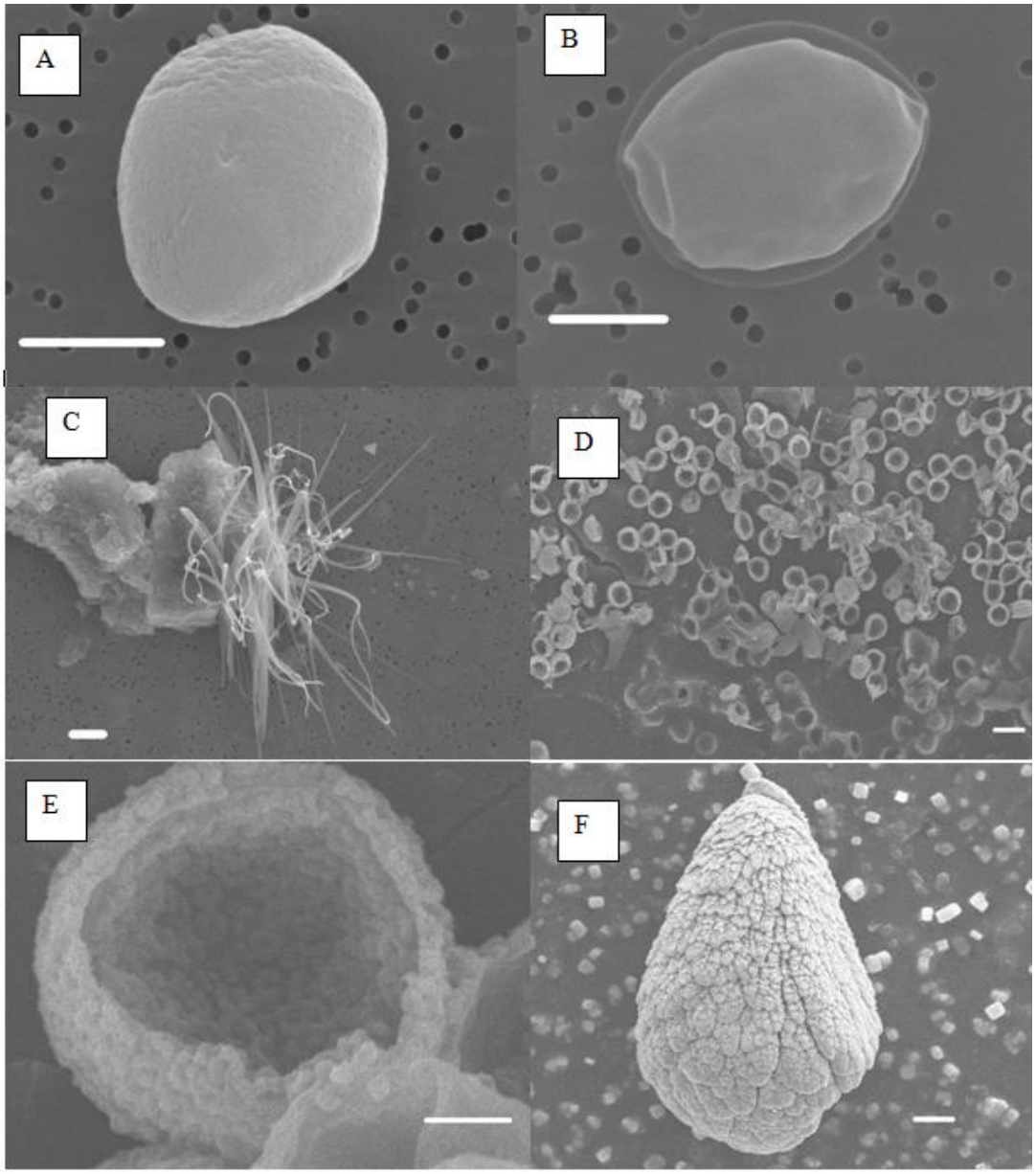


Figure 2.7: Examples of non-diatom taxa isolated from *Amphistegina* specimens. Possible dinoflagellate isolated from *A. gibbosa* from Looe Key 14 m, June 2005 (A). Unknown microbes isolated from *A. radiata* from Tutum Bay 13 m (B) and Killi Bob 12 m (C). Unknown microbes isolated from *A. gibbosa* from Navassa Island, November 2004 (D and E) and Carysfort Reef 10m, May 2005 (F). Scale bars equal 2 µm for A, B and E, and 5 µm in C, D, and F.

CHAPTER 3: SPATIAL AND TEMPORAL DISTRIBUTIONS OF ALGAL ENDOSYMBIONTS IN *AMPHISTEGINA GIBBOSA* FROM THE FLORIDA KEYS: DNA EXTRACTION AND SEQUENCING

Abstract

Previous studies utilizing culturing techniques have reported a wide diversity of diatom endosymbionts within reef-dwelling foraminifers of the genus *Amphistegina*. Here, molecular techniques were used here to explore spatial and temporal distribution patterns of the predominant diatoms found in *Amphistegina gibbosa* specimens collected from throughout the Florida reef tract. The foraminifers were collected between December 1999 and July 2005 from various sites in the lower, middle, and upper Florida Keys at depths ranging from 3–75 m. DNA was extracted from the foraminifers, and portions of two genes (the small subunit of the ribosomal RNA gene, *18S*, and the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase [i.e., RubisCO] gene, *rbcL*) were amplified, sequenced, and analyzed. The BLAST searches and phylogenetic analyses of more than 1200 diatom sequences isolated directly from *A. gibbosa* specimens from the Florida Keys revealed that >99% of all the diatom *18S* and *rbcL* sequences were nearly identical (97-99% BLAST search similarity) to the *18S* and *rbcL* sequences of an unnamed diatom in GenBank labeled Fragilariaceae sp. (Accession # JX413542.1 for *18S* and JX413559.1 for *rbcL*), which was isolated from subtidal sand grains collected at the Florida State University Coastal and Marine Laboratory in October 2010. A small but consistently observed intra-specific cluster of many of the sequences from the deepest 75 m site was noted. Otherwise, no significant spatial or temporal variations were observed.

Among >20 diatom species previously reported from culture studies as endosymbionts of *Amphistegina*, *Nanofrustulum shiloi* (previously *Fragilaria shiloi*) was most closely related (92-95% BLAST search similarity) to the sequences isolated directly from *A. gibbosa*. My findings support observations from earlier ultrastructural studies that reported evidence for specific and stable host-symbiont relationships in other species of *Amphistegina*. Thus, most, if not all, of the diatoms that have previously been reported as endosymbionts of *A. gibbosa* based upon culture studies were likely epiphytic or undigested prey that thrived in the culture media.

Introduction

In the late 1970s and early 1980s, ultrastructural studies that utilized transmission electron microscopy (TEM) revealed that the endosymbionts within the reef-dwelling foraminifers of the genus *Amphistegina* are diatoms (Leutenegger 1977, 1983, 1984; Berthold 1978). Further classification was not possible because the *in hospite* diatom cells lacked their characteristic frustules, which was the primary means of classification at that time. To attempt to solve this problem, Lee et al. (1979a, b) developed protocols to meticulously clean the host foraminifers, then crush them to expel their symbionts, which were then grown in enriched culture media. The diatoms in culture produced frustules, which were then used for identification and classification. The process appeared to be straightforward and, over the past 40 years, more than two dozen species of diatoms have been isolated, identified and reported to be endosymbionts within *Amphistegina* and other diatom-bearing foraminifers (e.g., Lee et al. 1979a, 1980a, 1995a, 2011a, b; Lee and Correia, 2005; see Table 1.1).

The evidence from culture studies that benthic foraminifers, particularly those of the genus *Amphistegina*, harbored a diverse array of diatom endosymbionts appeared to be an

integral part of the foraminifers ability to survive and thrive at extreme ends of photic scale from depths of <1 m down to >100m (Lee and Hallock, 1987). Since the 1970s, numerous publications have focused and expanded on the hypothesis of non-specificity of diatom endosymbionts among and within the larger benthic foraminifers that host them (e.g. Lee, 2011 a, b, and references therein). The working hypothesis that the endosymbionts of diatom-bearing foraminifers are diverse and non-specific spawned further hypotheses and speculations, such as the potential for different symbiont assemblages to drive the morphology, physiology, and evolution of these foraminifers or to provide adaptive advantages with changing climate or environments (e.g., Lee and Hallock, 1987; Lee, 2011a, b).

Although Lee et al. (1995a) isolated and cultured diatoms from 50 *A. gibbosa* specimens from the Florida reef tract, a systematic study of the distribution of symbionts in space and time has not previously been reported. Therefore, the original goal of my study was to use molecular methods of taxonomy to quantify how the taxa of algal symbionts in populations of *A. gibbosa* differed among reefs, depths, or seasons. This research was considered a critical "next step" in the continued development and application of *Amphistegina* as bioindicators of reef condition (e.g., Hallock et al., 2003; Hallock, 2012). Understanding spatial and temporal variability of the endosymbiont taxa, and the role of those symbionts in the variability in responses of hosts to specific stressors, are essential to interpreting results of previous and ongoing field studies and laboratory experiments.

Methods and Materials

Sample collection and preparation for DNA extraction

Amphistegina gibbosa specimens used in this research were opportunistically obtained from then ongoing field studies (e.g., Fisher et al., 2007; Baker et al., 2009). Specimens were collected from depths of 3–75 m at 27 reef sites in the lower, middle, and upper Florida Keys, between December 1999 and July 2005 (Table 3.1). Specimens from water depths <50 m were collected by Scuba divers, who collected pieces of reef rubble into plastic bags, brought the bags to the surface, and transported them to a field laboratory where the foraminifers and associated meiobiota were brushed from the rubble into seawater (see methods described by Hallock et al., 1995 or Williams et al., 1997). Samples from sites ≥50 m were collected with dredge buckets. The samples were transported in insulated containers to the University of South Florida Reef Indicators Laboratory in St. Petersburg, Florida, for further processing, which included microscopic evaluation and sorting using fine forceps. Up to 25 *A. gibbosa* specimens from each site were selected and individually cleaned by placing in 0.2 µm filtered and autoclaved seawater containing an antibiotic solution (Fisher #: BW17-745E) at 1ml/100ml and agitated with alcohol sterilized fine artist brushes 10 times, as described in previous studies (see Lee, 2011a and references therein). After June 2002, single foraminifers were placed inside a bleach-cleaned and alcohol-sterilized syringe-filter holder (Fisher # 09-753-10A) without a filter inside and pressure washed by forcing 20 ml of sterile seawater containing antibiotics ten times through the filter-holder unit. Typically, individual foraminifers were then crushed and approximately one third of the specimen was immediately placed in culture medium (see Chapter 2), while the remaining two thirds was preserved by freezing for DNA extraction at a later date.

DNA Extractions from Amphistegina *Hosts*

The Qiagen DNeasy® Plant Mini Kit was used with some modifications to extract DNA from the *A. gibbosa* specimens (Table 3.1). Initially, DNA extractions (and sequencing) utilized individual whole specimens as protocols were being developed. Then, DNA was extracted from half of the portion of each specimen remaining from the prior culture isolations studies, reserving the other one third of each specimen in case of mishap. Eventually, ten (or as many as available if less than ten, see Table 2.2 and 2.3) of these specimen portions were combined and used for a single DNA extraction. Ultimately, the data from individuals, portions of individuals, and from groups of the partial specimens all from the same sites and dates, were pooled for the final analysis. Thus, each DNA extraction (and sequences that followed) included the same specimens used in culture isolation studies (see Chapter 2), as well as some individual whole specimens used during the early stages.

For each extraction, an entire host specimen, or individuals or groups of one-third portion(s) thereof, was added to a sterile 1.5–2 ml centrifuge tube with conical bottom and screw caps with O-ring, then pulverized using an alcohol-washed, alcohol-flame sterilized, rounded-tip steel hex driver as a pestle. Approximately 300 µl of 1 mm heat sterilized glass beads (as described by Wawrik et al., 2003) and 400 µl of buffer AP1 (i.e., the lysis buffer) from the DNeasy® Plant Mini Kit were added to the microtubes and shaken vigorously for either 90 s in a Savant FastPrep Homogenizer, 3 min in a Biospec Min-Bead-Beater 8, or 15 min in the Scientific Industries Disruptor Genie. Modifications were made over the course of the study to adequately disrupt the cells of the foraminifers and diatoms for efficient DNA extraction. Steps 2 through 10 of the DNeasy® Plant Mini Kit were followed (product manual available at www.qiagen.com). The optional step in step 3 was included to remove the glass beads in

addition to any detergent, protein or polysaccharide precipitates from the DNA preparation. Steps 11 and 12 were modified to eluting three times in separate collection tubes with volumes of 50 µl, 100 µl, and 100 µl. Aliquots of the final 100 µl eluate were added to sterile, nuclease-free water (Promega) to make concentrations of 1/2, 1/5, 1/10, 1/100, and 1/1000 of the final eluate. These modifications were made to effectively elute DNA bound to the silica column and to dilute PCR inhibitors in the eluate.

DNA Extractions of Diatom Reference Cultures

The Qiagen DNeasy® Plant Mini Kit was also used with some modifications to extract the DNA from cultures of *Navicula viminoides, Nitzschia laevis,* and *Amphora sp*., provided by John J. Lee of City University of New York, to use as references for this study. The DNA extraction protocol for these algal cultures was identical to that used with the foraminiferal hosts, except that the algal cultures were not pulverized with the round-tipped hex driver and the algae were concentrated in microtubes by centrifugation.

To concentrate the algae in the growth media, the sides of the culture tubes were scraped with a sterile, disposable culture loop to dislodge diatom mats adhering to the sides of the tubes. The culture tubes were then shaken vigorously and 2 ml of the culture media was added to sterile 2 ml microtubes with screw caps. The microtubes were spun in a microcentrifuge for 2 min at maximum speed to pellet the diatoms previously suspended in the culture. If a visible pellet (>500 nm) was not formed, the top 95% of the liquid media was removed by pipetting and replaced by another 2 ml of media and centrifuged again at max speed for 2 min. If a pellet was visible, all of the liquid was removed by pipetting, leaving the pellet behind, otherwise the top 95% of the liquid was removed and remaining liquid used in the DNA extraction. Approximately

300 µl of 1 mm heat sterilized glass beads and 400 µl of buffer AP1 were added to the micotubes, and all other subsequent steps were the same as with the DNA extractions directly from the foraminiferal hosts, as described above.

Polymerase Chain Reaction (PCR) of DNA Extracts

Eight PCR reactions were done for each DNA extraction (i.e., the three elutions and the five dilutions of the final eluate 1/2, 1/5, 1/10, 1/100, and 1/1000). All PCR reactions were done with 12.5 µl of Promega GoTaq Green PCR Master Mix containing 0.5 µl of the forward and reverse primers with concentrations of 10 µM, 5 µl of DNA extract, and 6.5 µl Promega Nuclease-Free Water added to get to 25 µl. Previously designed primer sets for portions of two genes, the large subunit of the ribulose-1,5-bisphosphate carboxylase/ oxygenase[i.e., RuBisCO gene (*rbcL*)] and the small subunit of the ribosomal RNA gene (*18S*), were amplified by PCR and used for molecular identification in this study.

The *rbcL* primer set (forward primer, 5′-GATGATGARAAYATTAACTC-3′; reverse primer, 5′-ATTTGDCCACAGTGDATACCA-3′) amplified a 554 bp fragment of the large subunit of the RuBisCO gene (*rbcL*) and was adapted from Paul et al. (2000). This primer set was chosen because it was specific to diatoms, which were previously identified as the endosymbionts of *Amphistegina* through *in situ* cell ultrastructure using Transmission Electron Microscopy (e.g., Berthold, 1978; Leutenegger 1983, 1984) and other microalgae that could also be potential algal endosymbionts. This avoided amplifying the DNA of the hosts or non-diatom taxa such as contaminants, food particles, etc. The *rbcL* gene is also a conserved, protein-encoding gene, which allows unambiguous alignment at the amino acid level. Although this gene is conserved, it is less so than the *18S* gene, and it has greater resolving power and has been

shown to allow differentiation down to species level (Paul et al., 2000; Paul, 2001). The PCR conditions for generating the *rbcL* amplicons were: 2 min at 95°C; 50 cycles of 1 min at 95°C, 30 s at 50°C, and 45 s at 72°C; 5 min at 72°C, and hold at 4°C.

Primers for a portion of the small subunit of the ribosomal RNA gene (*18S*) were also used in this study (forward primer, 5′-AACCTGGTTGATCCTGCCAGT-3′; reverse primer, 5′-GATCCTTCTGCAGGTTCACCTA-3′). These primers were adapted from Medlin et al. (1988). The *18S* gene was used because it is one of the most commonly sequenced genes, thus having the largest data set available for comparison. The *18S* primers used are believed to be "universal" among eukaryotic organisms. Therefore, it amplifies not only DNA of potential diatom endosymbionts, but also the host's DNA and other eukaryotic microorganisms associated with the host. The PCR conditions for generating the *18S* amplicons were: 2 min at 95°C; 50 cycles of 1 min at 95°C, 30 s at 56°C, and 2 min at 72°C; 5 min at 72°C; and hold at 4°C.

PCR Purification

To verify the presence and correct size of PCR amplicons, 5 µl of all PCR products were run in 1% agarose gel with ethidium bromide and appropriate markers using an Owl Model A2 Gator Large Electrophoresis System. The gels were photographed on a UV illuminator using the Kodak Gel Logic 100 Molecular Imaging Systems. If primer-dimers were visible after electrophoresis, the remaining PCR products were run in a new gel and the targeted amplicons excised and purified using the Qiagen QIAquick Gel Purification Kit according to the manufacturer's directions. If no primer-dimers were visible, the PCR products were purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer's directions. However, the optional 35% guanidine HCL wash step was added as described in the manual to

remove residual primer-dimers not visible on the gel but that would readily ligate into vectors in subsequent cloning reactions.

Cloning of Purified PCR Products

The purified PCR products were ligated into the pCR®4-Topo vector and cloned by TOP10 Chemically Competent *E. coli* cells using the Invitrogen TOPO TA Cloning Kit for Sequencing with a few modifications of the manufacturer's directions. To reduce cost, the volumes of all reagents (except S.O.C. medium) were reduced to one third of what was described in the manufacturer's protocol. The amount of transformation media used to inoculate plates was then increased to three times the directed amount (i.e., from 10-50 µl to 30-150 µl). Additionally, three different volumes (usually 30, 90, and ~130 µl) of the transformation media were plated. These modifications still yielded hundreds of positive colonies, yet tripled the amount of cloning reactions possible.

Screening Clones by Direct PCR

Up to 96 clones per transformation were analyzed for the correct size PCR product inserts by direct PCR of the colonies. PCR primers (forward primer, 5′-CGCCAAGCTCAGAATTAACC-3′; reverse primer, 5′-TAAAACGACGGCCAGTGAAT-3′) for the vector regions flanking the inserted PCR product were designed using the Primer3 web-based program (Rozen and Skaletsky, 1999). These primers were also just upstream of the T3 and T7 sites on the vector for later sequencing of PCR products generated. PCR reactions were done in 25 µl solutions of Promega GoTaq Green PCR Master Mix containing 0.5 µl of each primer at a concentration of 10 µM and Promega Nuclease-Free Water added to get to 25 µl.

Colonies were gently touched with sterile pipette tips and gently swirled in PCR solution. The PCR conditions for amplifying the inserted PCR products were: 10 min at 94°C, 1 min at 95°C, 35 cycles of (1 min at 95°C, 30 s at 63°C, and 2 min at 72°C), 10 min at 72°C, and hold at 4°C. Then 2 µl of the PCR products were run on a 1% agarose gel with appropriate markers and checked for the correct size inserts as described previously.

Sequencing of Clones

PCR products of the correct size that were generated directly from clones were purified using the Eppendorf Perfectprep Cleanup 96 Kit, Promega Wizard SV 96 PCR Clean-Up Kit, or Qiagen Qiaquick PCR Purification Kit, and sent to Macrogen (Seoul, South Korea) or Polymorphic DNA Technologies, Inc. (Alameda, California, USA) for sequencing. Unpurified PCR products were also sent to Macrogen and Agencourt Bioscience Corporation (Beverly, Massachusetts, USA) for purification and sequencing. Since the *rbcL* amplicons were only 554 bp long and were flanked by the T3 and T7 primer sites, the T3 or T7 primers were used to sequence the entire amplicon in one extension. The 18S sequences were approximately 1800 bp, which usually could not be sequenced in a single extension. Therefore, the 18S forward PCR primer was used for sequencing as many bases as possible in a single run.

Post Sequence Editing

All sequence chromatograms files (ABI) were imported into an Invitrogen Vector NTI Advance 10 database. The program Invitrogen ContigExpress was used to trim all sequences of erroneous or ambiguous base calls, vector contamination, base calls with Phred scores less than 20, or sequences beyond 700 bp. Since the *rbcL* sequences were sequenced with vector primers

and the orientation of the inserted amplicons into the vector was random, the *rbcL* sequences were further analyzed and oriented.

BLAST Search and Comparison to Prior Studies

GenBank BLAST searches (highly similar sequences [megablast]) were done on trimmed *rbcL* sequences greater than 400 bp and *18S* sequences greater than 450 bp in length. Since previous *in hospite* TEM ultrastructural studies established that the symbionts within *Amphistegina* were diatoms (Leutenegger 1977, 1983, 1984; Berthold 1978), the BLAST search results were used to remove all non-diatom sequences from further analyses, particularly from the *18S* sequences where "universal" PCR primers were used. The most similar diatoms from the BLAST results for the queried sequences were compared between sites, dates and depths, and to diatoms cultured from the same species and general location in a previous study (Lee et al., 1995a).

Alignment and Phylogenetic Analysis of Sequences

The trimmed sequences (*rbcL* >400 bp and *18S* >450 bp, with Phred scores > 20) obtained from the hosts and from the reference cultures were aligned with other diatom *rbcL* sequences from GenBank using the MUSCLE program (Edgar, 2004) integrated in the MEGA version 7 phylogenetic software (Kumar et al., 2016) and with diatom *18S* sequences from the SILVA ribosomal RNA database using the online SINA alignment tool (Pruesse et al., 2012). The diatom sequences from GenBank and SILVA were chosen to include all species of diatoms previously reported as endosymbionts of foraminifers using culture isolations (e.g., Lee et al., 1979a, 1995a; Lee and Correia, 2005) and molecular methods (Holzmann et al., 2006; Schmidt

et al., 2015), as well as diatom endosymbionts in other organisms (Chesnick et al., 1996, 1997; McCoy, 2004). If sequences from those diatom species were not available in the databases, sequences from diatoms of the same genus were included if available. Several sequences from the genus *Bolidomonas* were chosen as outgroups because of their taxonomically close relationship to diatoms, and their use in previous studies as outgroups for diatoms (e.g., Theriot, 2010; Medlin, 2014; Guo et al. 2015). Some poorly aligned and aberrant sequences (<5% of data) were removed from the alignment. The alignments for all sequence data used throughout the dissertation are provided in FASTA form in supplemental material listed in Appendix for both the *rbcL* and *18S* data sets.

MEGA version 7 software (Kumar et al., 2016) was used for all phylogenetic analyses presented throughout the dissertation for its multiple functionality, speed, stability with large data sets, comparable results, and ease of use compared to more specialized program (e.g., Kumar et al., 2004; Tamura et al., 2007, 2011, 2013). The best fit substitution models and additional parameters for both data sets (*18S* and *rbcL*) were computed and used to construct neighbor-joining (NJ), maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) phylogenetic trees for each. Additionally, the NJ method was used to construct phylogenetic trees for various subsets of each data set in order to determine if there were any spatial and temporal patterns among the samples. The NJ method was chosen because of its speed and accuracy with large data sets of similar sequences (Saitou and Imanishi, 1989). Additionally, the veracity of the branches within these large NJ phylogenetic trees could be statistically tested using the bootstrapped method of the interior-branch test to calculate the confidence probability (CP) for each node, where CP>95% is considered statistically significant. This test of phylogeny is less affected by large numbers of sequences than the traditional

bootstrap statistical test, which become increasingly conservative with more sequences and thus mask true differences (Sitnikova et al., 1995; Sitnikova, 1996). Since the number of sequences affects the statistical analyses (Sitnikova et al., 1995; Sitnikova, 1996), the following comparison were made, using various smaller subsets of the *rbcL* and *18S* data:

1) upper Florida reef tract (Carysfort Reef 10 m, 25m samples) versus lower Florida reef tract (Looe Key 10 m, 25 m samples);

2) shallow (Carysfort Reef 10 m samples) versus deep (Carysfort Reef 75 m samples) sites; and

3) samples collected in the winter versus summer months.

The explanations for the abbreviated sequence data labels are provided in Table 3.1. In some of the phylogenetic trees, the diatom sequence data from DNA extracted directly from foraminiferal hosts are in green and those from species (or closely related groups of taxa) that were cultured and presumed to be endosymbionts are in red. Some sequence data were compressed into groups, which preserved the horizontal scale but the size of the compressed groups are proportional to the number of sequences (1 pixel per sequence). Poorly aligned and aberrant sequences were removed from the phylogenetic analyses.

Results

BLAST Search of 18s and rbcL Sequences

A total of 722 partial *rbcL* sequences (with lengths greater than 400 bp after trimming and Phred scores > 20) were obtained from amplifying, cloning, and sequencing DNA extracted directly from *Amphistegina gibbosa* specimens, which had been collected from 27 reef sites throughout the Florida reef tract, at depths from 6 m to 75 m. A total of 864 partial *18S*

sequences (with lengths greater than 450 bp after trimming) were obtained from 25 reef sites throughout the Florida Keys. BLAST search results of all the acceptable sequences showed that 721 of 722 *rbcL* sequences were from diatoms. A total of 480 *18S* diatom sequences were among the 864 sequences obtained using the non-specific *18S* PCR primers.

Over 99% of all the diatom sequences (both *rbcL* and *18S*) obtained directly from the foraminiferal hosts were most similar (97-99% BLAST search similarity) to the *rbcL* (accession # JX413559.1) or *18S* (accession # JX413542.1) sequences of an unnamed Fragilariaceae species (Figure 3.1) "isolated from some subtidal sand grains collected at the FSU marine station in October 2010" (personal discussions with Matt Ashworth and Chris Lobban, December 2015).

Only five sequences were most similar to diatoms not within the family Fragilariaceae (Table 3.1). Of those five sequences only one was alignable and suitable in the final analysis. This sequence, an *rbcL* sequence most similar to diatoms of the genus *Diploneis*, came from a specimen collected at a 50 m site at Tennessee Reef in May 2005. No seasonal or geographical differences were observed among the BLAST search results.

18S and rbcL Phylogeny of DNA extracted from Amphistegina gibbosa

Just like the BLAST search, the NJ phylogenetic analyses for both genes (*rbcL* and *18S*) showed that almost all of the diatom DNA extracted, amplified, and sequenced directly from the *A. gibbosa* hosts belong to a single and very specific group of diatoms within the family Fragilariaceae that is most similar to the aforementioned unnamed Fragilariaceae species isolated from sand grains (Figures 3.2-3.3). Of the few sequences (<5%), that fell outside this clade, all but one had to be removed from the phylogenetic analyses because they could not be properly aligned with the other sequences.

Among the many diatoms previously reported as endosymbionts of *Amphistegina*, *Nanofrustulum shiloi* was the closest taxonomically (Figures 3.4-3.5). However, it was not identical to any of the DNA extracted from *A. gibbosa* specimens from the Florida Keys and several other species were more closely related to diatom DNA extracted directly from the hosts. The unclassified Fragilariaceae species isolated from sand grains that was most similar in the BLAST searches and several others from the Fragilariaceae group were more closely related to the diatom DNA directly from the hosts than was *N. shiloi*.

The sequences obtained from the three reference cultures of diatoms received from Prof. Lee, originally identified as *Nitzschia laevis*, *Navicula viminoides*, and *Amphora* sp., clustered with previously identified diatom taxa from culture studies. In Figure 3.2, the *Amphora* sequences are shown, while the *Nitzschia laevis and Navicula viminoides* sequences are included in compressed sequence data sets under their respective genera. Data for the latter species does appear in the unabridged trees in the supplemental files described in the Appendix.

Within the large group of diatom sequences directly from the *A. gibbosa* specimens there were several clusters slightly different from the majority of the other sequences. The most consistent clusters among all the different phylogenetic methods were many diatom sequences from *A. gibbosa* specimens collected from deepest site, Carysfort Reef 75 m. The difference was not significant (CP < 95%) within the large trees, but when the number of sequences was reduced to compare just diatom sequences from Carysfort Reef 75 m to sequences from specimens collected at Carysfort Reef 10 m, the differences were statistically significant based on the bootstrapped method of the interior-branch test (Figure 3.6). There were no significant differences between the diatom sequences from the upper versus lower Florida reef tract (Figure 3.7) nor within hosts collected in the summer versus winter months (Figure 3.8).

The ML, ME, and MP phylogenetic analyses of the entire data sets for both genes (*rbcL* and *18S*) yielded results consistent with those from the NJ method (Figures 3.9-3.10).

A small number of poorly aligned and aberrant sequences (less than 5% of each data set) were removed from the phylogenetic analyses. These sequences often contained portions of the cloning vectors, misreads, chimeras, or multiple signals that were not detected and removed by the Vector NTI software. Such "bad" sequences were the cause of almost all of the contrary results in the BLAST searches and phylogenetic analyses.

Discussion

The Forgotten TEM Studies of Symbiosis in Amphistegina

Since the late 1970s, the overwhelming majority of publications on presumed endosymbionts within the larger foraminifers have come from studies that utilized culture techniques (e.g., Lee et al., 1979a, 1980a, 1995a, Lee and Correia, 2005; Lee, 2011a, b; see also Table 1.1). A few early ultrastructural studies utilizing TEM demonstrated that the endosymbionts *in hospite* were indeed diatoms (Leutenegger 1977, 1983, 1984; Berthold 1978), though identifications were not possible without frustules, which were the primary means of classification at that time. Lee et al. (1979a, b) addressed this problem using culture methods, observing that, in culture, the diatoms developed frustules and could be identified. Over the past 40 years, such culture methods have been used to "discover" more than two dozen species of diatoms isolated from *Amphistegina* and other larger foraminifers that host diatom endosymbionts (e.g., Lee, 2011a, b, and references therein).

In the early 1980s, Leutenegger (1983, 1984) published evidence for the specificity and stability of host-algal relationships in several species of larger foraminifers, including several species of *Amphistegina*, though not *A. gibbosa*, the species examined in this study. Leutenegger (1983, 1984) utilized TEM to compare the ultrastructure of the diatom cells *in hospite* with cultures of diatoms she received from John J. Lee, which had been isolated from the hosts. She observed a very consistent host-symbiont relationship within individual species of *Amphistegina* that did not change with depth, season, or locations. Moreover, she noted that the endosymbionts within the hosts of two Indo-Pacific species of *Amphistegina* shared internal characteristics with only one of the diatoms, *Fragilaria shiloi* (now *Nanofrustulum shiloi*) cultured from hosts, and all others were different. Leutenegger's observations and its conclusions have subsequently been largely ignored.

My results, based upon both the partial *18S* and *rbcL* genes, unequivocally demonstrated that the diatoms extracted from *A. gibbosa* specimens from sites throughout the Florida reef tract, were nearly identical across depths, seasons and years. Furthermore, of all the diatom species cultured from *Amphistegina* spp. worldwide (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a, Lee and Correia, 2005; Lee et al., 2011a), DNA from *Nanofrustulum shiloi* is most similar (92-95% BLAST search similarity) to the DNA extracted directly from the *A. gibbosa* specimens. While my results are consistent with Leutenegger's (1983, 1984) observations, the molecular findings expand upon Leutenegger's observations by clearly showing that, although based on the *18S* and *rbcL* phylogeny *N. shiloi* is closely related to the "true" endosymbiont of *A. gibbosa*, they are not the same. The *18S* and *rbcL* sequences in GenBank from *N. shiloi* were not the most similar to the diatom sequences extracted directly from *A. gibbosa*, as revealed by the BLAST search.

Additionally, the sequences from *N. shiloi* never fell within, nor formed an immediate sister group to, the large group of sequences isolated from *A. gibbosa* hosts in any of the phylogenetic trees, which were constructed using both *18 S* and *rbcl* genes and three different statistical tests. Of the sequences in GenBank of the >20 species of diatoms previously reported as symbionts based on culture studies (or members of their genera where sequences of a species were not available in GenBank), none fell within the large group formed by *18S* or *rbcL* sequences isolated directly from the *A. gibbosa* specimens from the Florida reef tract. The uniformity of the diatom sequences (based on BLAST search) obtained directly from *A. gibbosa* was markedly different from the diverse assemblage of diatoms reported by Lee et al. (1995a) based upon cultures isolated from *A. gibbosa* from the Florida reef tract (Figure 3.1). My results provide very strong evidence that the "true" diatom endosymbiont of *A. gibbosa* is a single species (perhaps with some intra-specific variability) and is not among the species previously reported as diatom endosymbionts of *Amphistegina* based on culture studies (e.g., Lee et al., 1979a, 1980a, 1995a, Lee, 2011a; see also Table 1.1).

Rather, my findings strongly support those of Leutenegger (1983, 1984), who also observed a very consistent host-symbiont relationship within individual species of *Amphistegina* that did not change spatially or temporally. Moreover, she noted that the endosymbionts within two Indo-Pacific species of *Amphistegina* shared internal characteristic only with *Fragilaria shiloi* (né *N. shiloi*) cultured from hosts, while all other diatoms identified from cultures were ultrastructurally distinct from the *in hospite* diatoms.

The Identity of the "True" Diatom Endosymbionts of Amphistegina gibbosa

Communications with Matt P. Ashworth and Chris Lobban (December 2015), the researchers who submitted the sequences to GenBank that were most similar to 98% of the *18S* and 99% of the *rbcL* sequences isolated from *A. gibbosa* in this study, have yielded some interesting new insights. The unclassified diatom DNA labeled Fragilariaceae sp. (Accession # JX413542.1 for *18S* and JX413559.1 for *rbcL*) were "isolated from some subtidal sand grains collected at the FSU marine station in October 2010 . . . and presumed to be free-living" (personal communication with Matt P. Ashworth, December 2015). Furthermore, Ashworth has isolated the strain in active culture and provided SEM images of the small, unclassified pennate diatom, tentatively being referred to by member of his group as "staurosiroids." Further clarification and communication with Drs. Ashworth and Lobban will determine if their diatom sequences from the subtidal sands and the diatom isolated in culture are in fact the same and from free living diatoms on the sand grain or were diatoms endosymbionts of "living sands" (e.g., Lee, 1995, 1998, 2002; Lee et al., 1995b), which were mistaken for sand grains.

Intraspecific Spatial and Depth Trends in Diatom Endosymbiont Assemblages

A small, but significant, difference was observed between endosymbiont sequences obtained from *A. gibbosa* specimens from shallower (<50 m) sites compared to the deepest site (75 m) off Carysfort Reef. Most of the deeper-water endosymbionts clustered within subgroups of the larger groups of both the *18S* sequences and *rbcL* sequences. This separation was much more pronounced when comparing smaller subsets of the data. Holzmann et al. (2006) similarly observed a small but significant depth trend in the diatom endosymbionts of nummulitid foraminifers. These observations indicate that there may be some intra-specific (or intra-generic)

variations among the symbionts with depth, but nothing remotely close to the variability previously reported from culture isolation studies.

The only "other" non-aberrant (and non-Fragilariaceae) diatom sequence isolated directly from a host specimen also came from a 50 m site. This sequence was most similar to *rbcL* sequences from diatoms of the genus *Diploneis*. Lee et al. (1995a) isolated diatoms of this genus from 8 of 50 *Amphistegina* specimens collected in the Florida Keys. There is some possibility that *Diploneis* could be a rare, deeper-water endosymbiont, but there is a more likely explanation. The *A. gibbosa* specimens from the deeper sites were much smaller than typical adult specimens from the shallow sites, which made them harder to clean, thereby increasing the likelihood of sequencing a contaminant. In addition, more sequences per foraminifer were obtained from the DNA extracts of these deeper-water specimens because fewer deep-water samples were available. Therefore, I sequenced more clones from each deeper-water sample than from the readily available shallow-water samples. Since a higher percentage of their clones were sequenced, this increased the probability of sequencing stray diatoms that were epiphytic on, or preyed upon by, the host. In essence, the few deep-water *A. gibbosa* specimens available were both dirtier and inspected more thoroughly. Hence, stray non-endosymbiotic diatoms adhering to, or recently consumed by, the host were more likely to have been sequenced.

Implications of a Specific Diatom Symbiont within Amphistegina *on Past and Future Studies*

Amphistegina gibbosa are among the larger benthic foraminifers used as bioindicators of stressors influencing coral reefs (Hallock, 2000a, 2012; Hallock et al., 2003). Such studies into the physiological responses of *Amphistegina* to various stresses have provided insights into why corals and other keystone reef organisms are influenced by variations in physical and chemical

factors such as temperature, pH, contaminants, and water transparency. A key concern regarding the reliability of *Amphistegina* spp. as bioindicator was the potential that specimens at different sites or times harbored different symbionts that could potentially respond differently. My findings, that both resurrect the importance of the observations of Leutenegger (1983, 1984) and document the specificity of the diatom endosymbionts of *A. gibbosa*, bolster confidence in the usefulness of *Amphistegina* as bioindicators and minimize the possibly that bleaching and other physiological responses observed in their populations could be related to temporal or spatial differences in symbiont populations (Hallock et al., 1995; Talge and Hallock, 2003; Williams and Hallock, 2004).

Sequences of "Other" Organisms Isolated from Amphistegina gibbosa

The algal-specific *rbcL* primers used to isolate sequences from *A. gibbosa* specimens yielded only diatoms. However, about 45% of the sequences obtained using the "universal" *18S* primers were from non-diatom organisms associated with *A. gibbosa* (possibly parasites, epiphytes, food particles, etc.) or potentially even from the host, whose 18S sequences to date have yet to be published. The non-diatom sequences were largely excluded from detailed analyses mainly because this study focused on the symbionts, which were already determined *in situ* by ultrastructure studies to be diatoms (Leutenegger 1977, 1983, 1984; Berthold 1978).

Furthermore, finding the DNA of *A. gibbosa* would be like searching for a needle, or more appropriately a few needles, in a haystack. Since *Amphistegina* and other foraminifers are unicellular but often multinucleate (McEnery and Lee, 1981), there are only a few copies of each gene for the host compared to the many hundreds to thousands of copies for the diatom endosymbionts (e.g., Talge and Hallock, 2003) within the cell of a host specimen. Nevertheless,

BLAST searches were done on the "other" sequences and these sequences were used to build simple broad phylogenetic trees using a phylum-level variety of other taxa and the available foraminiferal sequences available in GenBank, such as species of the genus *Ammonia*, the closest morphological relative to *Amphistegina* that have *18S* sequences in GenBank. None of these sequences formed definitive foraminiferal groups or clusters with the *Ammonia* sequences. However, the "other" non-diatom sequences associated with *Amphistegina gibbosa* are worthy of further examination in a future study.

Table 3.1: BLAST search results showing the most similar diatom groups (genus or family level) to the rbcL and 18S sequences obtained from DNA extracted directly from *Amphistegina gibbosa* collected from various sites and depths in the Florida reef tract between December 1999 and July 2005. Diatoms isolated in culture from Florida reef-tract specimens Lee et al. (1995a) in an earlier study were added for comparison. The results only include diatom sequences with Phred values greater than 20 and lengths greater than 400 bp for *rbcL* and 450 bp for *18S*. The data for sequences obtained from DNA extracted from individual foraminifers at the same sites were combined. *The group Fragilariaceae sp. contains several unclassified Fragilariaceae sp. along with the genera *Staurosira*, *Opephora*, and *Nanofrustulum*.

Sequence Labels on Figures 1-6	Location	Site Name	Date of Collection	Collection Depth (m)	Total # Sequences	%Fragilaria-ceae sp.*	%*Diploneis* sp.	%*Navicula* sp.	%*Cocconeis* sp.	%*Nitzschia* sp.
3sis6m022003	Upper Keys	Site 2: SW Three Sisters	February 2003	6	23	100.0%	0.0%	0.0%	0.0%	0.0%
3sis6m052005	Upper Keys	Site 2: SW Three Sisters	May 2005	6	16	100.0%	0.0%	0.0%	0.0%	0.0%
algae6m052005	Upper Keys	Site 6: Algae Reef	May 2005	6	15	100.0%	0.0%	0.0%	0.0%	0.0%
algae6m072004	Upper Keys	Site 6: Algae Reef	July 2004	6	4	100.0%	0.0%	0.0%	0.0%	0.0%
algae6m072005	Upper Keys	Site 6: Algae Reef	July 2005	6	24	100.0%	0.0%	0.0%	0.0%	0.0%
cary10m072005	Upper Keys	Carysfort Reef 10m	July 2005	10	41	100.0%	0.0%	0.0%	0.0%	0.0%
cary10m052005	Upper Keys	Carysfort Reef 10m	May 2005	10	58	98.3%	0.0%	1.7%	0.0%	0.0%
cary25m052005	Upper Keys	Carysfort Reef 25m	May 2005	25	50	100.0%	0.0%	0.0%	0.0%	0.0%
cary50m052005	Upper Keys	Carysfort Reef 50m	May 2005	50	77	98.7%	0.0%	1.3%	0.0%	0.0%
cary50m092004	Upper Keys	Carysfort Reef 50m	September 2004	50	39	100.0%	0.0%	0.0%	0.0%	0.0%
cary75m052005	Upper Keys	Carysfort Reef 75m	May 2005	75	104	98.1%	0.0%	1.9%	0.0%	0.0%
conch10m072004	Middle Keys	Conch Reef 10m	July 2004	10	5	100.0%	0.0%	0.0%	0.0%	0.0%
conch20m072004	Middle Keys	Conch Reef 20m	July 2004	20	12	100.0%	0.0%	0.0%	0.0%	0.0%
conch30m121999	Middle Keys	Conch Reef 30m	December 1999	30	24	100.0%	0.0%	0.0%	0.0%	0.0%
kl9m072004	Upper Keys	Site 3: Between Molasses and Pickels	July 2004	9	10	100.0%	0.0%	0.0%	0.0%	0.0%

Table 3.1 (Continued)

Sequence Labels on Figures 1-6	Location	Site Name	Date of Collection	Collection Depth (m)	Total # Sequences	%Fragilaria-ceae sp.*	%*Diploneis* sp.	%*Navicula* sp.	%*Cocconeis* sp.	%*Nitzschia* sp.
kl9m082002	Upper Keys	Site 3: Between Molasses and Pickels	August 2002	9	1	100.0%	0.0%	0.0%	0.0%	0.0%
lk6m052005	Lower Keys	Looe Key 6m	May 2005	6	29	100.0%	0.0%	0.0%	0.0%	0.0%
lk10m052005	Lower Keys	Looe Key 10m	May 2005	10	58	100.0%	0.0%	0.0%	0.0%	0.0%
lk14m062005	Lower Keys	Looe Key 14m	June 2005	14	30	100.0%	0.0%	0.0%	0.0%	0.0%
lk25m052005	Lower Keys	Looe Key 25m	May 2005	25	59	100.0%	0.0%	0.0%	0.0%	0.0%
mr12m072005	Upper Keys	Molasses Reef 12m	July 2005	12	38	100.0%	0.0%	0.0%	0.0%	0.0%
mr18m022003	Upper Keys	Site 4: SW Molasses	February 2003	18	24	100.0%	0.0%	0.0%	0.0%	0.0%
mr25m05200518s	Upper Keys	Molasses Reef 25	May 2005	25	17	100.0%	0.0%	0.0%	0.0%	0.0%
mr27m072005	Upper Keys	Molasses Reef 27m	July 2005	27	48	100.0%	0.0%	0.0%	0.0%	0.0%
mr50m052005	Upper Keys	Molasses Reef 50m	May 2005	50	63	98.4%	0.0%	1.6%	0.0%	0.0%
tn6m052005	Middle Keys	Tennessee Reef 10m	May 2005	10	55	100.0%	0.0%	0.0%	0.0%	0.0%
tn10m052005	Middle Keys	Tennessee Reef 10m	July 2005	10	10	100.0%	0.0%	0.0%	0.0%	0.0%
tn10m072005	Middle Keys	Tennessee Reef 25m	May 2005	25	56	100.0%	0.0%	0.0%	0.0%	0.0%
tn27m072005	Middle Keys	Tennessee Reef 27m	July 2005	27	30	96.7%	0.0%	3.3%	0.0%	0.0%
tn30m052003	Middle Keys	Tennessee Reef 30m	May 2003	30	43	100.0%	0.0%	0.0%	0.0%	0.0%
tn30m072004	Middle Keys	Tennessee Reef 30m	July 2004	30	9	100.0%	0.0%	0.0%	0.0%	0.0%
tn50m052005	Middle Keys	Tennessee Reef 50m	May 2005	50	29	96.6%	3.4%	0.0%	0.0%	0.0%

Table 3.1 (Continued)

Sequence Labels on Figures 1-6	Location	Site Name	Date of Collection	Collection Depth (m)	Total # Sequences	%Fragilaria-ceae sp.*	%*Diploneis* sp.	%*Navicula* sp.	%*Cocconeis* sp.	%*Nitzschia* sp.
tn6m052005	Middle Keys	Tennessee Reef 6m	May 2005	6	15	100.0%	0.0%	0.0%	0.0%	0.0%
tn8m121999	Middle Keys	Tennessee Reef 8m	December 1999	8	40	100.0%	0.0%	0.0%	0.0%	0.0%
wb6m022003	Upper Keys	Site 5: White Bank Dry Rocks	February 2003	6	24	100.0%	0.0%	0.0%	0.0%	0.0%
wb6m072004	Upper Keys	Site 5: White Bank Dry Rocks	July 2004	6	21	95.2%	0.0%	4.8%	0.0%	0.0%
				Totals	1201	99.4%	0.1%	0.5%	0.0%	0.0%
Sequences from Reference Diatom Cultures from Prof. John J. Lee					Total # Sequences					
Nitzschia laevis					8					100.0%
Navicula viminoides					3			100.0%		
Amphora sp.					2	100.0% *Amphora* sp.				
Cultured Diatoms Identified by Microscopy					#Culture Isolation	%Fragilaria-ceae sp.*	%*Diploneis* sp.	%*Navicula* sp.	%*Cocconeis* sp.	%*Nitzschia* sp.
Diatoms isolated in culture from *A. gibbosa* from the Florida Keys (Lee et al. 1995a)					50	6.6%	16.0%	22.0%	8.0%	54.0%

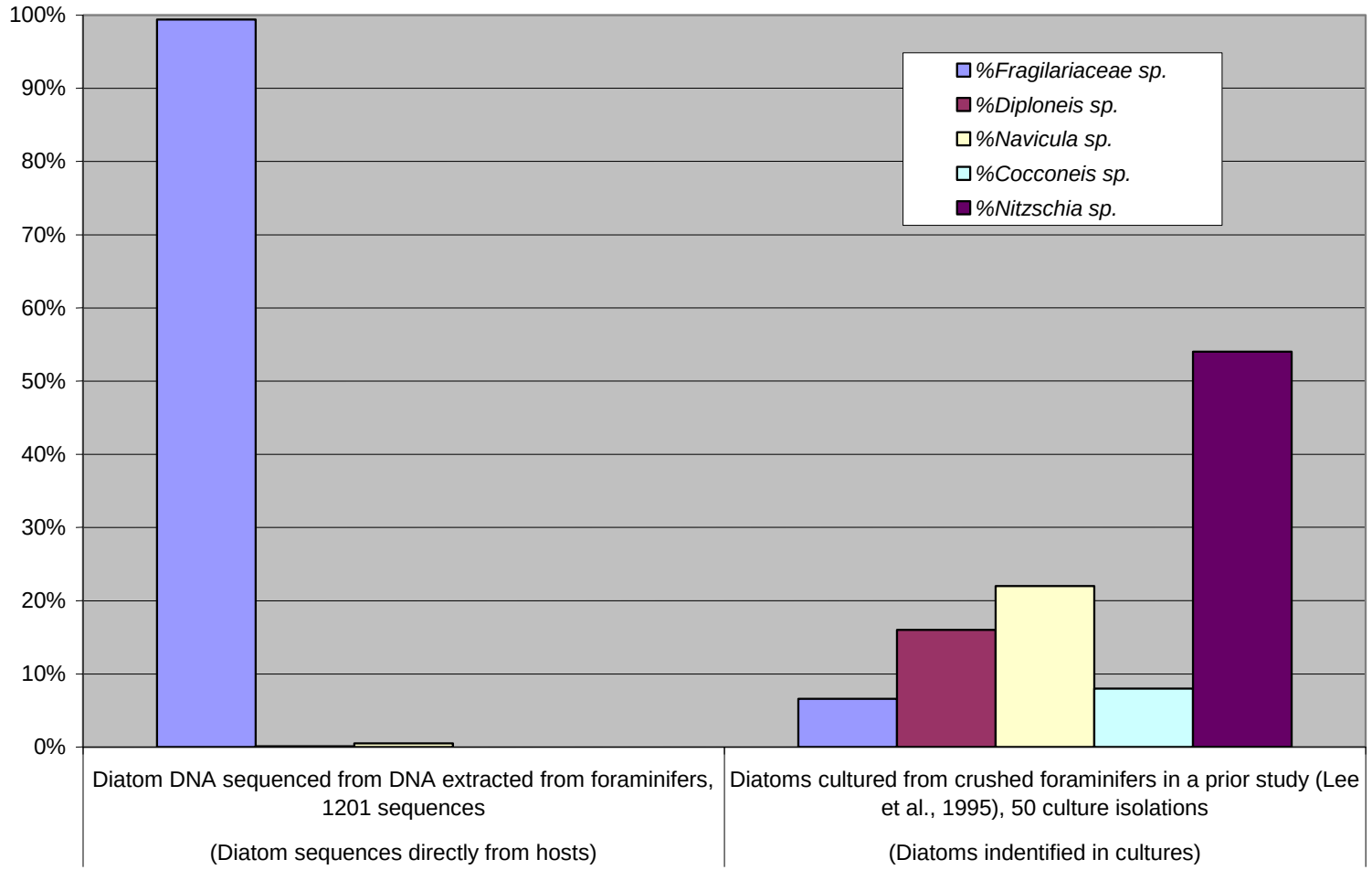


Figure 3.1: A comparison of diatom sequences obtained from DNA extracted directly from Amphistegina gibbosa collected from various sites and depths in the Florida reef tract to morphological identifications of diatoms isolated in culture by Lee et al. (1995a).

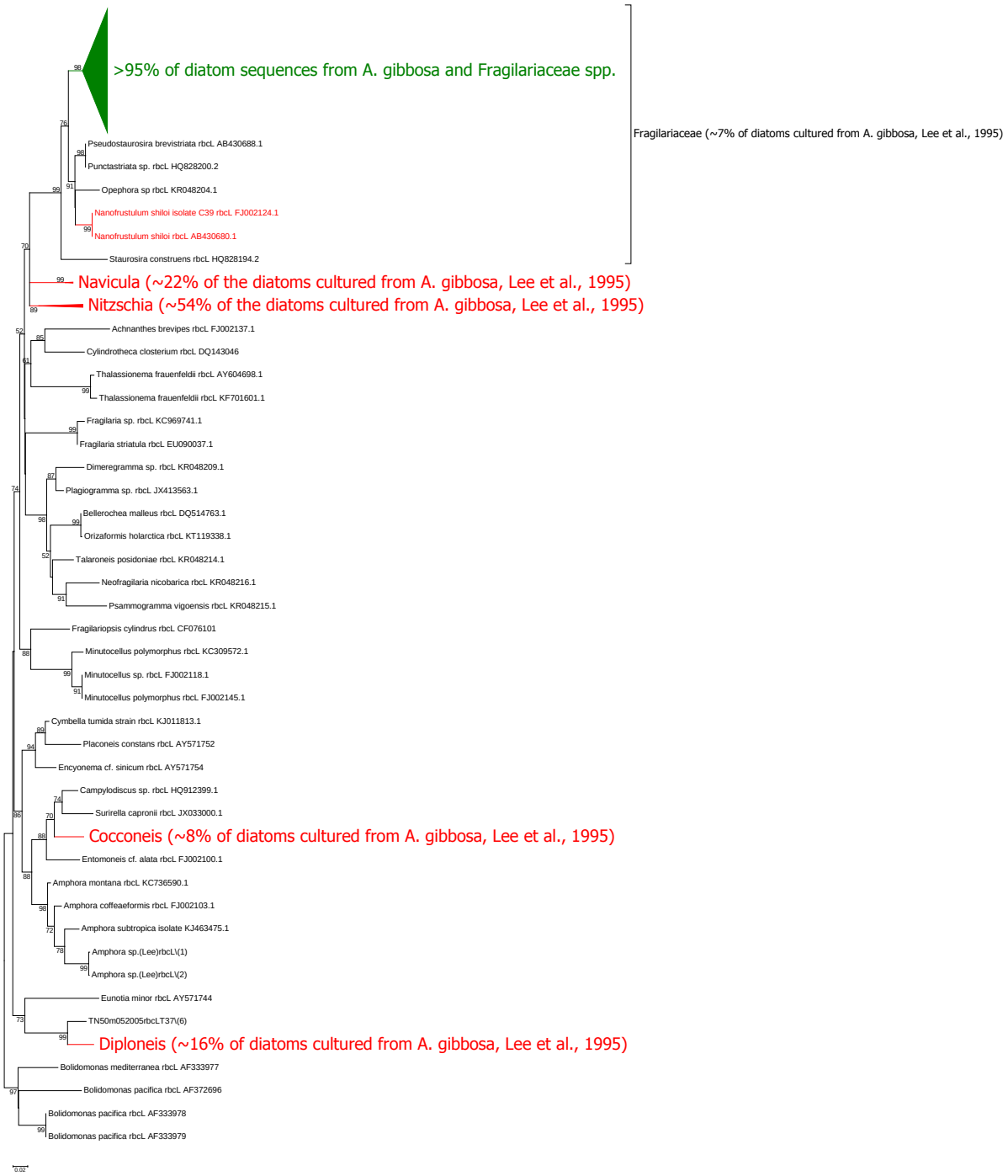


Figure 3.2: The *rbcL* phylogeny (Neighbor-Joining method) of diatom DNA extracted directly from *A. gibbosa* specimens (in green) collected throughout the Florida reef tract compared to species or genera of diatoms isolated in cultures (in red) in earlier study (Lee et al., 1995a). Phylogenetic analyses were conducted using MEGA7 (Kumar, 2016). The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the best fit model (Tamura 3-parameter method) available for the Neighbor-Joining method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.4054). The analysis involved 712 nucleotide sequences from the hosts and 56 references. The bold green and red branches are multiple sequences compressed into groups. The full tree is available in Supplemental Material as described in the Appendix.

Figure 3.3: The *18S* NJ tree of diatom DNA extracted directly from *A. gibbosa* specimens (in green) collected throughout the Florida reef tract compared to diatoms isolated in cultures (in red) from earlier study (Lee et al., 1995a). Phylogenetic analyses done with MEGA7 (Kumar, 2016). The confidence probability that the interior branch length is greater than 0 (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the best fit model (Tamura 3-parameter method) available for the Neighbor-Joining method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.6556). The analysis involved 448 nucleotide sequences from the hosts and 72 references. The bold green and red branches are multiple sequences compressed into groups. The full tree is available in Supplemental Material as described in the Appendix.

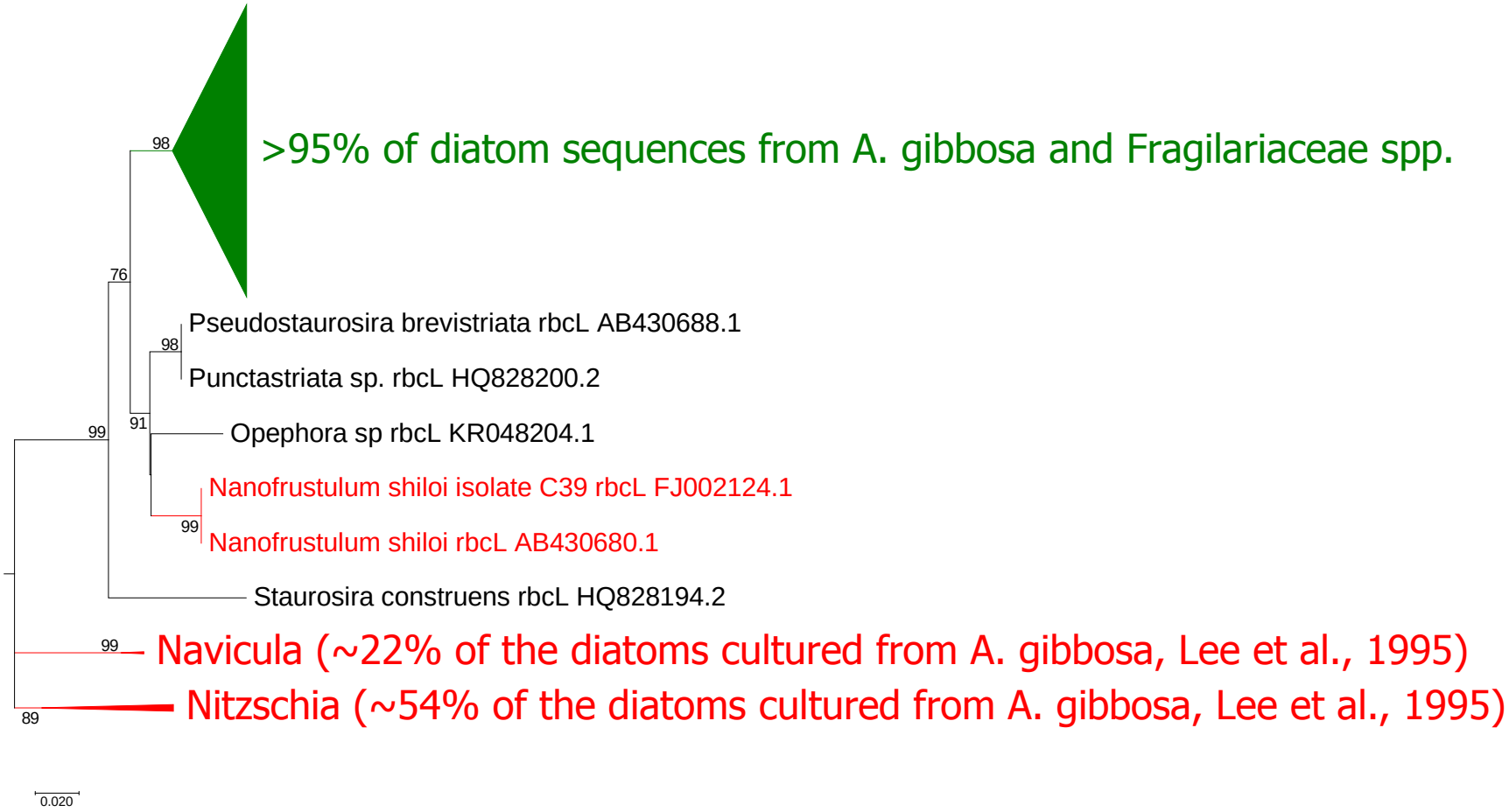


Figure 3.4: A close-up of the *rbcL* phylogeny (Figure 3.2) of diatom DNA extracted directly from *A. gibbosa* specimens (in green) collected throughout the Florida reef tract. Among the diatoms previously reported as endosymbionts, *Nanofrustulum shiloi* was the nearest relative. The full tree is available in Supplemental Material as described in the Appendix.

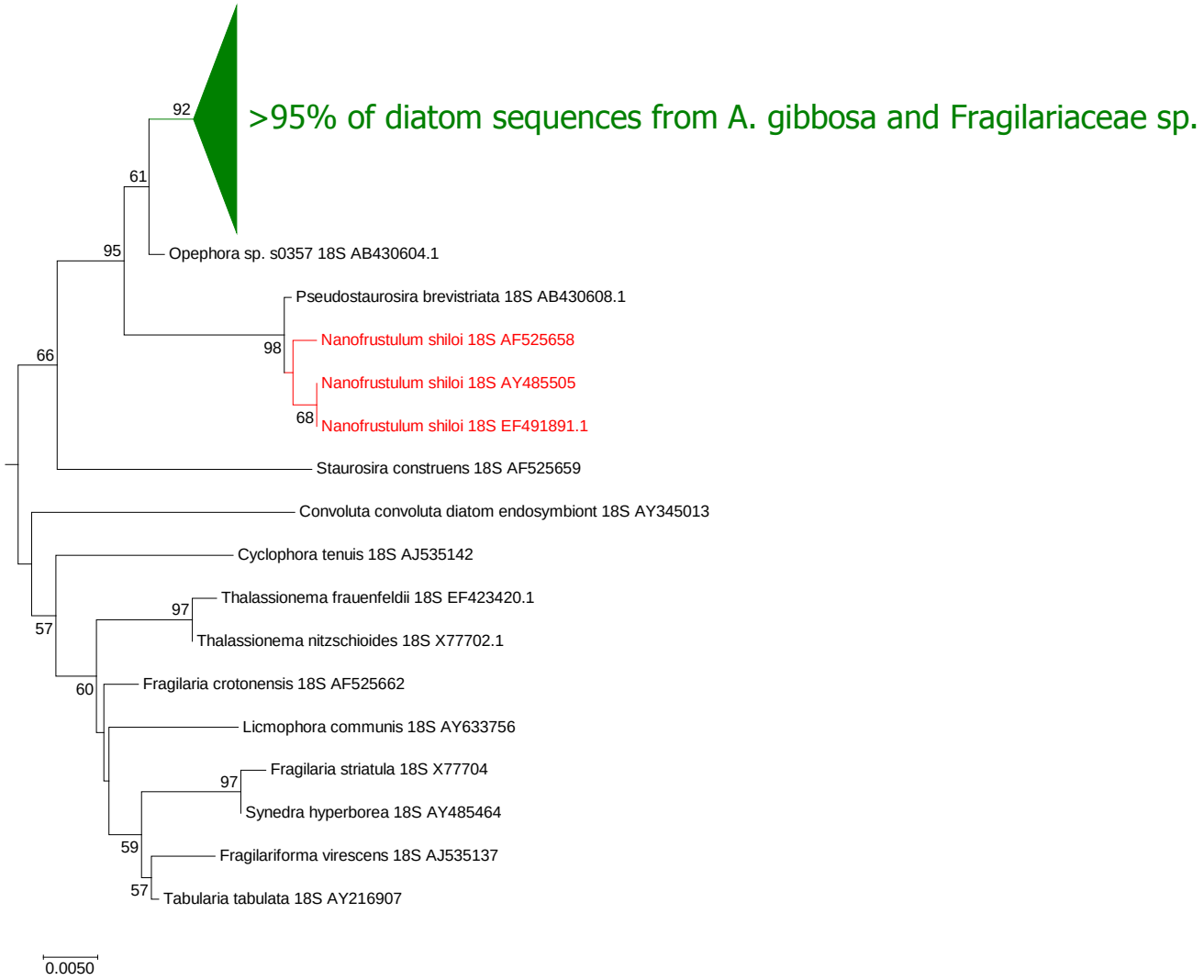


Figure 3.5: A close-up of the *18S* phylogeny (Figure 3.3) of diatom DNA extracted directly from *A. gibbosa* specimens (in green) collected throughout the Florida reef tract. Among the diatoms previously reported as endosymbionts, *Nanofrustulum shiloi* was the nearest relative. The full tree is available in Supplemental Material as described in the Appendix.

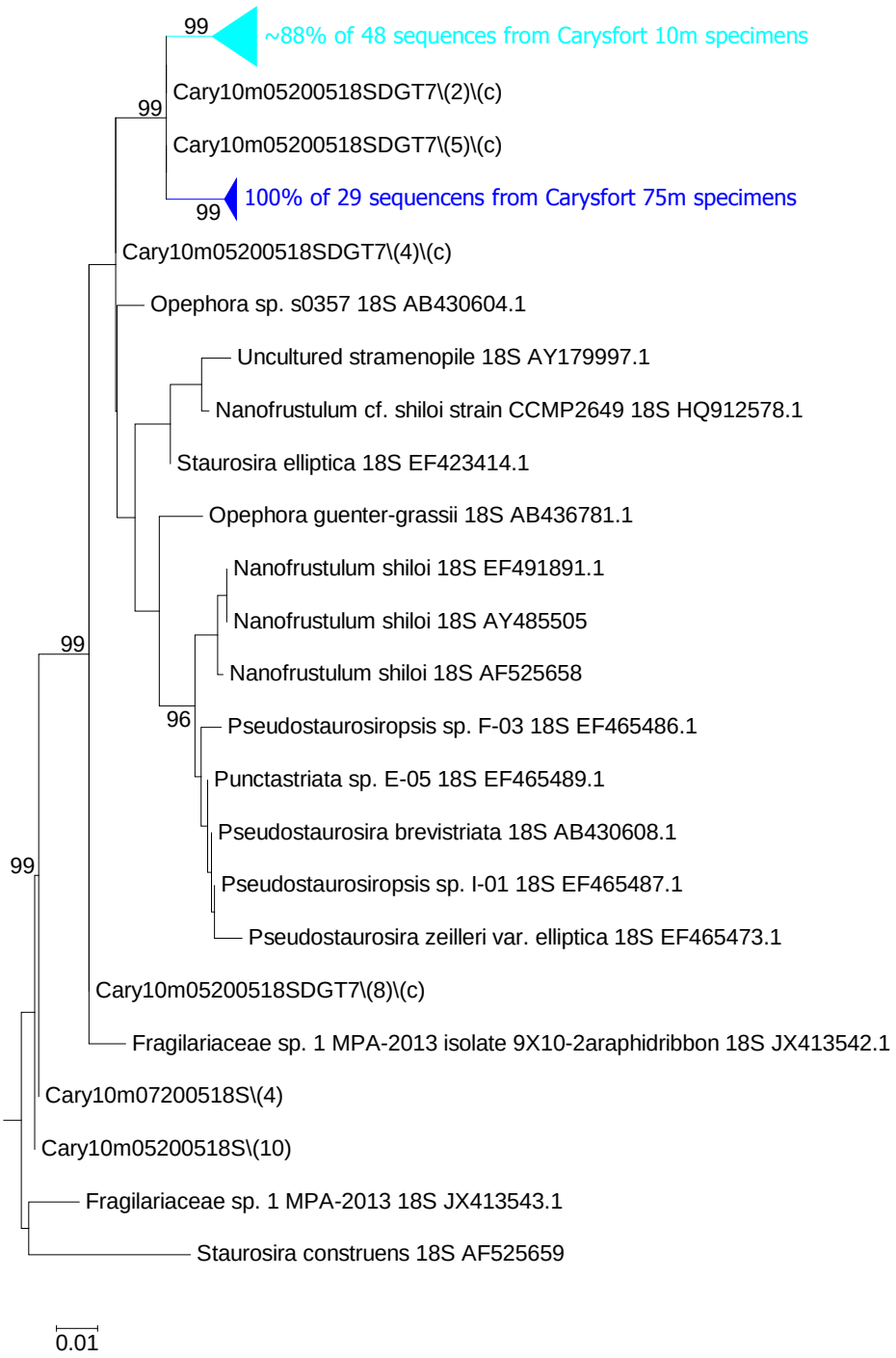


Figure 3.6: The 18S phylogeny of diatom sequences from *A. gibbosa* specimens collected at the deepest site (Carysfort 75m) compared to a shallower site in the same location (Carysfort 10m). All the sequence from hosts from the deepest site and most (88%) from the shallower site formed distinct subclades supported by high confidence probabilities.

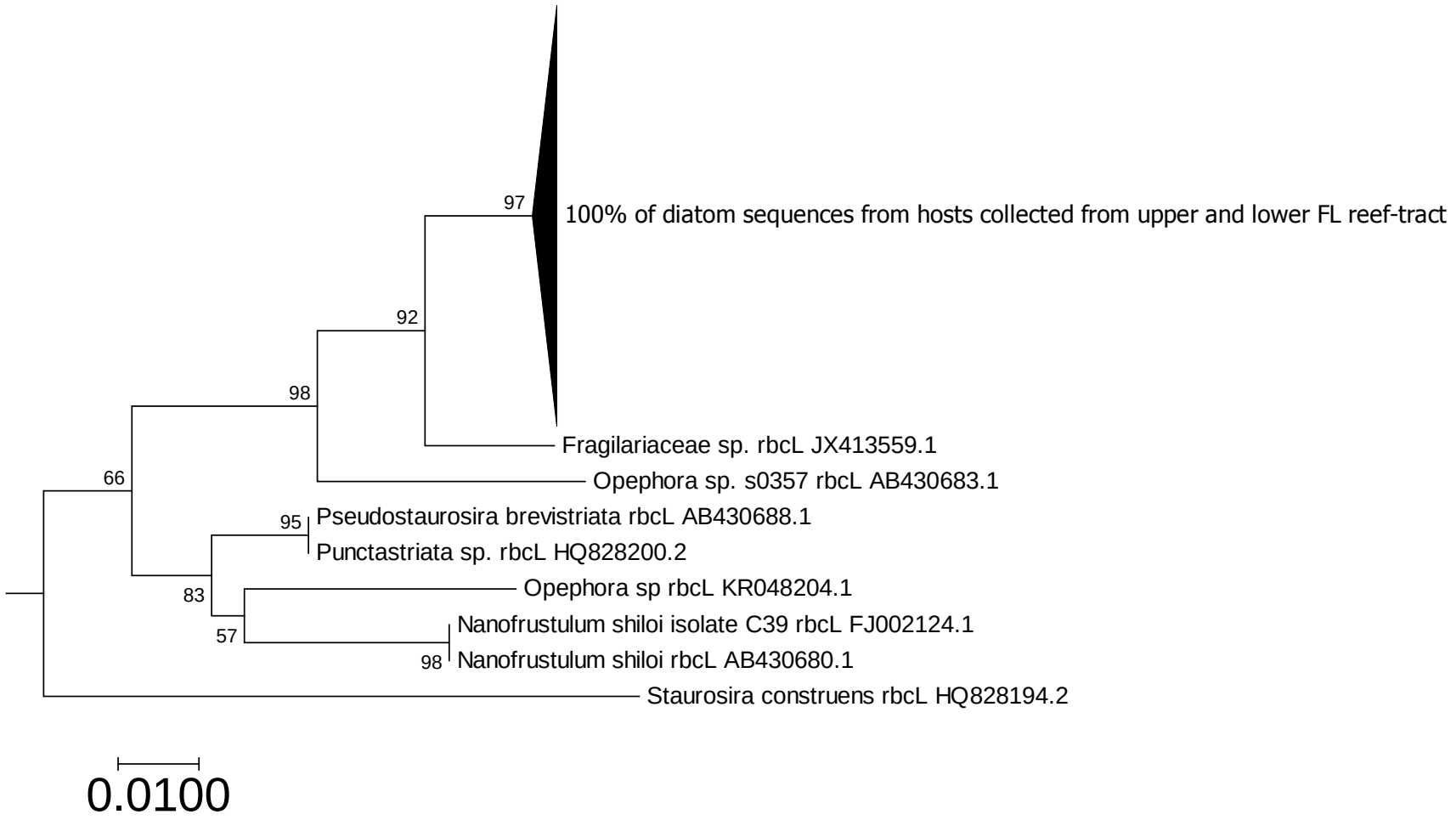


Figure 3.7: Comparison of diatom sequences from hosts collected in upper and lower FL Keys. The full tree is available in Supplemental Material as described in the Appendix.

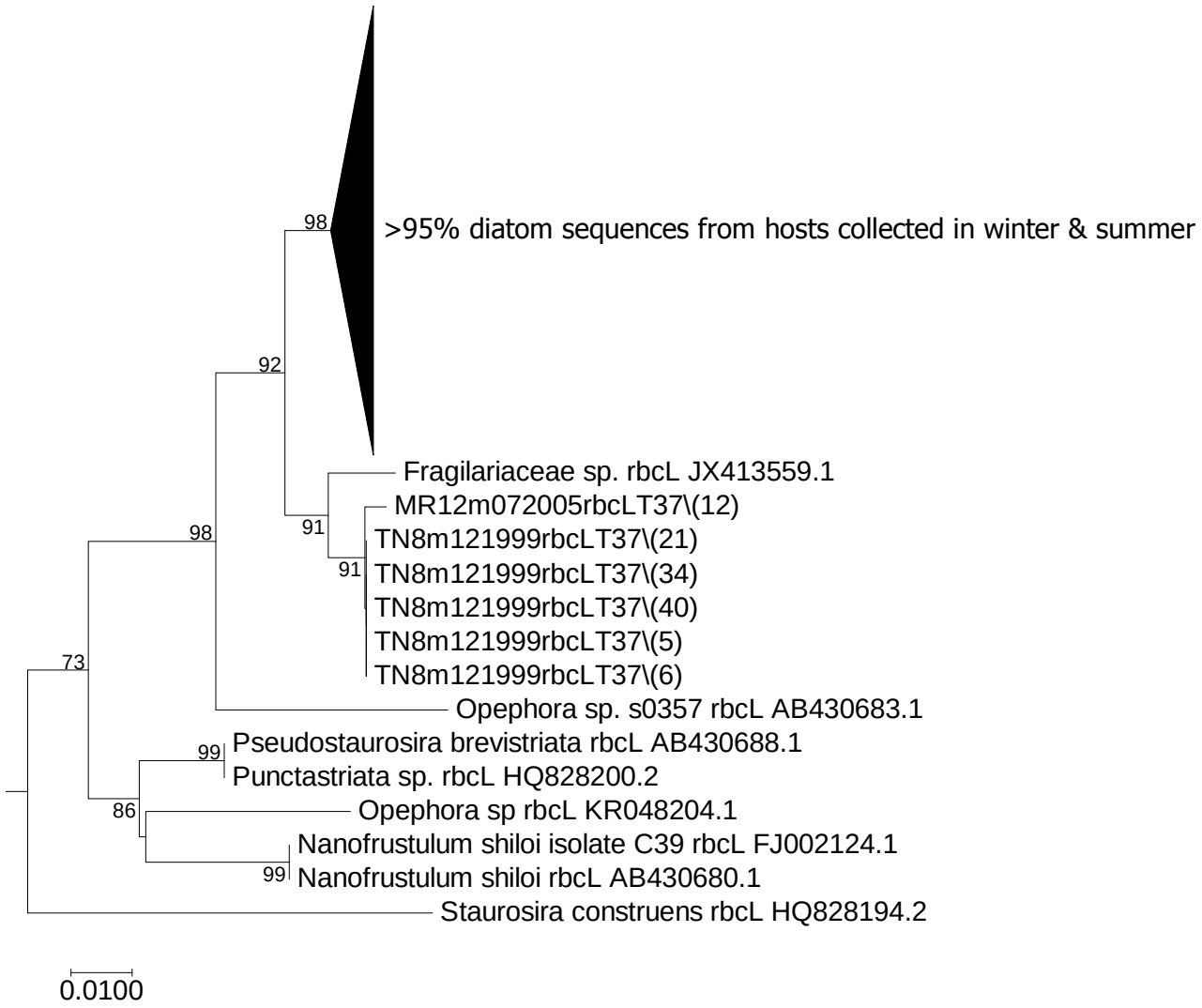


Figure 3.8: Comparison of diatom sequences from hosts collected in winter and summer. The full tree is available in Supplemental Material.

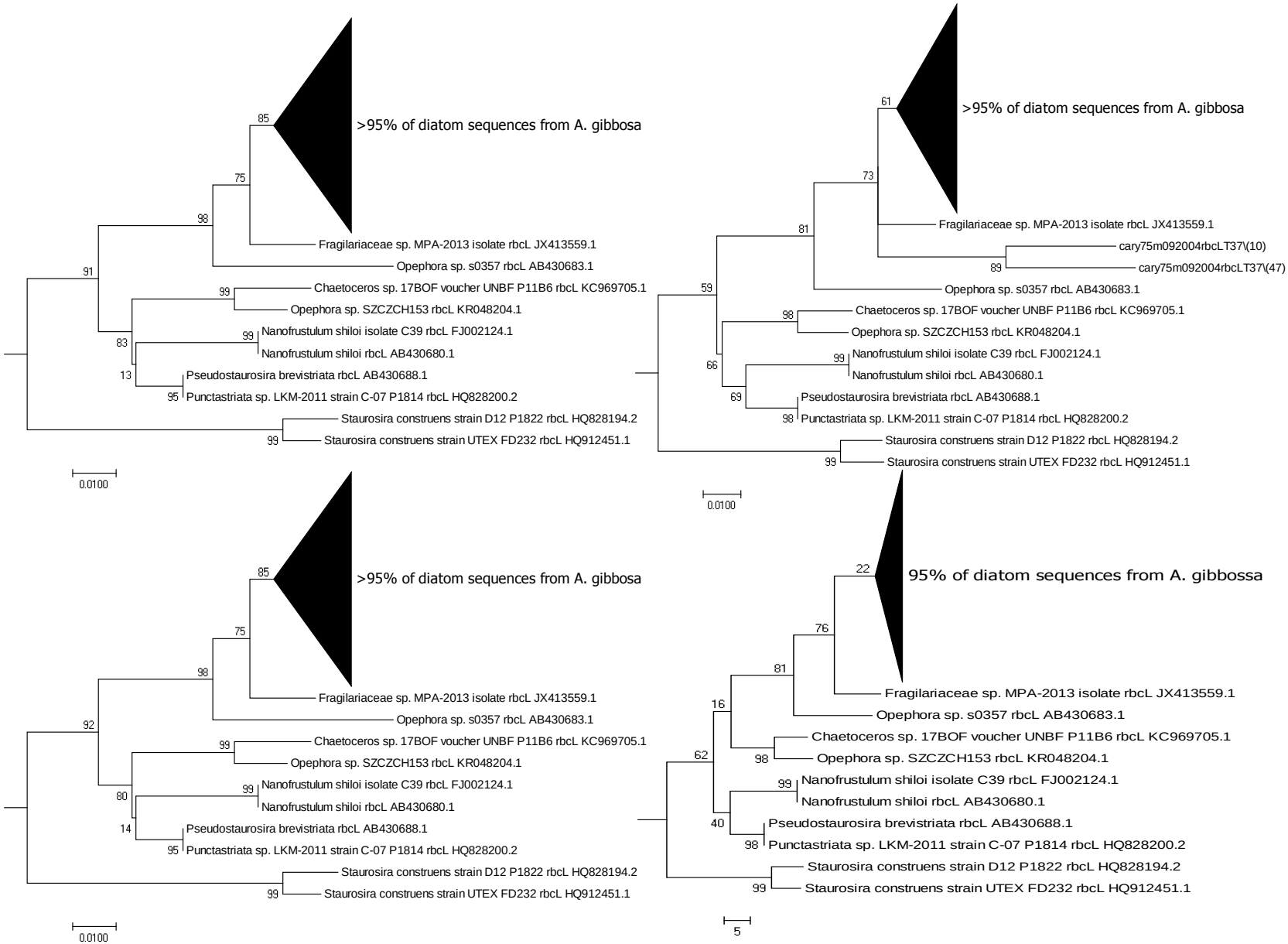


Figure 3.9: Comparison of the *rbcL* phylogeny (all data) using NJ, ML, ME, and MP methods. The full tree is available in the Supplemental Material.

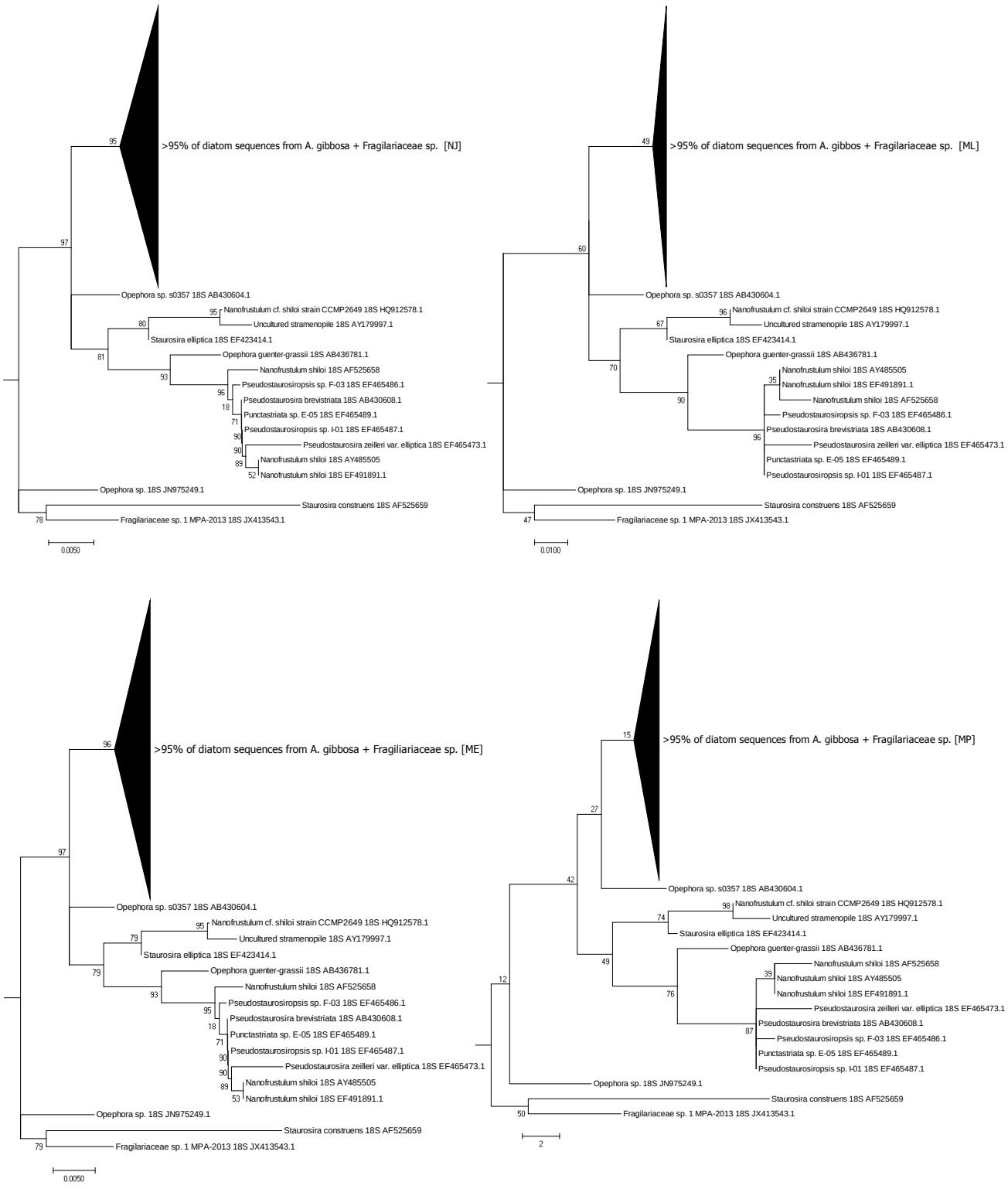


Figure 3.10: Comparison of the *18S* phylogeny (all data) using NJ, ML, ME, and MP methods. The full trees are available in the Supplemental Material.

CHAPTER 4: A COMPARISON OF MOLECULAR AND MORPHOLOGICAL METHODS FOR DETERMINING ALGAL ENDOSYMBIONTS WITHIN *AMPHISTEGINA* SPP.

Abstract

The identity and distribution of diatoms associated with reef-dwelling foraminifers of the genus *Amphistegina* determined by a) microscopic identification of diatoms cultured from the hosts, and b) DNA sequencing of two genes (*18S* and *rbcL*) from DNA extracted directly from the host and from cultures of presumed symbionts, were compared. As in previous culture isolation studies, multiple species of diatoms, predominantly pennate diatoms of the genera *Nitzschia*, *Navicula*, *Fragilaria* (including *Nanofrustulum*), and *Amphora*, were isolated from over 900 host specimens, and identified both morphologically and using DNA sequencing. In contrast, the diatom sequences obtained from DNA extracted directly from the *Amphistegina* hosts were species specific and different from the sequences from all of the cultured diatoms. The diatom sequences obtained directly from *Amphistegina gibbosa* specimens collected from various Caribbean and Western Atlantic sites, and from mixed samples of *A. lessonii* and *A. lobifera* specimens collected from Oahu, Hawaii, were most similar to those of diatoms in the family Fragilariaceae. The diatom sequences obtained directly from *A. radiata* specimens collected from sites in Papua New Guinea were most similar to diatoms of the family Plagiogrammaceae. The diatom sequences from *A. gibbosa* were almost all identical with only some very small and weakly supported differences among host specimens collected from

different depths and geographic locations. The more limited data set from *A. radiata* also exhibited minor differences between specimens from different geographic locations. More defined and statistically significant differences were observed in the diatom sequences from the mixed samples containing both *A. lessonii* and *A. lobifera* specimens, although all were still within the diatom family Fragilariaceae. These findings indicate that the approximately two dozen diatom taxa, which were previously identified as endosymbionts of *Amphstegina* spp. based upon culture studies, were likely contaminants such as stray epiphytes or undigested prey that thrived in the culture media. My findings are consistent with *in situ* TEM ultrastructural studies of the endosymbionts of *Amphistegina* and other diatom bearing foraminifers from the early 1980s and more recent molecular studies with other diatom-bearing taxa.

Introduction

Nearly 40 years ago, cytological examinations using transmission electron microscopy (TEM) revealed that benthic foraminifers of the genus *Amphistegina* host diatoms that live within the foraminiferal endoplasm (Leutenegger, 1977; Berthold, 1978). Further classification of the endosymbiotic diatoms was not possible because, while inside the hosts, the diatoms lack their characteristic frustules, which at the time was the primary means of identification and classification.

To address this problem, Lee et al. (1979a, b) developed methods aimed at liberating the diatom endosymbionts from the host and growing them in culture, where they could produce their characteristic frustules, which could then be used for identification. The initial success in cleaning the foraminiferal host, then isolating and growing the associated diatoms resulted in many additional publications in which more than 20 diatom species were described from culture

studies (e.g., Lee, 2011a,b and references therein; see Table 1.1). These results indicated a fluid relationship between the host foraminifers and multiple species of diatoms, sometimes even within a single host.

However, the findings from culture studies contrasted with observations reported by Leutenegger (1983, 1984), who examined the cytological structures within the diatoms inside the hosts using TEM and compared them to the samples of cultured diatoms from foraminiferal host specimens. She concluded that the symbiont-host relationship was very specific and that the characteristic cytological features of most of the diatoms observed in hospite did not match most of the species identified from culture studies. Only one of the symbionts appeared to match the symbionts of two host species. Despite these early findings, culture studies have remained standard procedure and have been widely published, as noted above.

The goals of my study were: 1) to identify the diatom endosymbionts within different species of *Amphistegina* from multiple sites in the Atlantic and Pacific using molecular techniques, and 2) compare the morphological and molecular results from culture studies with molecular results from diatoms extracted directly from host specimens, in many cases from the same host specimens.

Methods and Materials

To compare the morphological and molecular identities of cultured diatoms to the molecular identity of endosymbionts within different *Amphistegina* spp. hosts, DNA was extracted directly from *Amphistegina* specimens and from diatom cultures grown from selected corresponding hosts. The host specimens used in this study were obtained from researchers working at or visiting sites listed in Table 2.1, Table 3.1, and also in supplemental materials

described in the Appendix. *Amphistegina gibbosa* specimens were obtained from sites along the Florida reef tract (see Chapter 3), from the West Florida Shelf and off southeast Florida, and from several locations around the Caribbean including the Cayman Islands, Jamaica, and St. Vincent and the Grenadines. A mixed sample containing *A. lessonii* and *A. lobifera* was obtained from a previously studied site (Muller, 1974) on the southwest point of Oahu, Hawai'i. Samples of *A. radiata* were collected at Ambitle Island and Kimbe Bay, Papua New Guinea (Pichler et al., 2006).

As described in Chapter 3, the extracted DNA was amplified, cloned, and multiple clones sequenced. The sequences were trimmed of cloning vectors and ambiguous or uncertain base calls, and short sequences were removed. BLAST searches (nucleotide megablast) were performed on the trimmed sequences. The diatom sequences (based on BLAST searches) were used to construct phylogenetic trees for comparison between depths, locations, seasons, host species, direct extracts, and cultures. In addition to the large phylogenetic trees with all of the data for both genes, the following were compared, using various subsets of the *rbcL* and *18S* data:

1) diatom sequences from *A. gibbosa* collected from the Florida Middle Grounds in the Gulf of Mexico (western-most site) were compared to those from the eastern-most site, Young Island, St. Vincent and the Grenadines;

2) diatom sequences from *A. radiata* from Ambitle Island, a small island on the Pacific Ocean side of Papua New Guinea, were compared with sequences from Kimbe Bay, New Britain, a region of Papua New Guinea on the Bismark Sea; and

3) diatom sequences from a mixed *A. lessonii–A. lobifera* sample collected from Makapu'u rockpools on the southeast corner of Oahu, Hawai'i.

The software program MEGA 7 (Kumar et al., 2016) was used to determine the best fit substitution models and parameters for Neighbor-Joining (NJ) phylogenetic analyses with interior branch test of both the *18S* and *rbcL* data sets. The best fit models and parameters calculated by MEGA are provided in the figure captions for each phylogenetic tree and the confidence probabilities (CP) displayed above the nodes. Maximum likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) phylogenetic analyses of the entire data sets with bootstrap statistical analyses were also done.

The explanations for the abbreviated sequence data labels in the phylogenetic trees are provided in Table 3.1 and in supplemental material listed in Appendix. In some of the phylogenetic trees the sequence data from DNA extracted directly from foraminiferal hosts are in green and those from species (or closely related groups of taxa) that were cultured and presumed to be endosymbionts are in red. Some sequence data were compressed into groups which preserved the horizontal scale but the size of the compressed groups are proportional to the number of sequences (1 pixel per sequence). Some poorly aligned and aberrant sequences were removed from the phylogenetic analyses.

Results

BLAST Search Results of 18S and rbcL Sequences

I obtained a total of 2538 diatom sequences with Phred values greater than 20 (i.e., >99% accuracy) and lengths greater than 400 bp for *rbcL* and 450 bp for *18S*. Of those, 2377 diatom sequences were from DNA extracted directly from the *Amphistegina* host, and 161 sequences were from diatom cultures grown from diatoms associated with *Amphistegina* hosts. I obtained

2034 sequences from *A. gibbosa*, 197 sequences from *A. radiata*, and 146 sequences from a combined sample of *A. lessonii* and *A. lobifera* (Table 4.1).

The BLAST search results were surprisingly uniform for the diatom sequences obtained directly from host specimens. More than 99% of the sequences for *A. gibbosa* and 97% of the sequences from the mixed *A.lessonii–A. lobifera* sample were most similar to sequences from diatoms in the family Fragilariaceae (Figure 4.1, Table 4.1). In particular, these sequences were most or second most similar to the *18S* and *rbcL* sequences of an unnamed diatom in GenBank labeled Fragilariaceae sp. (Accession # JX413542.1 for *18S* and JX413559.1 for *rbcL*).

The BLAST search results of the diatom sequences from *A. radiata* were also very uniform, but very different from the sequences obtained from the other *Amphistegina* species. More than 98% of the diatom sequences from *A. radiata* were most similar to diatoms of the family Plagiogrammaceae (Figure 4.1, Table 4.1); no members of this family have been previously reported as endosymbionts in foraminifers.

The BLAST search results for the sequences obtained from the diatom cultures were completely different from those extracted directly from their corresponding hosts (Figure 4.1, Table 4.1). The sequences from the cultured diatoms were highly diverse, consistent with my finding based on morphological identifications of diatoms that grew in culture (Chapter 2) and with previous studies that have utilized culture methods (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correa, 2005; see Table 1.1). At least six different genera were observed in the 161 sequences from the cultures. Moreover, diatoms of the genus *Nitzschia* were the most frequent among the sequences from cultures (48%), the most commonly observed group using SEM of the cultures (55%), and also the most commonly observed in previous studies (62%) by Lee et al. (1995a). Diatoms from the genera *Navicula* and *Amphora*, and family Fragilariaceae,

were also commonly observed by microscopy of cultured diatoms (Chapter 2, also Lee et al., 1979a, 1989, 1992, 1995a; Lee and Correa, 2005), and among the sequences obtained from the cultures in my study (Figure 4.1, Table 4.1). None of the sequences in GenBank of diatoms previously reported as endosymbionts, nor any of the sequences from my cultures, nor from diatom cultures provided by J.J. Lee (as described in Chapter 3), were identical or nearly identical to the sequences obtained directly from host foraminifers.

18S and rbcL Phylogeny

All phylogenetic analyses, regardless of method, for both the *rbcL* (1440 sequences) and *18S* (1041 sequences) genes, showed that the diatom DNA sequences obtained directly from *A. gibbosa*, *A. lobifera*, and *A. lessonii* host specimens were closely related to diatoms within the family Fragilariaceae. The sequences from *A. radiata* were most similar to diatoms of the family Plagiogrammaceae. All sequences directly obtained from host specimens were clearly different from the diverse assemblage based on DNA sequenced from cultures of presumed symbionts (Figures 4.2–4.3). Almost all (>95%) of the diatom sequences from both *rbcL* and *18S* genes from *A. gibbosa* and *A. radiata* appear to belong to very specific monophyletic groups of diatoms within the family Fragilariaceae and Plagiogrammaceae (Figures 4.4–4.5), respectively.

Nevertheless, there were clusters of sequences (subgroups) within those groups when the number of sequences was reduced. For instance, many of the *rbcL* diatom sequences from *A. gibbosa* specimens collected from Young Island, St. Vincent and Grenadines, the most eastern Atlantic site, formed a consistent but weakly supported cluster in the trees of the entire data sets (see supplemental material listed in Appendix). This difference was more pronounced, but not statistically significant (CP≈80%), when the data set was reduced to direct comparison to diatom

sequences from a single other group, such as those from the Florida Middle Grounds, the western-most site from which *A. gibbosa* specimens were obtained (Figure 4.8). There was also a very small and weakly supported (CP≈80%) difference observed in *rbcL* diatom sequences from *A. radiata* collected from the two geographically separated sites in Papua New Guinea, i.e., Ambitle Island on the Pacific Ocean versus Kimbe Bay on the Bismarck Sea, when only those samples were compared along with the reference samples (Figure 4.9).

The *18S* and *rbcL* diatom sequences from the mixed *A. lessonii–A. lobifera* samples from the Makapu'u site in Hawai'i formed different subgroups within the family Fragilariaceae, which was evident even in the large trees of the entire data set (Figures 4.6–4.7). Those differences were even more pronounced and supported by some CP>95% when just those sequences were analyzed (Figure 4.10–11). The *18S* and *rbcL* diatom sequences from the mixed *A. lessonii–A. lobifera* samples formed two or more significantly different subgroups depending on the type of analysis. Nevertheless, almost all (>95%) of diatom sequences from these host specimens fell within the diatom family Fragilariaceae.

The overall results from the ML, ME, and MP phylogenetic analyses of the data were consistent with those from the NJ method (see Figure 4.12 and also supplemental material listed in Appendix). A small amount of poorly aligned and aberrant sequences (less than 5% of each data set) were removed from the phylogenetic analyses. These sequences often contained portions of the cloning vectors, misreads, chimeras, or multiple signals that were not detected and removed by the Vector NTI software. These "bad" sequences were the cause of almost all of the contrary results in the BLAST searches and phylogenetic analyses.

Discussion

Diatom Endosymbionts of Amphistegina *are Species Specific*

The *18S* and *rbcL* diatom sequences obtained from the *Amphistegina* specimens examined in my study were species specific. Sequences extracted from *Amphistegina radiata* revealed a single group of diatoms most closely related to the family Plagiogrammaceae. Sequences from *A. gibbosa* belonged to a single monophyletic group within the family Fragilariaceae. The sequences from the mixed sample of *A. lessonii–A. lobifera* specimens separated into at least two different groups, also within the family Fragilariaceae, that were slightly different from the sequences from *A. gibbosa* hosts. These different subgroups likely correspond to the fact that DNA was extracted from two different species with clear morphological differences. Although I did not differentiate between the two species prior to DNA extractions, given that *A. lobifera* attains substantially larger adult sizes and is about 3–5 times more abundant at the sample site than *A. lessonii*, a testable hypothesis for future studies is that the more common sequences came from *A. lobifera*.

The family Fragilariaceae includes *Nanofrustulum shiloi*, a common diatom previously identified in cultures from *Amphistegina* spp. Of the sequences obtained from cultured diatoms, those from *Nanofrustulum shiloi* were most similar, but clearly distinct from the sequences that came directly from three of the four *Amphistegina* species in my study. Moreover, none of the sequences that I obtained directly from *Amphistegina* spp. matched sequences of any of the diatoms frequently reported as endosymbionts from culture studies (e.g. Lee, 1992, 1998, 2006, 2011b; see also Table 1.1), nor the sequences from cultured diatoms from my study. However, sequences from my cultured diatoms did match those reported from previous culture studies.

The specific relationships between the host species and their particular endosymbionts, as indicated by the consistency in *18S* and *rbcL* diatom sequences obtained directly from host specimens, indicates that the symbionts and hosts co-evolved. Moreover, the minor differences between sequences from different geographic locations indicate the potential for local specialization. This interpretation is further supported by the strong similarity among the three most similar species of *Amphistegina*. The oldest of the three similar species is likely *A. lessonii*, as similar specimens are common in early Miocene facies from, e.g., Australia (e.g., Hallock et al., 2006a). A common assumption is that *A. gibbosa* diverged from *A. lessonii* when the Caribbean/Atlantic populations were genetically isolated from Pacific populations with the closure of the Central American Seaway in the Pliocene, at least 4 million years ago (e.g., Smith et al., 2013). When the shallow-dwelling *A. lobifera* diverged from *A. lessonii* is not known, but was probably later as indicated by greater similarity between the subgroups isolated from the mixed-species sample. A possibility would be during the Pleistocene, when major changes in sea level isolated and reconnected tropical seas, and is postulated to have driven extensive speciation in coral taxa, especially *Acropora* spp. in the Indo-Pacific region (Veron, 1995, 2000).

The major difference in diatom sequences between the *A. lessonii* group and *A. radiata* is similarly consistent with morphological differences between the two groups and their much longer, distinct fossil records of more than 20 million years or more (e.g., Hallock et al. 2006a).

On the other hand, my findings clearly do not support the conclusions based on previous culture-isolation studies that *Amphistegina* spp. can host multiple species of diatom symbionts and that their ability to swap out symbionts allows them to adapt and thrive under various environmental conditions (e.g., Lee, 1992, 1998, 2006, 2011a, b). My findings suggest a stable, specific relationship between the host and symbiont, consistent with previous ultrastructural

observations by Leutenegger (1983, 1984) and the molecular findings in diatom-bearing nummulitid Foraminifera by Holzmann et al. (2006).

Leutenegger's Ignored Findings

In the early 1980s, Leutenegger (1983, 1984) used TEM to examine *in situ* the ultrastructure of the diatom endosymbionts from three species also used in my study, *A. lessonii*, *A. lobifera*, and *A. radiata*. The main conclusion from her observations was that the symbionts of all the *Amphistegina* specimens she observed were specific and consistent over different locations, depths, and seasons. For the past 30+ years, her work has been largely ignored. Instead, the sheer number of publications based on culture studies (e.g., Lee et al. 1979a, 1980a, 1995a, 2011a, b; Lee and Correia, 2005; see list in Table 1.1) has dominated thinking about symbioses in the larger foraminifers.

My findings are remarkably consistent with those of Leutenegger's (1983, 1984). The cell ultrastructure of the diatom symbionts she examined *in situ* in *A. lessonii* and *Amphistegina lobifera* specimens were highly specific and similar to only *Nanofrustulum shiloi* from among all of the other previously reported diatom endosymbionts. Furthermore, Leutenegger (1984) found that the symbionts of *A. radiata* were also highly specific but not similar to any of the diatoms previously reported as symbionts from culture studies. Based on the sequences I extracted directly from *A. lessonii* and *A. lobifera* specimens, the actual diatom endosymbiont is a species within the family Fragilariaceae, which includes *N. shiloi*, a diatom commonly cultured from *Amphistegina* and other symbiont bearing foraminifers in this and previous studies. However, despite the taxonomically close relationship between the diatom consistently sequenced from those *Amphistegina* spp. and *N. shiloi*, they are not the same. None of the *N. shiloi* sequences in

GenBank (nor any of the other cultured diatoms assumed to be endosymbionts) was the most similar to the diatom sequences isolated directly from the hosts, using the BLAST search methods or any of the subsequent phylogenetic analyses.

Further evidence that Cultures Don't Match Molecular Identification

Holzmann et al. (2006) in their molecular study of the identities of the diatom endosymbionts of nine species of nummulitid foraminifers from various locations, depths, and collection years showed that their endosymbionts were monophyletic and most similar to diatoms of the genus *Thalassionema*. Their study indicated a stable, specific host-symbiont relationship, just as Leutenegger's (1983, 1984) TEM studies did. Prior culture-isolation studies (e.g., Lee et al., 1989, 1992, 1995a) with many of these same nummulitid species and subsequent reviews of symbiosis in foraminifers (e.g., Lee et al., 2010, Lee, 2011a, b) were directly inconsistent with those studies' findings, which both used more direct approaches to identify the diatom endosymbionts.

Schmidt et al. (2015), in their study of the invasion of the eastern Mediterranean by symbiont-bearing foraminifersof the genus *Pararotalia calcariformata*, isolated in culture four different species of diatoms (*Minutocellus polymorphus*, *Navicula* sp., *Amphora bigibba*, *Amphora* sp.) from just five specimens of *P. calcariformata*. However, when they extracted and sequenced the DNA obtained directly from the foraminifers, they only obtained sequences for a single species, *M. polymorphus*, which is not among any of the species of diatom previously identified as endosymbionts in foraminifers.

Schmidt et al. (2015) directly sequenced the PCR products of a portion of the *18S* gene. Surprisingly, their chromatograms showed DNA from only a single species, despite culturing

four different species of diatoms spanning three genera from just five foraminifers. Schmidt et al. concluded that their findings point to *M. polymorphus* being the "dominant" endosymbiont within *P. calcariformata*. Considering the finding in my study, it is likely that *M. polymorphus* is the only symbiont of *P. calcariformata*. For Schmidt et al. (2015) to have generated useable sequences directly from the PCR products, the original template needed to be very close to 100% pure, otherwise the chromatogram and sequence would be noisy and in most cases unusable. Based on the fact that they were able to get clean sequences from PCR products with a signal for only a single species indicates either: 1) their primers do not work for the *Navicula* and *Amphora* diatoms (however, this is unlikely, since they designed the primers after knowing the species they were targeting and would have likely tested it to confirm it worked), or 2) the *Navicula* and *Amphora* diatoms observed in culture were stray epiphytes or food particles that thrived in the culture media to sufficient quantities so they were able to observe them in 60% and 40% (respectively) of the isolated cultures.

On a very small scale, this part of the Schmidt et al. (2015) study is exactly what I observed in my study of endosymbionts within *Amphistegina*. DNA extractions directly from a host species yielded only a single "species" of diatom. However, the cultures (and DNA extraction of those cultures) showed that diatoms of the genera *Navicula* and *Amphora* and others were common. Just like Schmidt et al. (2015), I was able to detect diatoms of the genera *Navicula* and *Amphora* in my cultures by microscopy (and I was able to extract and sequence their DNA to further verify their identity and the ability of my primers to work on them). However, these diatom sequences were absent from the sequences obtained directly from the hosts. This would only happen if copies of their gene were extremely low, such as a stray

epiphyte or food particle. If their numbers are so low that they don't show up after PCR, then they are certainly not in sufficient quantities to be considered endosymbionts.

Conclusions

1. The *18S* and *rbcL* diatom sequences obtained from hundreds of specimens of *Amphistegina* spp. document that host-symbiont associations are highly specific.

2. Sequences extracted from *A. radiata* revealed a single group of diatoms most closely related to the family Plagiogrammaceae.

3. Sequences from *A. gibbosa* belonged to a single monophyletic group within the family Fragilariaceae. Minor differences were found between sequences obtained from *A. gibbosa* specimens collected in the eastern Caribbean compared to those from the Gulf of Mexico.

4. Sequences from a mixed sample of *A. lessonii–A. lobifera* specimens separated into at least two different groups, also within the family Fragilariaceae, that were slightly different from the sequences from *A. gibbosa* hosts.

5. No sequences directly from the hosts matched any of the sequences from cultured diatoms, nor any of the sequences in GenBank from diatoms previously grown in culture from *Amphistegina* hosts. The closest match was the previously identified diatom species, *Nanofrustulum shiloi*, which also belongs to the family Fragilariaceae

6. The results from the *18S* and *rbcL* diatom sequences obtained from *Amphistegina* spp. strongly support observations from cytological studies carried out more than 30 years ago that concluded that the diatom endosymbionts were host specific.

Table 4.1: A comparison of the diatoms (grouped by genus or family) indentified as endosymbionts in foraminifers using TEM cellular ultrastructure and BLAST searches of DNA extracted and sequenced directly from the foramaniniferal host versus identification of diatoms cultured from cleaned and crushed specimens. [1]The group Fragilariaceae sp. contains several unclassified Fragilariaceae sp. along with the genera *Staurosira*, *Opephora*, and *Nanofrustulum*. [2]The group Plagiogrammaceae sp. contains the genera *Talaroneis*, *Plagiogramma*,, and *Dimeragramma*. [3]The diatoms identified by TEM in *A. bicurculta*, *A. lessonii*, *A. lobifera*, and *A. papillosa* were specific and consistent and shared characteristics of *Nanofrustulum* (previously *Fragilaria*) *shiloi*. [4]The diatoms indentified by TEM in *A. radiata* and the nummulitid and alveolinid Foraminifera were specific and consistent but not identified, but they were placed in the specific and consistent diatom groups observed in those foraminifers in subsequent molecular studies.

Diatoms Indentified Directly from Foraminiferal Hosts												
Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Plagio-gramm-aceae sp.*[2]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
DNA sequences directly from *A. gibbosa* specimens collected in Atlantic	2180	99.3%						0.4%				0.3%
DNA sequences directly from *A. lessonii*, and *A. lobifera* specimens collected in Hawaii	146	97.2%										2.8%
DNA sequences directly from *A. radiata* specimens collected in Papua New Guinea	197		98.5%					0.5%				1.0%
DNA sequences directly from 9 species of ummulitid Foraminifera (Holzmann et al., 2006)	30			100%								
DNA Sequences directly from *P. calcariformata* (Schmidt et al. 2015)	2				100%							
TEM *in hospite* cellular ultrastructural examination in *A. bicurculta*, *A. lessonii*, *A. lobifera*, and *A. papillosa* (Leutenegger, 1983, 1984)	25	100% [3]										

Table 4.1 (Continued)

Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Plagio-gramm-aceae sp.*[2]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
TEM *in hospite* cellular ultrastructural examination in *A. radiata*(Leutenegger, 1983, 1984)	4		100% [4]									
TEM *in hospite* cellular ultrastructural examination in 5 species of nummulitid Foraminifera (Leutenegger, 1983, 1984)	20			100% [4]								
TEM *in hospite* cellular ultrastructural examination in 2 species of nlveolinid Foraminifera (Leutenegger, 1983, 1984)	4			100% [4]								
TEM *in hospite* cellular ultrastructural examination in 3 species of calcarinid Foraminifera (Leutenegger, 1983, 1984)	6					100%						
Diatoms Identified from Cultures												
Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Plagio-gramm-aceae sp.*[2]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
DNA sequences from diatoms cultured from *Amphistegina* spp. in this study	161	8.1%	1.2%				48.4%	28.7%	7.5%		1.2%	5.0%
Diatoms isolated in culture from *Amphistegina* spp. in this study	953	35.6%					55.4%	17.4%	20.7%	0.5%	0.3%	7.9%

Table 4.1 (Continued)

Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Plagio-gramm-aceae sp.*[2]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
Diatoms isolated in culture from *Amphistegina* spp. in previous studies (Lee et al., 1992, 1995a; Lee and Correia, 2005)	1856	13.4%					63.7%	10.8%	8.2%	2.0%	0.4%	4.1%
Diatoms isolated in culture from 3 species of nummulitid Foraminifera (Lee et al., 1992, 1995a; Lee and Correia, 2005)	493	4.1%					51.1%	4.5%	23.7%	7.3%	0.8%	13.4%
Diatoms isolated in culture from 5 species of calcarinid Foraminifera (Lee et al., 1992; Lee and Correia, 2005)	485	7.4%					44.1%	5.6%	42.5%	9.7%		1.9%
Diatoms isolated in culture from *Neorotalia calcar* (Lee et al., 1992)	105	1.9%					57.1%	7.8%	25.7%	3.8%		
Diatoms isolated in culture from *Alveolinella quoyi* (Lee and Correia, 2005)	14						60%	40%	60%	40%		

Table 4.1 (Continued)

Method of Identification	# Sequences or Host specimens	*%Fragil-ariaceae sp.*[1]	*%Plagio-gramm-aceae sp.*[2]	*%Thala-ssionema*	*%Minu-tocellus*	% Specific unidentified diatom (Leutenegger, 1983, 1984)	*%Nitzs-chia sp.*	*%Navi-cula sp.*	*%Amp-hora sp.*	*%Coc-coneis sp.*	*%Dip-loneis sp*	%Other
Diatoms isolated in culture from *Parasorites orbitolitoides* (Lee and Correia, 2005)	10						50%	30%	40%	10%		
Diatoms isolated in culture from *Pararotalia. calcariformata* (Schmidt et al. 2015)	5				80%			60%	40%			

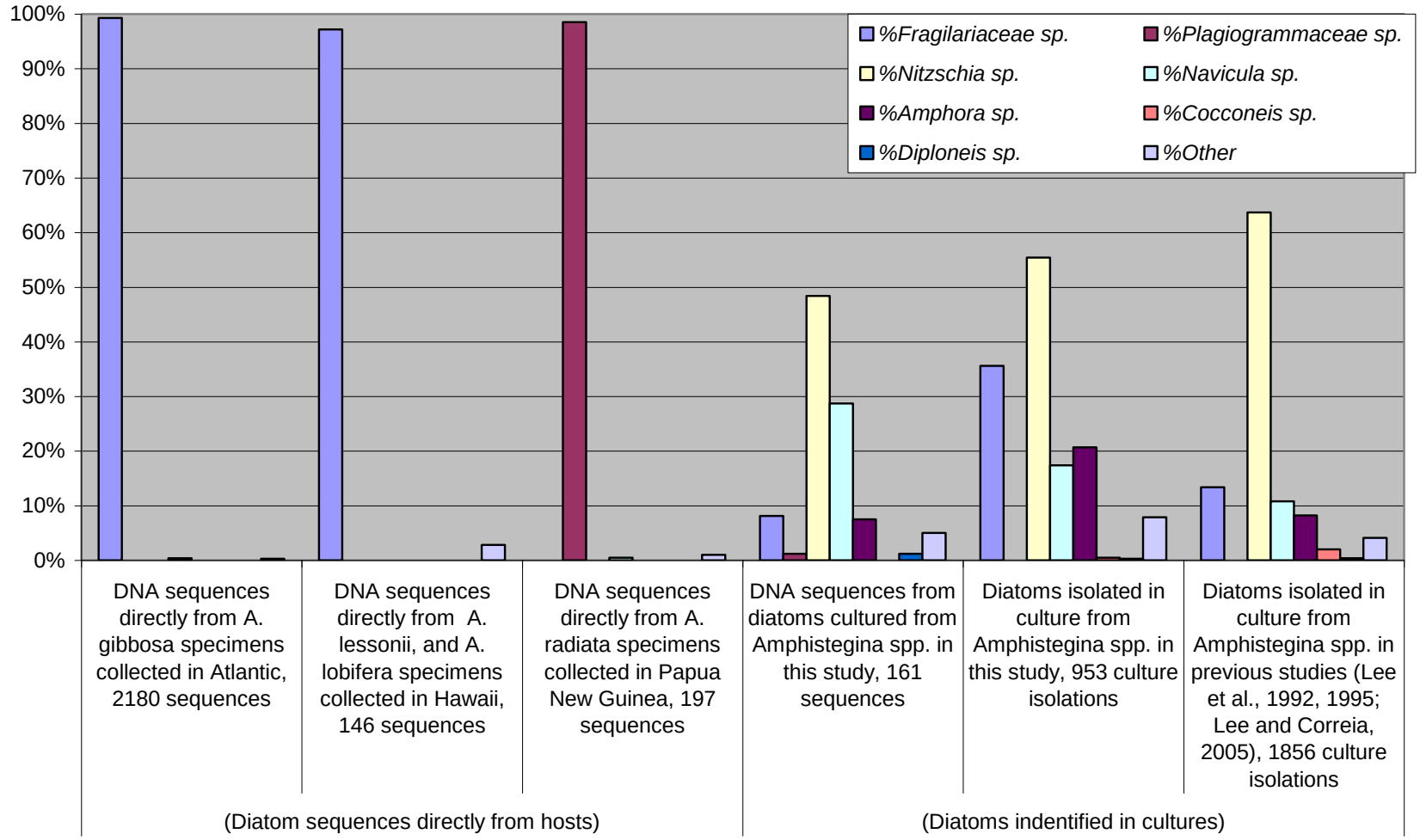


Figure 4.1: A comparison of the diatoms indentified as endosymbionts in *Amphistegina* spp. using molecular and morphological methods.

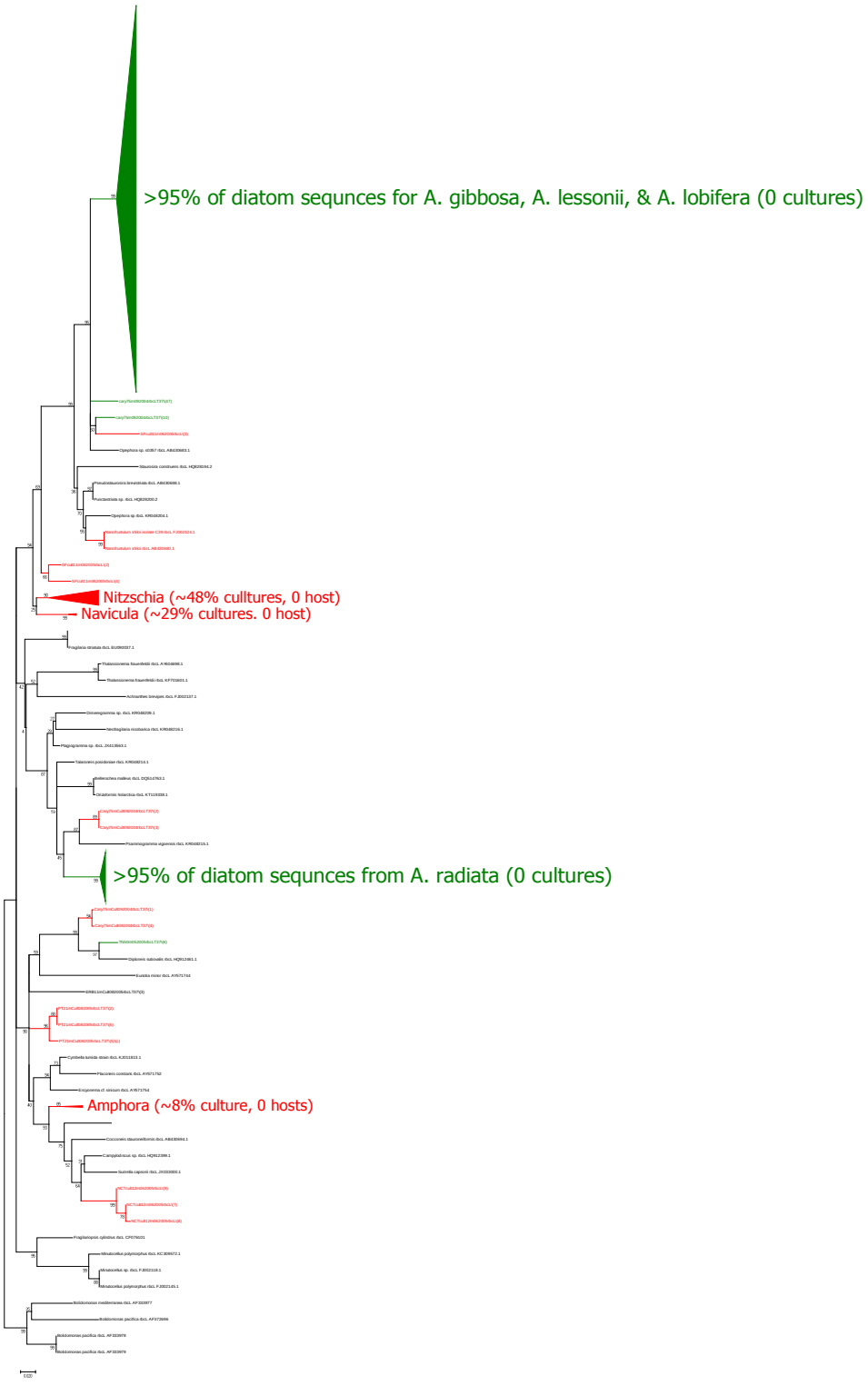


Figure 4.2: The *rbcL* phylogeny (Neighbor-Joining method) of diatom DNA extracted directly from *Amphistegina* spp. specimens (in green) compared to diatoms isolated in cultures (in red). The full tree is available in the Supplemental Material.

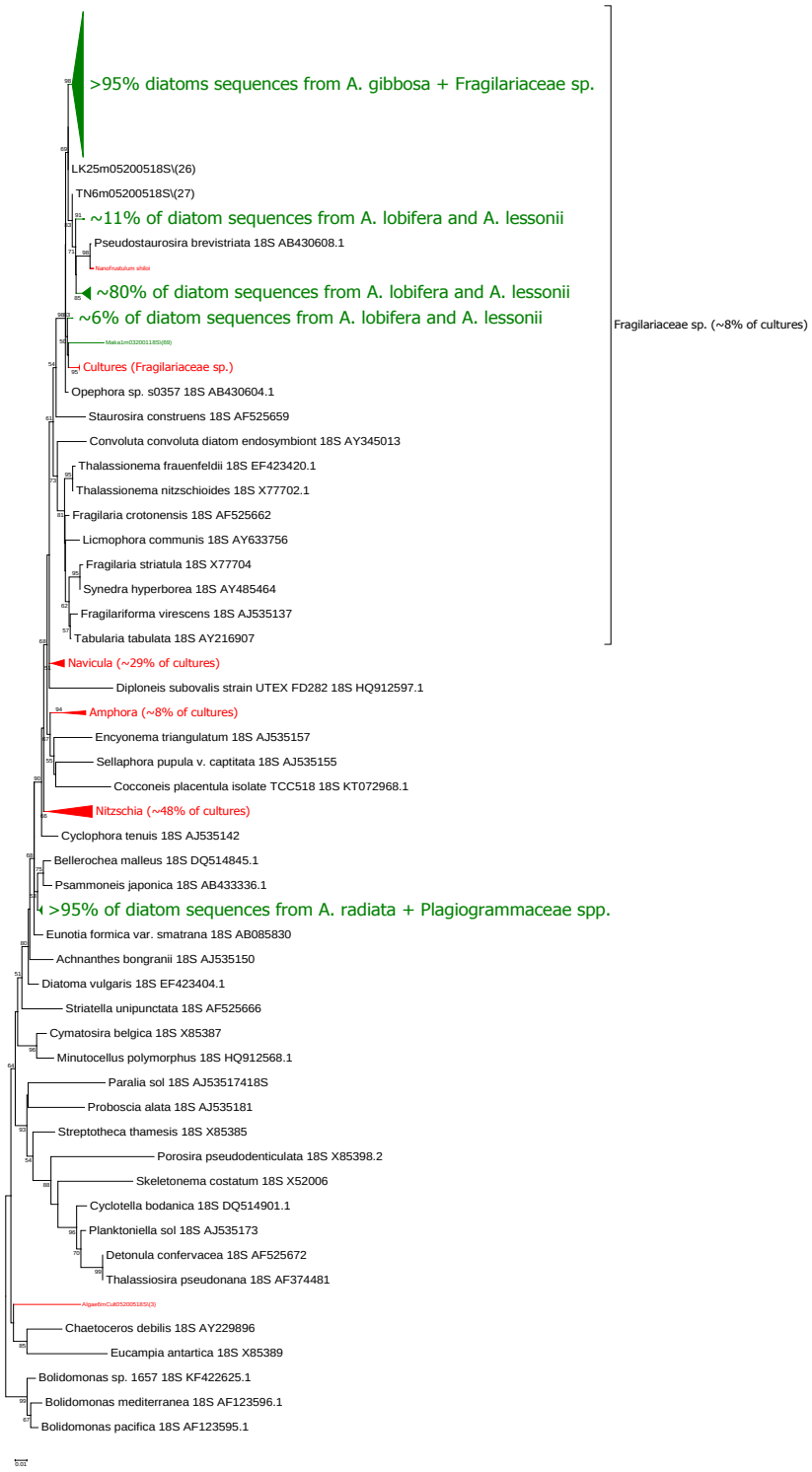


Figure 4.3: The *18S* phylogeny (Neighbor-Joining method) of diatom DNA extracted directly from *Amphistegina* spp. specimens (in green) compared to diatoms isolated in cultures (in red). The full tree is available in the Supplemental Material.

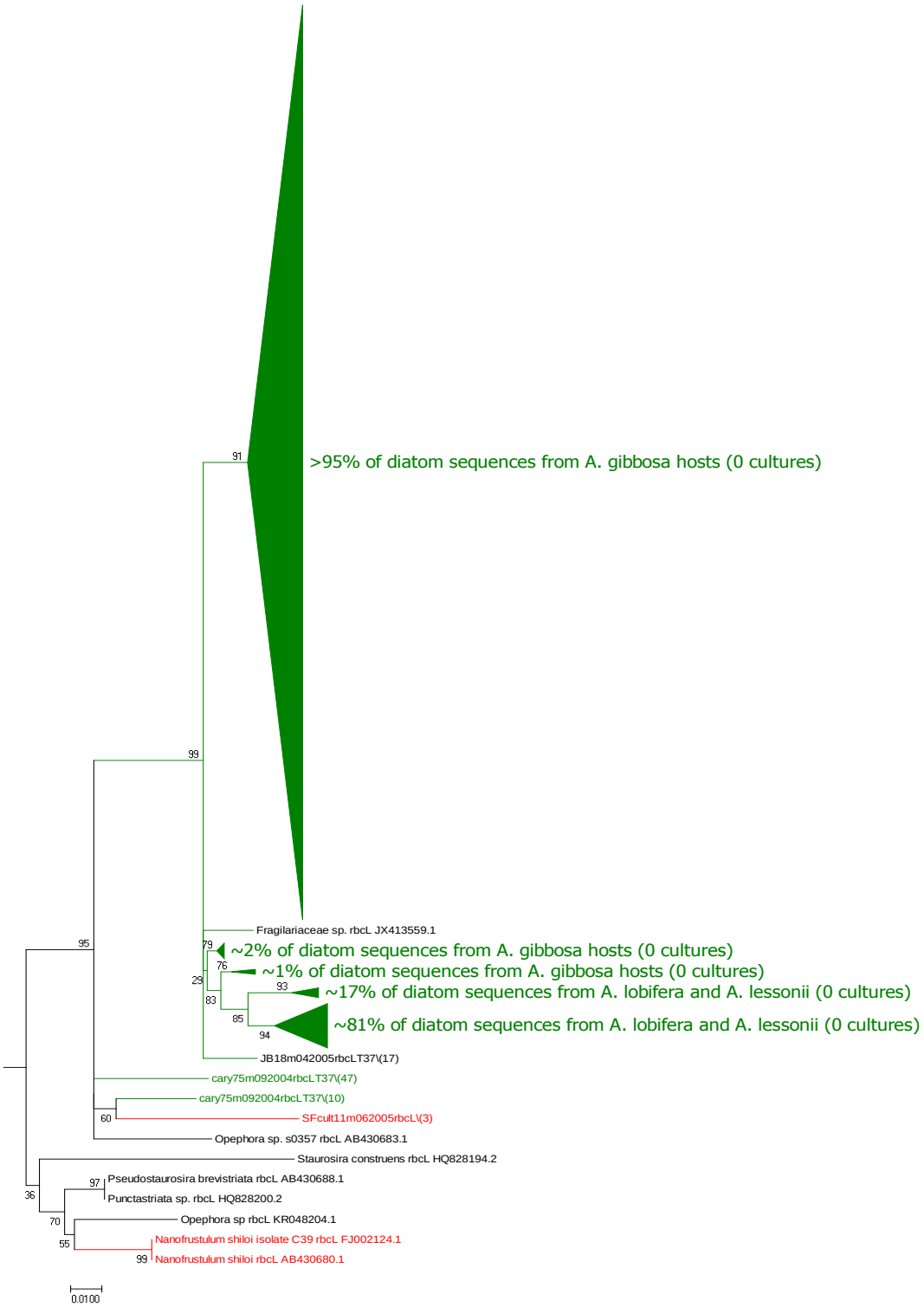


Figure 4.4: A close-up of the *rbcL* phylogeny (Figure 4.2) of diatom DNA extracted directly from *A. gibbosa, A. lobifera, and A. lessonii*. specimens (in green) compared to diatoms isolated in cultures (in red) and other closely related taxa in the family Fragilariaceae. The full tree is available in the Supplemental Material.

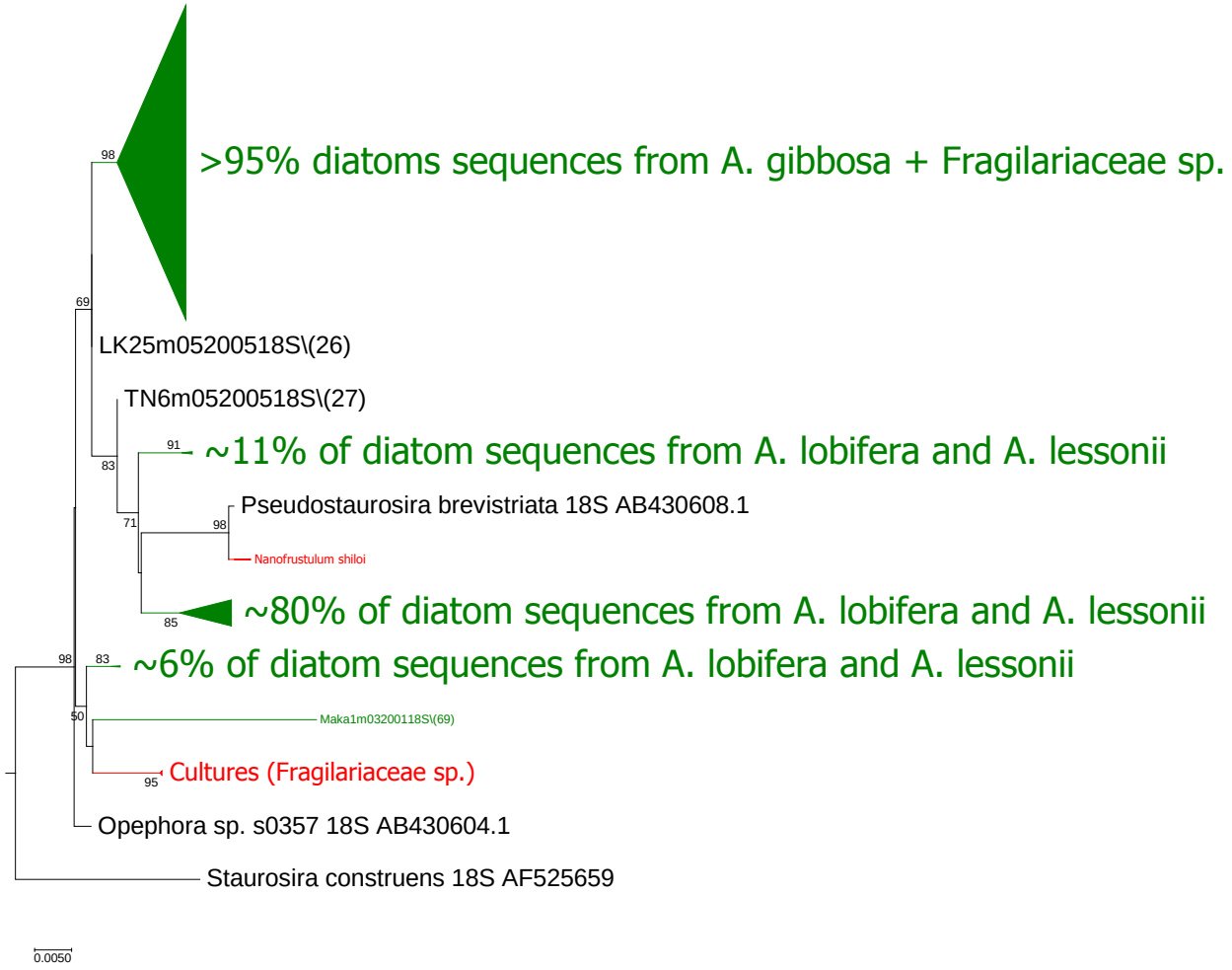


Figure 4.5: A close-up of the *18S* phylogeny (Figure 4.3) of diatom DNA extracted directly from *A. gibbosa, A. lobifera, and A. lessonii*. specimens (in green) compared to diatoms isolated in cultures (in red) and other closely related taxa in the family Fragilariaceae. The full tree is available in the Supplemental Material.

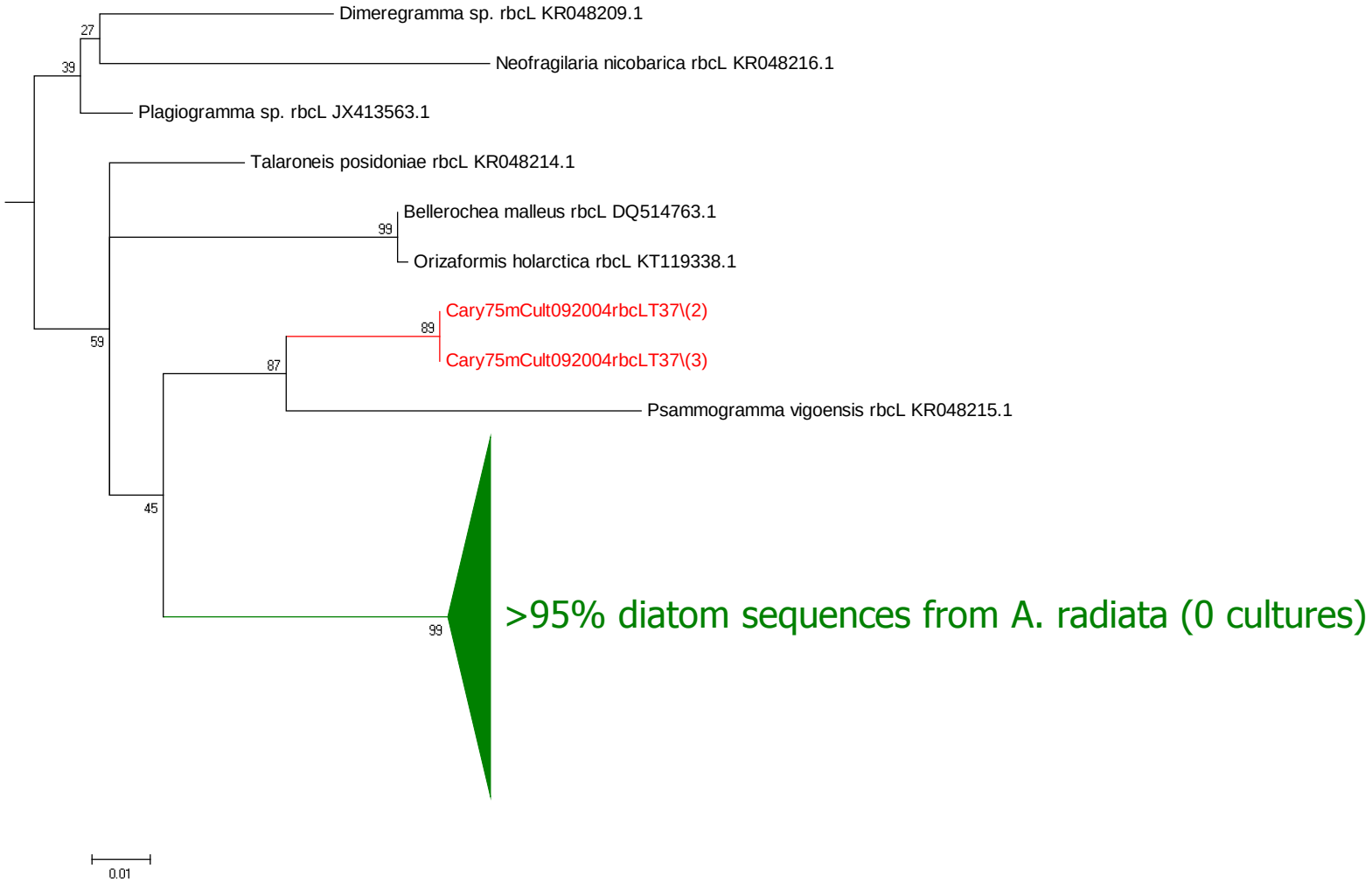


Figure 4.6: A close-up of the *rbcL* phylogeny (Figure 4.2) of diatom DNA extracted directly from *A. radiata* specimens (in green) compared to diatoms isolated in cultures (in red) and closely related taxa in the family Plagiogrammaceae.

Figure 4.7: A close-up of the *18S* phylogeny (Figure 4.3) of diatom DNA extracted directly from *A. radiata* specimens (in green) compared to diatoms isolated in cultures (in red) and closely related taxa in the family Plagiogrammaceae.

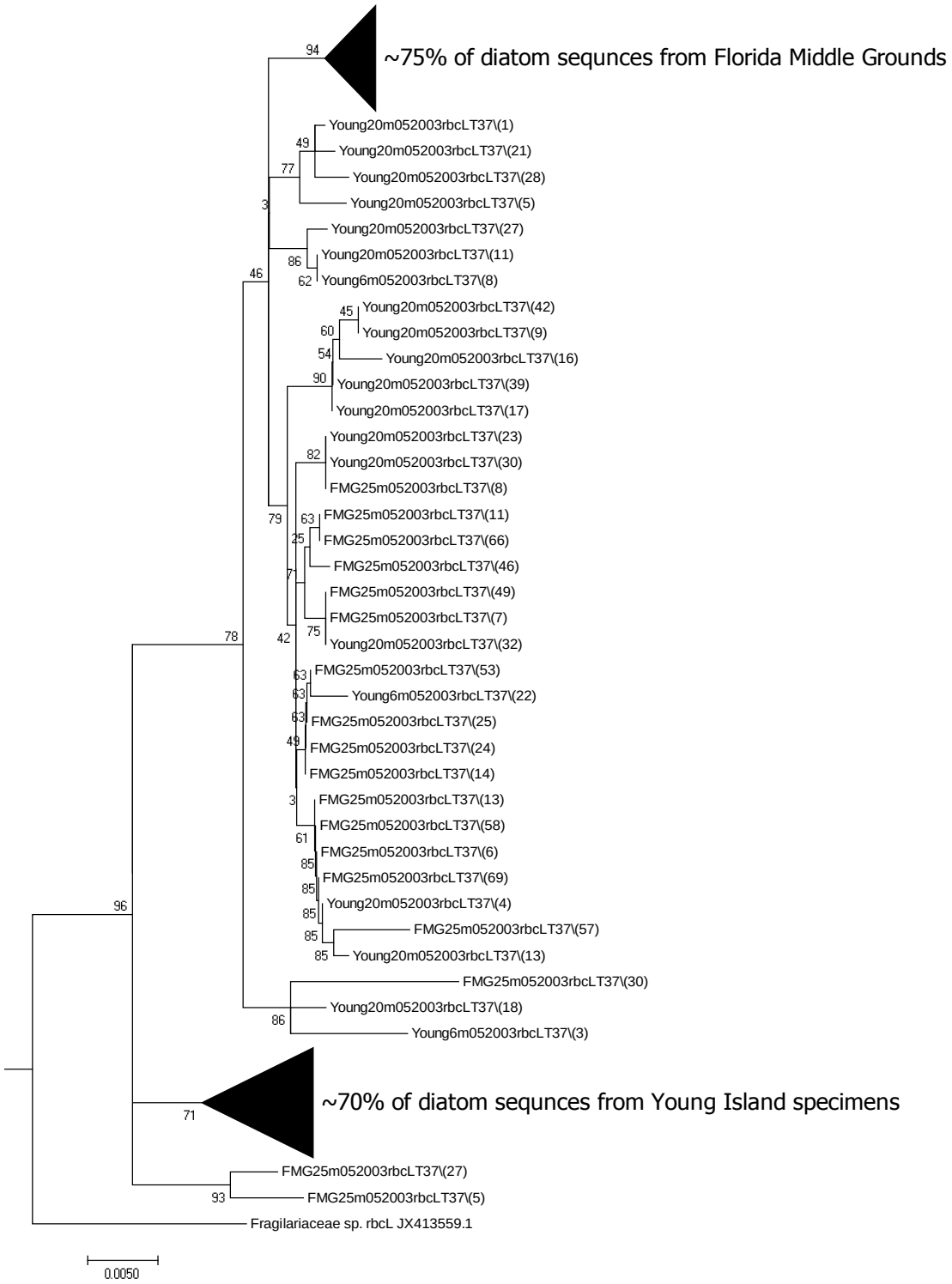


Figure 4.8: Part of *rbcL* phylogenetic tree (Neighbor-Joining method) comparing diatom sequences from *A. gibbosa* specimens collected from Young Island, St. Vincent, to those from Florida Middle Ground specimens. The full tree is available in the Supplemental Material.

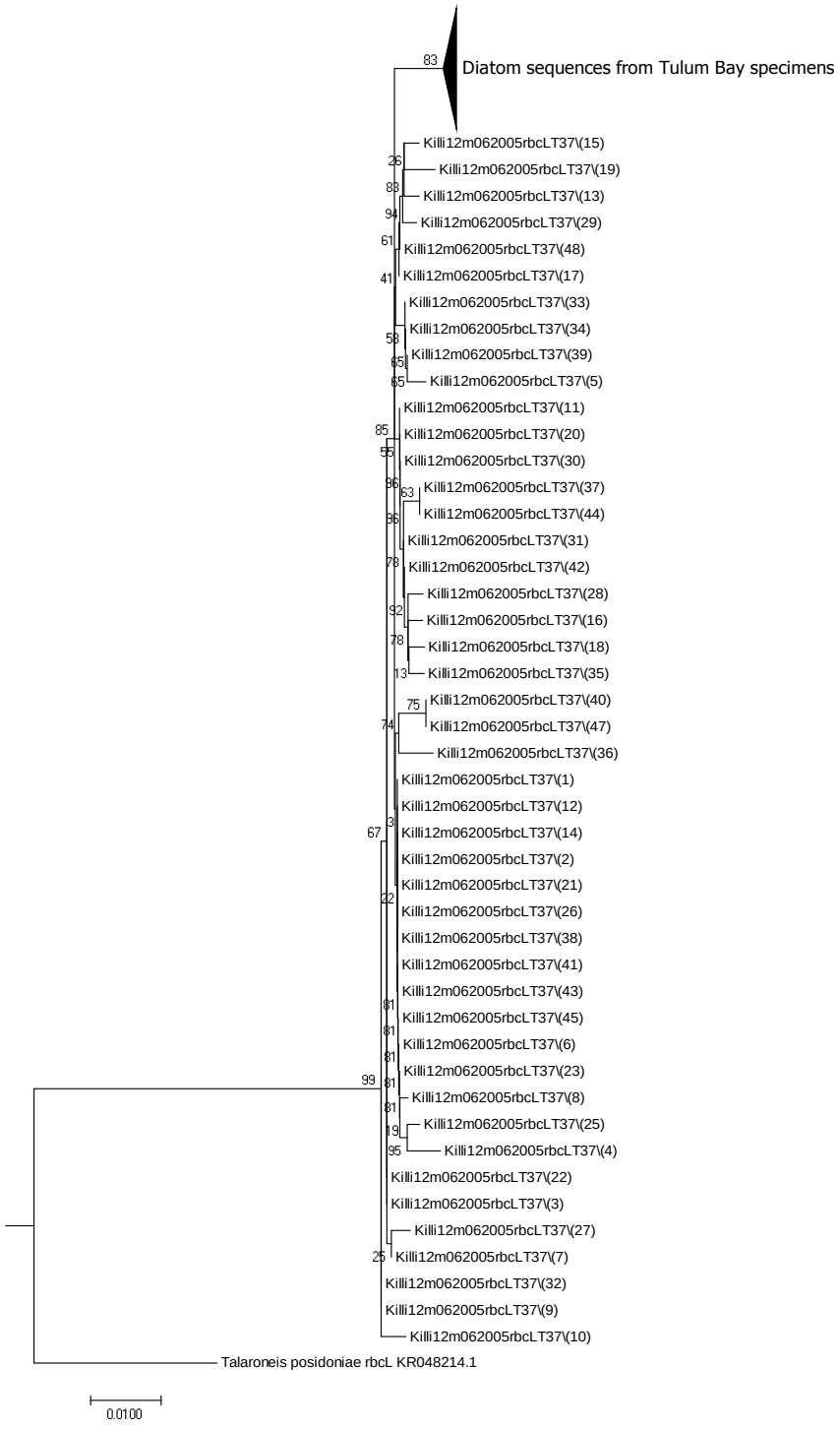


Figure 4.9: Part of *rbcL* phylogenetic tree (Neighbor-Joining method) comparing diatom sequences from *A. radiata* specimens collected from Killi Bob Bay and Tutum Bay in Papua New Guinea. The full tree is available in the Supplemental Material.

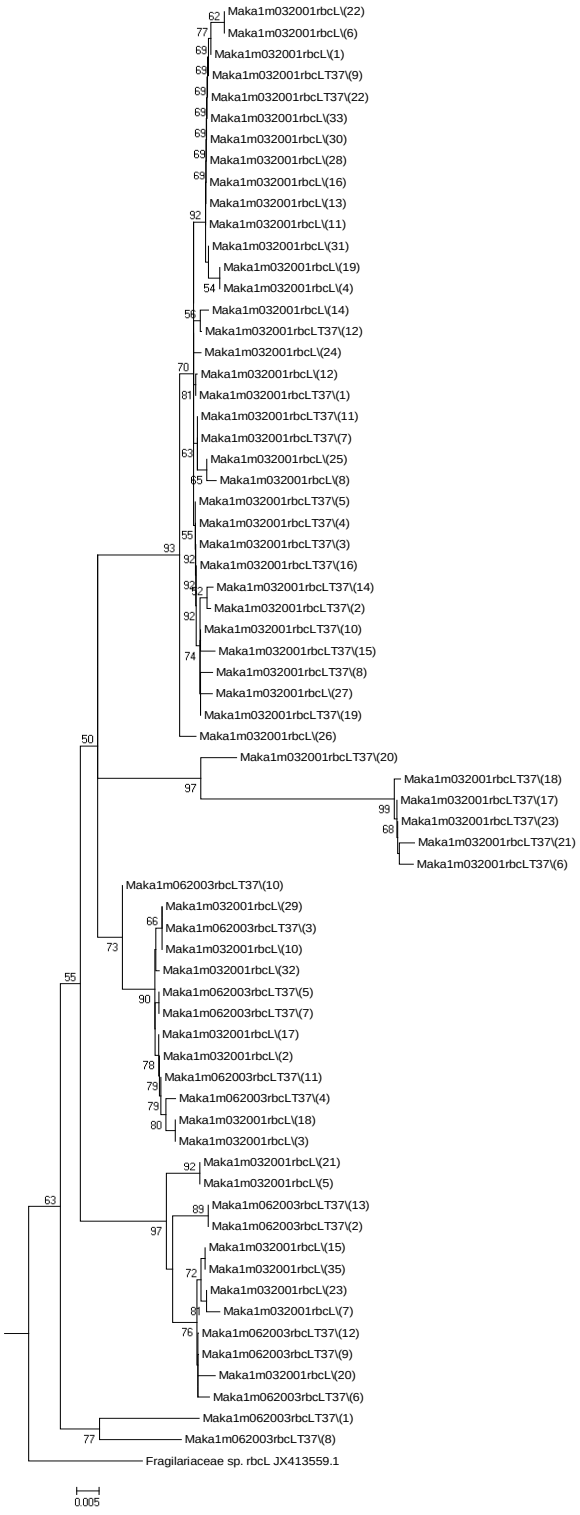


Figure 4.10: Part of *rbcL* phylogenetic tree (Neighbor-Joining method) comparing diatom sequences from the mixed *A. lobifera* and *A. lessonii* specimens collected from Makapuu Tide Pools on O'ahu, Hawai'i. The full tree is available in the Supplemental Material.

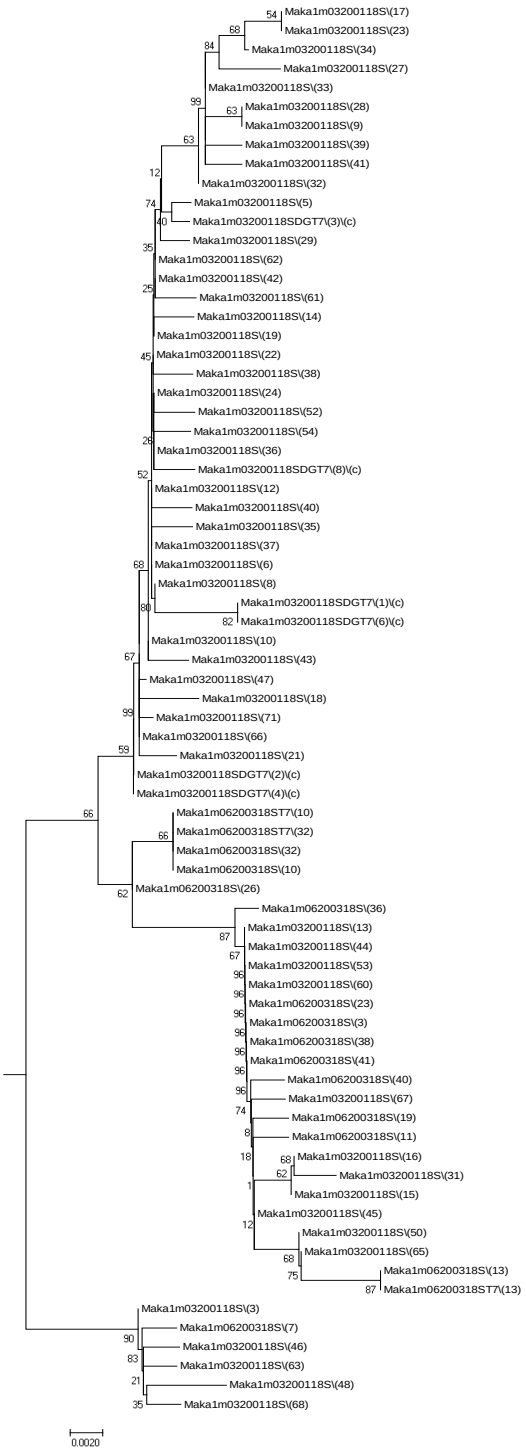


Figure 4.11: Part of *18S* phylogenetic tree (Neighbor-Joining method) comparing diatom sequences from the mixed *A. lobifera* and *A. lessonii* specimens collected from Makapuu Tide Pools on O'ahu, Hawai'i.. The full tree is available in the Supplemental Material.

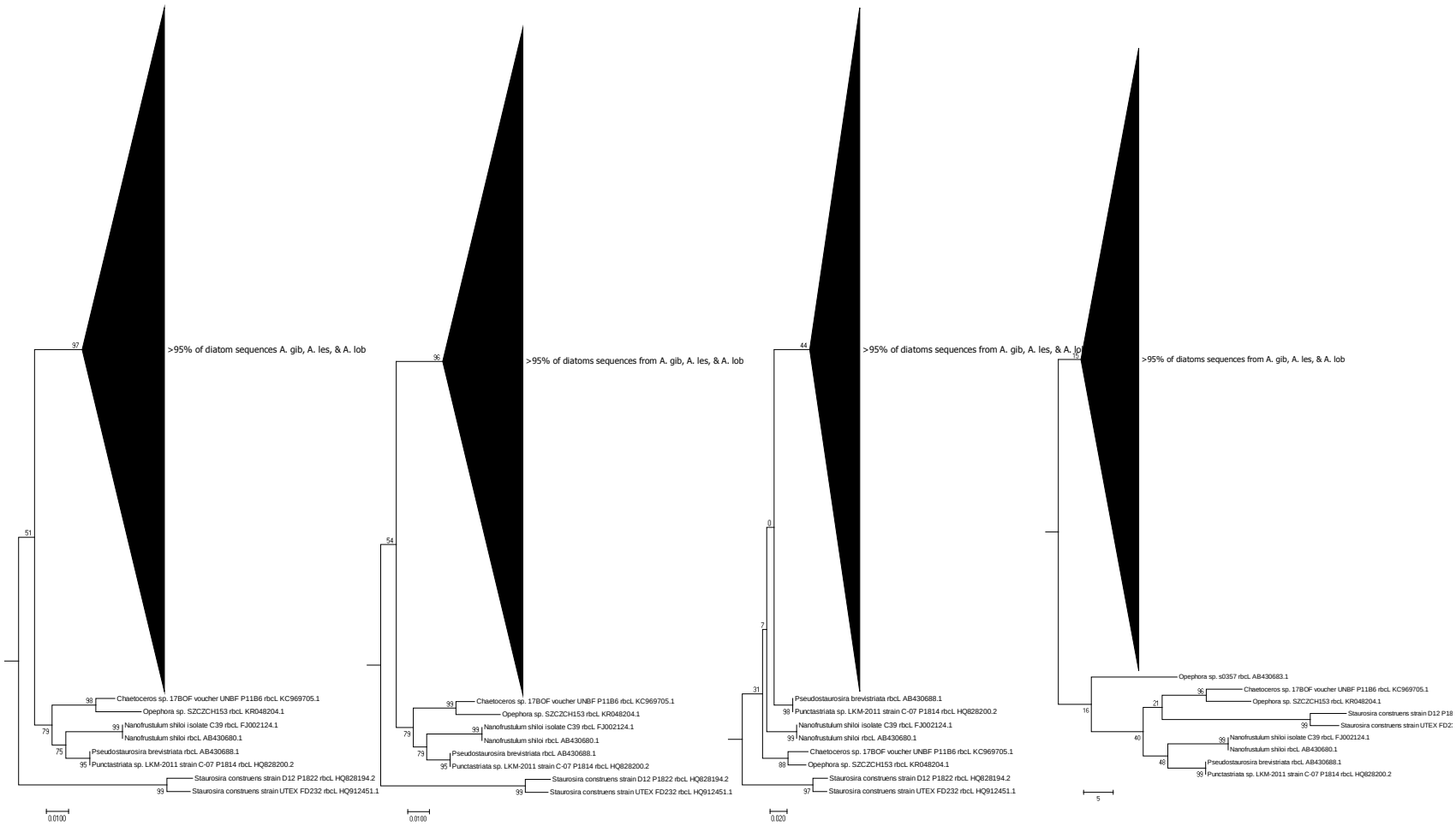


Figure 4.12: Comparison of part of the NJ, ML, ME, and MP trees for diatom DNA extracted directly from *Amphistegina* spp. specimens compared to sequences from diatoms isolated in cultures along with reference taxa. The full trees are available in the Supplemental Material.

CHAPTER 5: COMPARISON OF DIATOM SYMBIONTS WITHIN *AMPHISTEGINA* SPP. HOST SPECIMENS TO DIATOMS ISOLATED IN CULTURE: RESULTS FROM DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Abstract

Denaturing gradient gel electrophoresis (DGGE) was used to compare DNA extracted directly from specimens of *Amphistegina* spp. to DNA extracted from cultures of diatoms isolated from *Amphistegina* specimens. *Amphistegina gibbosa* specimens from a variety of locations around Florida and the Caribbean, as well as specimens of *Amphistegina* spp. from Hawai'i and Papua New Guinea, were compared. DNA was extracted directly from host specimens and from diatoms cultured from the same specimens using established protocols. Polymerase chain reaction (PCR) was used to generate amplicons of regions of the small subunit of the ribosomal RNA gene (*18S*), the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase [i.e., RubisCO] gene (*rbcL*), and the internal transcribed spacer 1 (*ITS1*) from the DNA extracts. The DGGE profiles of PCR amplicons of the foraminiferal hosts were different from those of diatoms cultured from corresponding hosts. The *rbcL* DGGE profile of DNA extracted and amplified from 80 foraminiferal hosts consisting of four different *Amphistegina* species were distinct from those of *Nitzschia laevis*, one of the most commonly cultured diatoms in past studies. The algal-specific *ITS1* and *rbcL* DGGE profiles from DNA extracted directly from the foraminiferal hosts contained only one prominent band per host species, indicating that each *Amphistegina* species has a single (or overwhelmingly dominant)

species of diatom endosymbiont. These results are consistent with ultrastructural observations in *Amphistegina* published more than 30 years ago, with more recent molecular findings from other diatom-bearing Foraminifera, and from my findings from sequencing both the *18S* and *rbcL* genes from DNA extracted directly from *Amphistegina* specimens.

Introduction

The presumed endosymbionts in larger benthic Foraminifera, particularly of the diatom-bearing members of the genus *Amphistegina*, have been identified from culture isolations from clean and crushed host specimens (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a, Lee and Correa, 2005; see Chapter 1, Table 1.1). Alternative methods, such as *in hospite* cytological examinations using transmission electron microscopy (Leutenegger, 1983; 1984), and more specialized but expensive methods such as DNA sequencing (e.g., Holzmann et al., 2006; Schmidt et al. 2015), have cast doubt on the reliability of culture isolation methods in identifying true endosymbionts as opposed to possible epiphytes or undigested algal prey that can thrive in culture media. In this study, I utilized denaturing gradient gel electrophoresis (DGGE) as an alternative molecular tool to assess and compare the identity and diversity of symbiont taxa within large numbers of foraminiferal hosts specimens from different locations, depths, and seasons and to compare them with presumed algal endosymbionts isolated in culture from their respective foraminiferal hosts.

Methods and Materials

As described in Chapters 3 and 4, DNA was extracted directly from several species of diatom-bearing foraminifers of the genus *Amphistegina* collected from different locations, as

well as from diatoms cultivated from cleaned and crushed foraminiferal hosts and presumed to be endosymbionts (Table 5.1). The DNA extracts from some sites and host species were combined and described in figure legends, where applicable.

Polymerase chain reaction (PCR) was used to generate amplicons of regions of the small subunit of the ribosomal RNA gene (*18S*), the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase [i.e., RubisCO] gene (*rbcL*), and the internal transcribed spacer 1 (*ITS1*) from the various DNA extracts. DNA extracts were sent by overnight courier to Todd LaJeunese at Florida International University to conduct the *ITS1* PCR and DGGE using his algal specific *ITS1* primers and DGGE protocols (LaJeunesse, 2007). The resulting gel images were digitized.

The "universal" *18S* and "algal-specific" *rbcL* DGGE were conducted with the assistance of Bina Nayak at the University of South Florida using methods developed during her doctoral dissertation research (Nayak, 2009), combined with other protocols (e.g., Paul et al., 2000; Diez et al., 2001). The *18S* DGGE profiles were generated using slightly modified protocols from Diez et al. (2001). The modifications involved substituting reagents and equipment for equivalent ones that were readily available, such as using Promega GoTaq Green PCR Master Mix for the PCR, a Bio-Rad DCode Universal Mutation Detection System (Cat. #170-9080) for electrophoresis for which the voltage reduced from 100 to 60 volts, SYBR Green I (Molecular probes, S-7567) for nucleotide dyes, and obtaining images using a Foto/Analyst Imaging System (Fotodyne Inc., Cat. #6-1500P). The *rbcL* DGGE profiles were generated using the *rbcL* primers from Paul et al. (2000) combined with the GC clamp from Diez et al. (2001) and combined procedures from Diez et al. (2001) and Nayak (2009). The forward primer was 5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGGATGATGARAAYATTAACTC-3′, and the reverse primer was 5′-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGATTTGDCCACAGTGD ATACCA-3′.

The PCR reactions to generate *rbcL* DGGE amplicons were done with 25 µl of Promega GoTaq Green PCR Master Mix containing 0.5 µl of the forward and reverse primers with concentrations of 10 µM, 10 µl of DNA extract, and 14 µl Promega Nuclease-Free Water added to get to 50 µl. The PCR conditions for generating the *rbcL* DGGE amplicon were: 2 min at 95°C, 50 cycles of (1 min at 95°C, 30 s at 56°C, and 45 s at 72°C), 5 min at 72°C, and hold at 4°C.

The *rbcL* DGGE utilized a Bio-Rad DCode Universal Mutation Detection System (Cat. #170-9080) with a 1mm thick 7% (wt/vol) polyacrylamide (acrylamide:bis acrylamide 37.5:1) gel containing a linear denaturing gradient of 25%-45% formamide and urea. The gels were electrophoresced using 40V at 60°C for 16 hours. The gels were stained with SYBR Green I (Molecular probes, S-7567) and images taken using a fluorescent green filter in a Foto/Analyst Imaging System (Fotodyne Inc., Cat. #6-1500P) as described by Nayak (2009).

Results

The *ITS* DGGE fingerprints from DNA extracted directly from eight groups of *A. gibbosa* specimens collected at different depths, locations, and seasons at numerous Western Atlantic sites were all nearly identical, showing a consistent pattern of a single prominent band (Figure 5.1). The *rbcL* DGGE profile of DNA extracted directly from the Atlantic species, *A. gibbosa,* contained one prominent band which was close in position to the two prominent bands from the DNA extracted from a mixed sample of *A. lobifera* and *A. lessonii* specimens collected in Hawai'i (Figure 5.2). The *rbcL* DGGE profile of DNA extracted from *A. radiata* specimens

collected in Papua New Guinea contained one prominent band, but its position was much further down the gel than the diatom DNA extracted from the other foraminiferal hosts species in this study (Figure 5.2). The *rbcL* DGGE profiles from DNA extracted directly from the foraminiferal hosts were clearly distinct from the profile produced by DNA extracted from *Nitzschia laevis*, one of the most commonly cultured diatoms from past culture isolation studies (Figure 5.2).

The *rbcL* DGGE profiles from DNA extracted directly from three groups of *A. gibbosa* specimens collected from different sites in the Western Atlantic produced strong, nearly identical bands. The profiles from DNA from diatoms cultivated from those same host specimens, revealed far more diverse and variable profiles, often containing multiple bands. None of the bands produced from the cultured diatom samples matched the single dominant band produced from the hosts (Figure 5.3).

The DGGE profiles produced utilizing the more general *18S* primer sets, which were not specific to algal DNA, produced highly variable and inconsistent results (Figure 5.4). Notably, however, the profiles from the hosts and cultures were consistently different.

Discussion

Contrary to previous reports from culture studies that reported a fluid and diverse group of diatoms as presumed endosymbionts within *Amphistegina* and other diatom-bearing foraminifers (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correa, 2005; see Table 1.1), the DGGE profiles clearly shows the opposite. The algal specific *ITS1* and *rbcL* DGGE profiles unequivocally showed that the taxa within *Amphistegina* species in this study were specific and consistent within each host species (Figures 5.1-5.3). The data strongly suggest that each *Amphistegina* species has a single (or overwhelmingly dominant) species of diatom

endosymbiont that is different from the numerous species cultured in previous studies and presumed to be endosymbionts. None of the *ITS1* or *rbcL* DGGE bands from cultured diatoms that were presumed to be endosymbionts, including *Nitzschia laevis*, one of the most commonly isolated diatom in past studies, matched the single dominant band in the *rbcL* DGGE profiles of DNA extracted from over 60 host specimens containing all the four species of *Amphistegina* in this study (Figures 5.2–5.3). These results are consistent with previous ultrastructural observations by Leutenegger (1983, 1984) and parallel to the molecular findings in diatom-bearing nummulitid foraminifers by Holzmann et al. (2006) and *Pararotalia calcariformata* by Schmidt et al. (2015). As in results reported in Chapters 3 and 4, the results from DGGE indicate a stable, specific relationship between the individual *Amphistegina* host species and its particular diatom endosymbiont. Moreover, these results indicate that the many diatom taxa, which were previously identified as endosymbionts of *Amphstegina* spp. based upon culture studies, consistent with the *rbcL* DGGE results in Figure 5.3, likely included minor associates or contaminants such as epiphytes or food particles that thrived in the culture media.

Most of the DGGE work was done with a "universal" *18S* primers set, which targets most eukaryotic DNA. This primer set was eventually abandoned because the *18S* DGGE bands contained many other species, making the fingerprint highly variable and often incoherent. Nevertheless, clear differences were observed between the DGGE fingerprints directly from hosts compared to cultures isolated from those hosts. Future DGGE studies of symbionts in foraminifers should consider utilizing algal-specific primers.

Conclusions

1. Results from DGGE analyses indicate that each *Amphistegina* species has a single (or overwhelmingly dominant) species of diatom endosymbiont that is different from the numerous, previously identified species cultured from *Amphistegina* hosts that were presumed to be endosymbionts.

2. Results from DGGE analyses support results from phylogenetic studies based on sequencing of 18S and rbcl DNA from A*mphistegina* host specimens and diatoms grown in culture from the same hosts, as well as earlier cytological studies that indicated a single morphotype of symbiont in each species of *Amphistegina* examined.

Table 5.1: Specimens used in DGGE analyses, including dates, locations and depths of collection.

Figure	PCR Primers	Lane #	Collection Location	Site Name	DNA Source	Collection Date	Collection Depth (m)	Total # Sample
5.1	*ITS1*	1	Upper Keys	Carysfort Reef 10m	*A. gibbosa*	May 2005	10	10
		2	Upper Keys	Carysfort Reef 75m		May 2005	75	10
		3	West Florida Shelf, Gulf of Mexico	Florida Middle Grounds		May 2003	25	10
		4	Southwest of Haiti	Navassa Island		Nov 2004	25	10
		5	Northern Jamaica	Pear Tree 29m		Aug 2005	29	10
		6	Northern Little Cayman	Nancy's Cup of Tea		Jun 2005	12	10
		7	Southeast Florida	Juno Beach		Apr 2005	18	10
		8	St. Vincent and the Grenadines	Young Island Reef 20m		May 2003	20	10
5.2	*rbcL*	1	Oahu, Hawaii	Makapuu Tide Pools	*A. lobifera* and *A. lessonii*	Mar 2001	1	20
						Jun 2003		
		2	Southwest of Haiti	Navassa Island	*A. gibbosa*	Nov 2004	25	30
			Upper Keys	Molasses Reef 27m		Jul 2005	27	
			Southeast Florida	Juno Beach		Apr 2005	18	
		3	Papua New Guinea	Killi Bob Reef, Kimbe Bay	*A. radiata*	Jun 2005	12	30
				Tutum Bay 120			13	
				Tutum Bay 300			28	
		4	*Nitzschia laevis* culture from John J.Lee					

Table 5.1 (Continued)

Figure	PCR Primers	Lane #	Collection Location	Site Name	DNA Source	Collection Date	Collection Depth (m)	Total # Sample
5.3	*rbcL*	1	Southwest of Haiti	Navassa Island	*A. gibbosa*	Nov 2004	25	20
		2	St. Vincent and the Grenadines	Young Island Reef 20m		May 2003	20	15
		3	Upper Keys	Molasses Reef 27m		Jul 2005	27	10
		4	Southwest of Haiti	Navassa Island	*A. gibbosa* Cultures	Nov 2004	25	7
		5						7
		6						6
		7	St. Vincent and the Grenadines	Young Island Reef 20m		May 2003	20	5
		8						5
		9						5
		10	Upper Keys	Molasses Reef 27m		Jul 2005	27	4
		11						3
		12						3

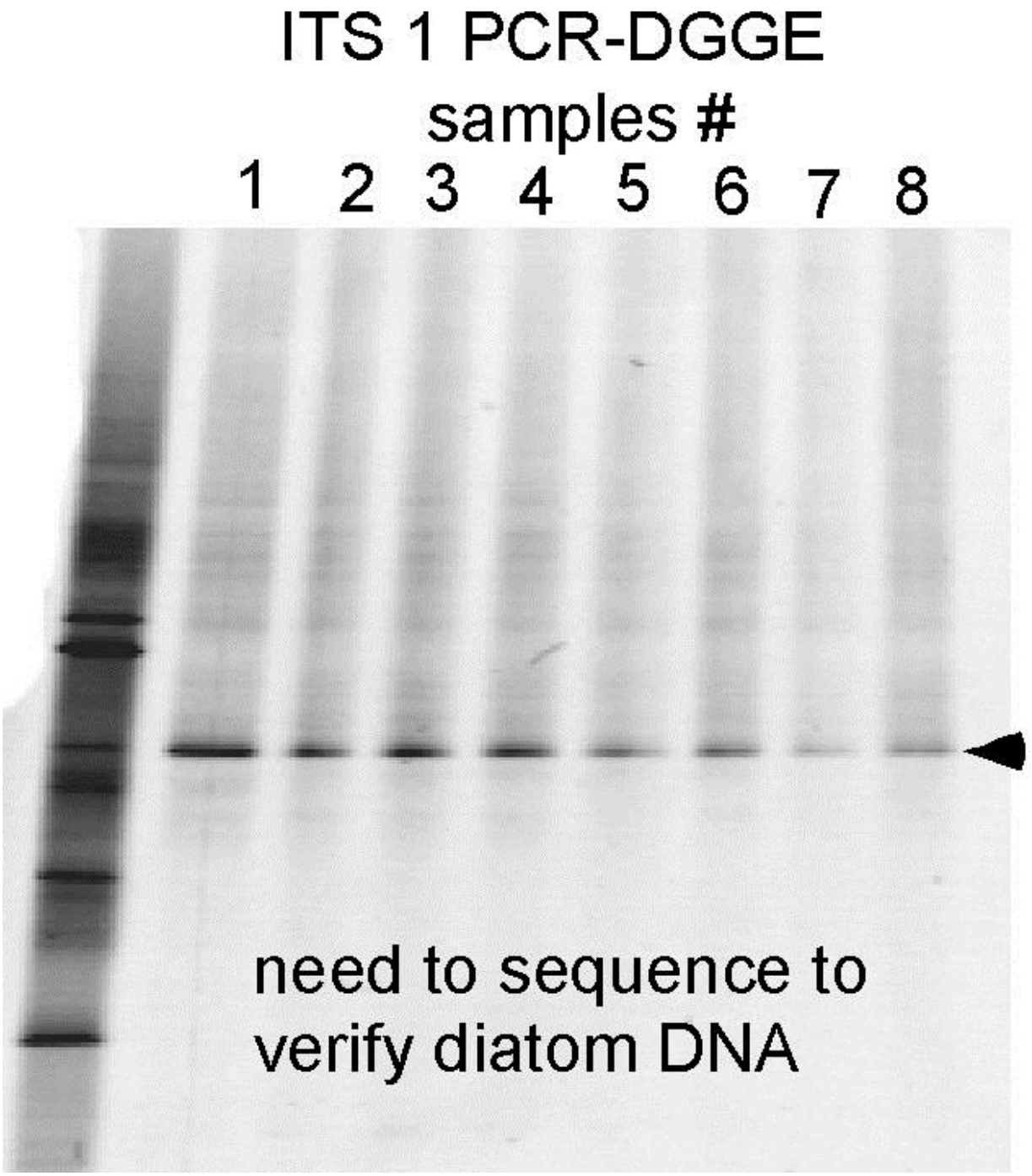


Figure 5.1: The *ITS1* DGGE fingerprint of *A. gibbosa* from different sites in the Western Atlantic. Each sample consisted of the DNA extracted from 10 host specimens. The lanes are: 1) Carysfort Reef 10m, 2) Carysfort Reef 75m, 3) Florida Middle Ground 25m, 4) Navassa Island 25m, 5) Pear Tree Reef, Jamaica 29m, 6) Nancy's Cup of Tea, Little Cayman 12m 7) Juno Beach 18m, and 8) Young Island 20m, St. Vincent and the Grenadines.

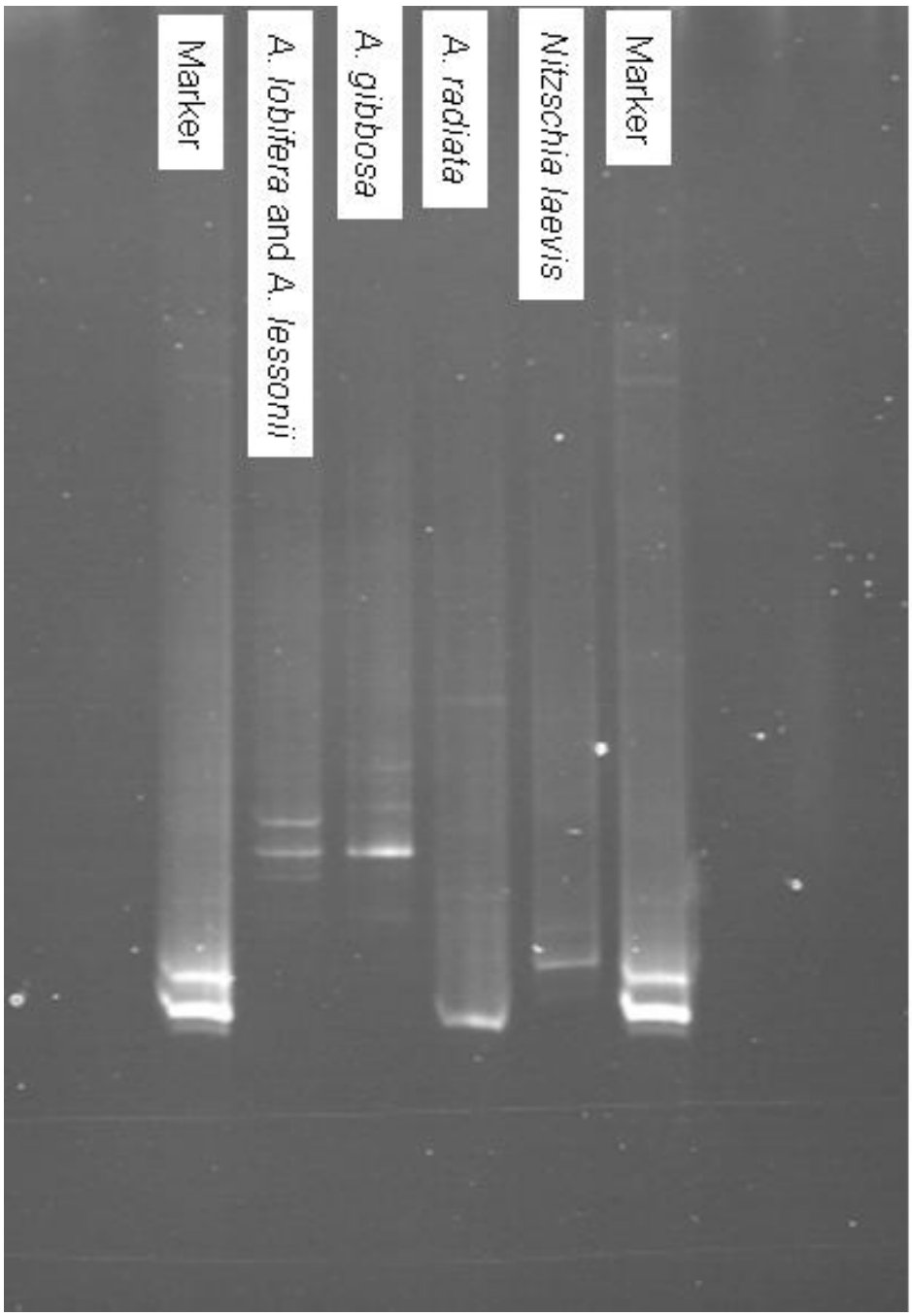


Figure 5.2: Figure 5.2: The rbcL DGGE fingerprint comparing the taxa within the different *Amphistegina* spp. in this study to *Nitzschia laevis*, one of the most commonly cultured diatoms from symbiont-bearing foraminifers. Each sample consisted of the DNA extracted from 10 host specimens. Lanes 1 and 6 are markers, lane 2 is a mixed sample of *Amphistegina lessonii* and *A. lobifera*, lane 3 is *A. gibbosa*, lane 4 is *A. radiata*, and lane 5 is *Nitzschia laevis* culture (provided by J.J. Lee).

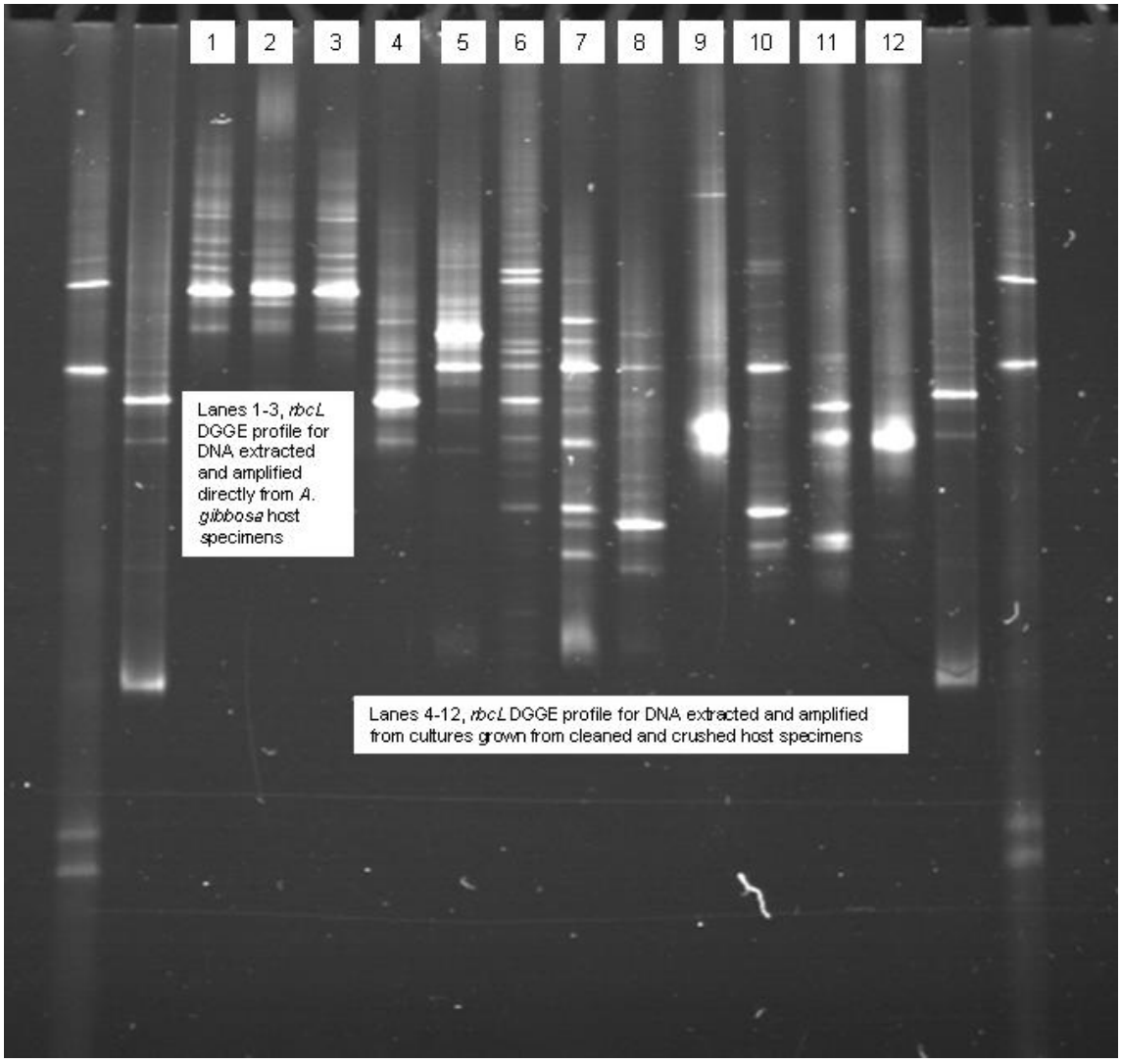
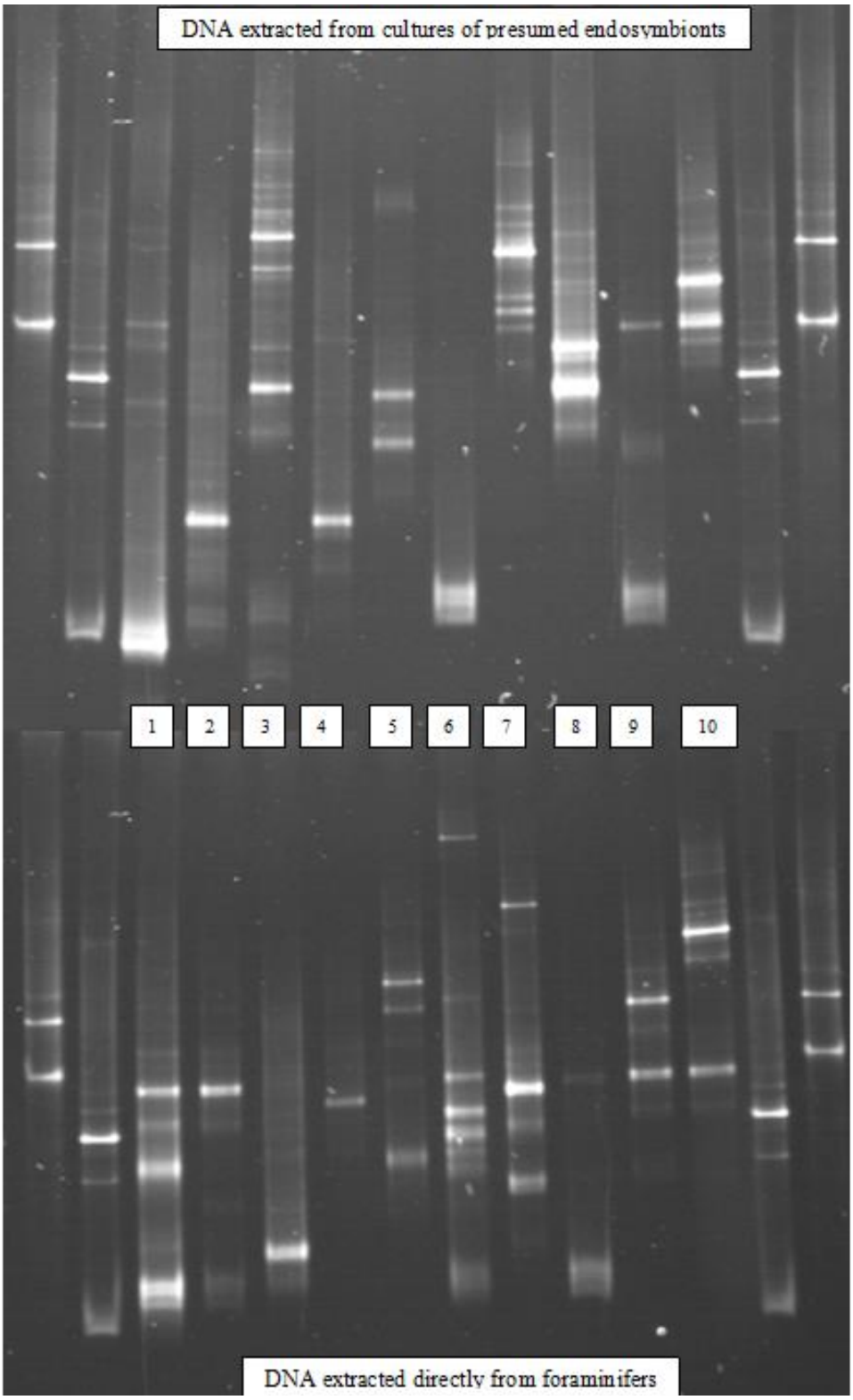


Figure 5.3: Figure 5.3: The rbcL DGGE fingerprint comparing the symbiont taxa within *A. gibbosa* host specimens to presumed symbionts isolated in culture. The first two and last two lanes are marker. The lanes labeled 1, 2, 3 are profiles from DNA extracted directly from 20 host specimens from Navassa Island, Haiti 25 m, 15 host specimens from Young Islands, St. Vincent and Grenadines 20m, and 10 host specimens from Molasses Reef, Florida Keys 27 m (respectively), lanes 4-12 represent three separate DNA extractions from the cultures from each respective site: lanes 4 and 5 are each from seven cultures and lane 6 from six cultures (total=20 cultures from 20 host specimens) from Navassa Islands; lane 7–9 each represent five cultures from Young Island, St. Vincent and the Grenadines (total=15 cultures); and lane 10 is from four cultures and lanes 11 and 12 are each from three cultures from Molasses Reef.



Figures 5.4: The 18S DGGE profiles of DNA extracted from cultures of presumed symbionts (top) compared to extractions directly from their corresponding *A. gibbosa* hosts (bottom). Samples are the same as Figure 5.1 plus 9) Looe Key 10m and 10) Molasses Reef 27m.

CHAPTER 6: DISCUSSION AND IMPLICATIONS FOR FUTURE STUDIES

Summary and Major Findings of Study

My study of the spatial and temporal distribution of diatom endosymbionts in *Amphistegina* spp. was prompted by nearly 40 years of culture-based studies of diatom-bearing species of foraminifers that reported >20 species of diatoms associated with *Amphistegina* spp. (e.g., Lee et al., 1979a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1). Following the discovery and world-wide documentation of bleaching in *Amphistegina* species in the 1990s (Hallock et al., 1993, 2006b; Hallock, 2000b and references therein) and the development of a biotic index based on the abundances of the shells of larger foraminifers, especially *Amphistegina* spp., as an environmental assessment tool (Hallock et al., 2003; Hallock, 2012 and references therein), the importance of understanding if the diatom endosymbionts of *Amphistegina* spp. varied over time, especially with seasons, depth, or across regions, became essential to further understanding how *Amphistegina* populations respond to environmental stressors.

For my dissertation research, I examined *A. gibbosa* specimens collected from >20 sites in the western Atlantic, Caribbean and Gulf of Mexico ranging in depth from 3 m to 75 m between December 1999 to August 2005, *A. radiata* specimens collected from two sites in Papua New Guinea in June 2006, and mixed samples of *A. lessonii* and *A. lobifera* collected from one site in Hawai'i in March 2001 and June 2003. To assess the types of symbionts present within these foraminiferal hosts, I used three different methods: 1) culture-isolation techniques based

upon those developed by Lee et al. (1979a, b) and used consistently since (e.g., Lee et al., 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1) to identify presumed endosymbionts, 2) DNA sequencing of portions of two genes (*rbcL* and *18S*) extracted directly from the foraminiferal hosts, 3) DNA sequencing of portions of *rbcL* and *18S* genes extracted from diatom cultures isolated from the foraminiferal hosts, and 4) PCR and DGGE fingerprinting of fragments of 3 genes (*18S*, *rbcL* and *ITS1*) obtained from DNA extracted directly from the foraminiferal hosts and from cultures. As with most exploratory research, I encountered complications with all methods, particularly in the initial stages.

In the culture isolations, I had problems consistently isolating diatoms from seemingly healthy host specimens, especially from those collected at depths >30 m. I also had problems identifying many of the diatoms (and other microorganisms) cultivated. The most common problems were deformed or unidentifiable specimens, and high densities of sticky organic matter adhering multiple species that prevented obtaining useful images of individuals. Dissolving this organic matter without also destroying the diatom frustules was a delicate balance.

The major problem that I encountered with DNA sequencing involved reliably extracting and amplifying diatom DNA from samples not stored at or below -40° C. Foraminifers and cultures stored for extended periods (> few months) at temperatures higher than -40° C often had a high percentage (or exclusively) non-diatom sequences as opposed to the opposite for fresh or properly stored samples. The DGGE protocol required numerous trials and errors to determine the best primers, concentration of denaturants in gel, amount of sample to load, voltage, and run times to obtain the defined separation between different sequences in the gels. Nevertheless, I was eventually able to produce consistent and repeatable results for all methods.

Based on a review of previous studies, analyses of over 900 cultures isolated, over 2000 DNA sequences from portions of two genes (*18S* and *rbcL*), and DGGE fingerprints of *18S, rbcL* and *ITS1* fragments from DNA extracted either from both hosts and cultures, I can conclude the following:

1) The diatom endosymbionts of *Amphistegina gibbosa, A. lessonii, A. lobifera*, and *A. radiata* are not any of the species of diatoms isolated in culture from *Amphistegina* or any other diatom-bearing foraminifers in previous studies (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; Table 1.1). This conclusion is supported by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences and the *rbcL* DGGE fingerprints of DNA extractions from the host versus those from culture.

2) The diatom endosymbionts of *A. gibbosa, A. lessonii,* and *A. lobifera* are all closely related and within the family Fragilariaceae. An unnamed Fragilariaceae sp. cultured by Matt Ashworth from sand grains collected at a subtidal site at the Florida State University Coastal and Marine Laboratory was the most (or in a few cases second most) similar *rbcL* and *18S* sequence in GenBank to all but a few (< 2%) of the diatom sequences from DNA extracted directly from these three host species. This conclusion is supported by the BLAST search of all the DNA sequences and by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences using NJ, ML, ME, and MP analyses.

3) The diatom endosymbionts of *A. radiata* are also very closely related but completely different from the endosymbionts from the other three species of *Amphistegina* in this study. The diatom sequences from *A. radiata* were most closely related to sequences previously reported from diatoms of the family Plagiogrammaceae. This group contained no species previously

reported as diatom symbionts of Foraminifera. This conclusion is supported by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences and the *rbcL* DGGE.

4) Although within the family Fragilariaceae, the diatom endosymbionts of *A. gibbosa* (Atlantic species) are slightly but significantly different from the symbionts of the two Pacific species, *A. lessonii* and *A. lobifera*, from Oahu, Hawai'i. This conclusion is supported by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences and the *rbcL* DGGE.

5) Depending on the data set and the type of molecular analysis used, there were at least two (DGGE) and up to 5 or 6 (DNA sequences) of slightly but statistically significantly different (interior branch test confidence probabilities, CP >95%) groups of diatoms from the mixed *A. lessonii–A. lobifera* samples. This result is supported by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences and evident in the *rbcL* DGGE, which has two prominent bands.

6) Many of the diatom sequences from *A. gibbosa* specimens collected at the deepest site, Carysfort 75 m, formed a consistent but not statistically significant (CP ≈ 85%) cluster within the entire data set from the Florida Keys. When I reduced the sequence numbers to compare just one set of shallow samples, for instance comparing sequences from hosts collected from the nearby Carysfort 10 m site to those from the Caryforts 75 m site, I saw a clear, statistically significant difference (CP >95%). However, as the number of sequences increased, significance plummeted. This phenomenon has been documented experimentally (Sitnikova et al., 1995; Sitnikova, 1996) as a major drawback of current tests of phylogenetic trees. As the number of sequences increase, the bootstrap values (and to a lesser extent, the interior branch confidence probabilities) become more conservative. This is supported by the phylogenetic analyses of the *rbcL* and *18S* diatom DNA sequences. However, this small difference is not visible in the less precise DGGE fingerprints.

7) Within the large group of nearly identical diatom sequences extracted from *A. gibbosa* specimens, many (but not all) of the *rbcL* sequences from the eastern-most Atlantic site, Young Island, St. Vincent and the Grenadines, formed a non-significant cluster (CP ≈ 80%). As with the samples from the deepest site, if I reduced the data set to just compare these samples to one other group of samples, for instance sequences from Young Island hosts compared to the sequences from hosts from the Florida Middle Grounds site in the Gulf of Mexico, these differences become more pronounced and the confidence values increase. This small difference could be noticed very faintly in the less precise *rbcL* DGGE with very close comparison to the adjacent lanes, but was undetectable with the more conserved *ITS1* profile.

8) No seasonal variations were observed when comparing sequences from hosts collected in the winter versus summer. This conclusion was supported by the phylogenetic analyses of the *rbcL* diatom DNA sequences; there were insufficient data for an *18S* comparison.

9) There was a small, though not statistically significant, difference observed in the *rbcL* diatom sequences from *A. radiata* collected in two geographically separated sites in Papua New Guinea, i.e., Ambitle Island on the Pacific side of PNG compared with Kimbe Bay, New Britain, which is on the Bismarck Sea. As with the small differences noticed between groups of *A. gibbosa,* that geographic-source difference in sequences was less prominent when the entire data set was analyzed.

10) My findings strongly support the *in situ* TEM cell ultrastructure observations reported by Leutenegger (1983, 1984), who noted stable and specific symbiont-host relationships and a similar *Nanofrustulum* (previously *Fragilaria*) *shiloi* -like symbiont in *A. lessonii* and *A. lobifera,* but a morphologically very different symbiont in *A. radiata*.

11) My findings were also congruent with two, more recent molecular studies. Holzmann et al. (2006) observed a stable and specific symbiont-host relationship in nine diatom-bearing nummulitid Foraminifera, in contrast to previous culture studies. Holzmann et al. (2006) noted that the diatom sequences from several species collected at sites >65 m depth formed a cluster, similar to what I observed in *A. gibbosa* collected at the Carysfort 75 m site in my study. Most recently, Schmidt et al. (2015) found only a single diatom from the direct sequencing of PCR products from the diatom-bearing *Pararotalia calcariformata*, despite culturing four different diatom species from these hosts.

Do the Symbionts Reflect the Evolutionary Histories of the Hosts?

The similarities and differences in lineages of endosymbionts of the four species of *Amphistegina*, revealed by molecular techniques, are consistent with what is known of their evolutionary histories. The basic morphological features of the *A. lessonii–A. gibbosa–A. lobifera* group includes prominent trochospiral coiling, a medium to large apertural face and approximately a dozen chambers in the final whorl; these characteristics were recognized in an Upper Eocene-Oligocene species, *A. waiareka* (Larsen, 1978). In contrast, the *A. radiata* morphology is characterized by a lower, tighter trochospiral coil, by smaller, more numerous chambers, a smaller aperture, and dorso-ventral symmetry, such that it appears nearly involute planispiral. Similar morphologies can be traced back to *A. eyrensis* species described from the Eocene of New Zealand (Larsen, 1978). Some time in the future, when the *Amphistegina* spp. can be characterized using molecular techniques, the possibility that these two distinct *Amphistegina* lineages do not merit classification in the same genus should be explored. The differences in their diatom symbionts are evident at the family level, presenting the possibility

that the two *Amphistegina* lineages independently acquired, and co-evolved with, their symbionts. The use of molecular clocks of the evolution histories of the symbionts and host would be an interesting tool to explore this hypothesis in future studies with additional data.

Are Symbionts a Reflection of Habitat?

The differences in the endosymbionts found between the host species might explain their differences in habitats. *Amphistegina radiata* tend to live at greater depths or deeper within reef rubble than is optimum for the *A. lessonii* group. Correspondingly, Leutenegger's study (1984) showed that the diatom symbionts of *A. radiata* had more than 12 chloroplasts per cell compared to 1–2 per cell in the symbionts of *A. lessonii* and *A. lobifera*. More chloroplasts translate to higher potential for light capture, an essential characteristic to thrive in deeper water (Hallock, 1999). At the same time, more chloroplasts could render *A. radiata* more sensitive to photo-inhibition, as noted by Walker et al. (2011). Such close and synergistic relationships between host and symbiont are very unlikely to have coevolved among/between multiple nonspecific and fluid partners as previously suggested (e.g., Lee and Hallock, 1987). Chai and Lee (1999, 2000) postulated that cell surface proteins on certain diatoms and receptors on the reticulopodia of diatom-bearing foraminifers help differentiate which species are friends and which are food, but the evidence was circumstantial.

Inherent Limitations of Culturing Methods

My findings are not surprising in the current realm of microbiology. Microbiologists have shifted away from utilizing culturing techniques, at least supplementing them with molecular and physiological techniques, for identifying and characterizing microbes, both prokaryotes and

eukaryotes (such as tiny endosymbiotic diatoms). As the potential for artificially selecting for some micro-organisms over others and, more importantly, the fact that some microbes are uncultivable using currently known methods, has become widely recognized and as molecular techniques have become more affordable, culture studies alone have become more suspect.

My findings clearly show that molecular identity of diatoms cultured in this and previous studies do not match the genetic identity of the diatom DNA extracted directly from the *Amphistegina* hosts. My findings, and those of Holzmann et al. (2006), indicate that the endosymbionts of the four *Amphistegina* species in this study and the nine nummulitid species their study have yet to be successfully cultured. McCoy (2004) had the same problem in his numerous attempts to culture the diatom symbionts of flatworms (platyhelminths) during his doctoral dissertation research.

Of the 2000+ diatom sequences I obtained directly from *Amphistegina* specimens, >98% of them were unequivocally different from all of the diatoms that have been reported as endosymbionts of Foraminifera, which have sequences available in GenBank or that I sequenced from reference cultures provided by J. J. Lee. Sequences for all of the taxa previously identified as diatom endosymbionts were used in the phylogenetic analyses. If *18S* or *rbcL* sequences were not available for species previously identified as diatom endosymbionts from culture studies, I included data for members of the same genus. Utilizing two different approaches (i.e., DNA sequencing of two genes and DGGE fingerprinting of three genes), I was able to determine that the DNA extracted directly from the host consisted of a single, as yet to be identified "species" each for *A. gibbosa* and *A. radiata,* with 2–5 closely related species or subspecies for *A. lessonii* and *A. lobifera*. None of the diatom sequences directly from these host specimens matched any

sequences of presumed symbionts in GenBank, our cultures, or the reference cultures provided by J.J. Lee.

The Case of the Missing Endosymbionts (Solved)

During the initial stages of my culture studies (Chapter 2), I was often unable to culture diatoms from seemingly healthy *Amphistegina* specimens. During that time, I vigorously and painstakingly cleaned each foraminifer before crushing it an attempt to expel and grow its endosymbionts, as described in previously established protocols, and to prepare and preserve the other portion for future DNA extraction and sequencing. After many failed attempts at culturing diatom endosymbionts, I attempted to grow "endosymbionts" from several uncleaned specimens and discovered that many of the same diatom "endosymbionts" observed in previous studies (e.g., Lee et al., 1979a, 1989, 1992, 1995a) finally grew when the foraminifers were not cleaned. According to prior studies, these diatoms were "absent" or "extremely rare" outside the hosts (e.g., Lee et al., 1989; Chai and Lee, 1999). So, I concluded at the time that my vigorous cleaning methods were destroying the cells of the diatom endosymbionts, which reside just below the surface of the test. When I reduced the cleaning intensity, I was able to successfully grow many of the same diatom taxa observed in prior studies (e.g., Lee et al., 1980a, 1989, 1992).

Moreover, I was unable to culture diatoms from all but a few of the *A. gibbosa* specimens collected from depths ≥50 m or greater, despite not cleaning some of these deepwater specimens. Epiphytic diatoms at these depths are likely less common because of reduced light conditions, which probably led to my lack of "symbiont" cultures from those samples. Nevertheless, I was able to readily and consistently extract and sequence the DNA of the Fragilariaceae symbiont

directly from these hosts, whether I obtained a culture or not. As the molecular evidence indicates, the endosymbionts were likely uncultivatable using the methods I employed, and my later "successful" cultures were likely epiphytic diatoms not removed by cleaning that thrived in the culture media.

Suggestions for Future Studies

Knowing what I know now, i.e., that the diatom endosymbionts are essentially a monoculture within each foraminiferal host, I could have skipped many time consuming and expensive steps in my process of determining the "assemblage" of symbionts within *Amphistegina*. I could have directly sequenced the PCR products from the host as Schmidt *et al*. (2015) did with *Pararotalia calcariformata*, and likely would have gotten clean sequence data just as they did. In hindsight, my protocol could have been essentially four simple steps: 1) clean the foraminifers vigorously with fine paint brushes as described previously, 2) extract the DNA by pulverizing, then bead beating in an extraction buffer, 3) PCR amplification of extract using an algal-specific primer, such as *rbcL*, and 4) send the unpurified PCR products to an outside lab, such as Macrogen, for purification and sequencing. This would have cut cost and time by more than 90%.

However, hindsight is 20/20, and I chose to start with an expensive DNA extraction kit (Qiagen Plant Mini kit) because other researchers (e.g., Pawlowski et al., 2001a, b) successfully used them with other species of Foraminifera. I also assumed, based on numerous publications utilizing culture isolations (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1) and my earlier culture results, that indeed I was dealing with multiple diatom endosymbionts even within a single individual. Therefore, I thought I needed to use

expensive cloning kits to separate these 20+ species of diatom "endosymbionts" to get good clean sequence data, because direct sequencing of PCR products containing multiple taxa would have only produced noisy, unusable data with multiple signals.

Based on my experiences, I recommend that a more streamlined molecular approach can be used as a starting point for future examinations of *Amphistegina* and other species of algal-bearing Foraminifera. The identity of the symbionts of any foraminifers primarily based on culture isolations should be re-examined using alternative methods, because culture data have proven to be highly questionable in light of this study in conjunction with others (Leutenegger, 1983, 1984; Holzmann et al., 2006; Schmidt et al., 2015). For instance, an assessment of an unstudied, potentially diatom-bearing species, such as *Asterigerina carinata*, could be carried out much more quickly and inexpensively.

Where is the Foraminiferal Host's DNA?

As an ancillary benefit of using "universal" *18S* primers, I had hoped to also find some foraminiferal DNA that I could use for the phylogeny of the various species of *Amphistegina* and to examine whether there were any biogeographical trends within *Amphistegina gibbosa*, for which I had the most data. I did not expect to find many host DNA sequences, because the foraminiferal hosts are single-celled (sometime multinucleate) organisms (McEnery and Lee, 1981), so they have only a few copies of the targeted gene from the host compared to the thousands of copies from the many symbionts cells. However, the BLAST search of all *18S* sequences revealed that none of them were similar to any foraminiferal DNA in GenBank. Most of the non-diatom sequences were most similar to slime molds, fungi, and "uncultured marine organisms." There are not very many foraminiferal sequences in GenBank, and most of the

phylogeny is based on morphology. So, the *Amphistegina* DNA sequences could very well be hidden among the "non-diatom" sequences that I excluded from my phylogenetic analyses. However, it is more likely that no foraminiferal DNA was sequenced, because it was relatively rare compared to that for the symbionts.

Another possibility is that the DNA extraction, PCR primers, or any other molecular method down the line failed to work with the foraminiferal DNA. There are examples of so-called "universal" *18S* primers failing to work on large conspicuous organisms (Meyers et al., 2010) and an even larger portion of many small understudied eukaryotes may be missed (Stoeck et al., 2006; Wang et al., 2014) by "universal" primers. If *Amphistegina* is among these groups, then PCR methods and subsequent sequencing would simply fail to detect it. The search for the DNA sequences of the *Amphistegina* hosts remains a topic of future research.

Why did the same diatom taxa keep showing up in "symbiont" cultures?

Despite over 200 genera and 10,000 species of diatoms (Round et al., 1990; Mann, 1999), in past studies, only about 25 species of diatoms were isolated from over 3000 culture isolations from *Amphistegina* and other diatom-bearing larger foraminifers (Lee, 2011a, b). Over 75% of those diatoms were in one of the four genera *Nitzschia*, *Navicula*, *Fragilaria* (including the *Nanofrustulum*), and *Amphora* (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a; Lee and Correia, 2005; see Table 1.1). With so many species, why were the same taxa being cultured so consistently from *Amphistegina* and other diatom-bearing Foraminifera, such as *Nitzschia frustulum* found in ≈30% of the isolations? The fact that those past studies only cultured a relatively small number of diatoms from *Amphistegina* and other diatom-bearing foraminifers

seemed to support the hypothesis that these diatoms were endosymbionts. However, there are numerous alternative explanations including:

1) The consistently cultured diatoms could be species that thrive in the artificially created environment. Cultures are known to select for particular species over others. We know very little about the micro-environment in which the symbionts have evolved to reside, much less about how to re-create it in the lab to culture the diatoms outside their host.

2) The consistently cultured diatoms could be common benthic diatoms in tropical oceans. The host specimens in this and previous studies were collected from similar tropical marine habitats. Therefore, it is reasonable that the benthic diatom assemblages also share similarities. Previous studies concluded that the diatoms cultured from *Amphistegina* and other diatom-bearing foraminifers are "extremely rare" in the environments where the host specimens were collected (e.g., Lee et al. 1989; Chai and Lee, 2000; Lee, 2011a, b). However, the diatoms presumed to be endosymbionts were grown in enriched media from crushed foraminifers but were compared to untreated substrate or scrapings from them, that were just simply prepared for SEM to determine what diatoms were present (Lee et al., 1989). For the comparison to be valid, the substrate or scrapings should also have been brewed in enriched media to determine if what was grown from the hosts can also be grown from the substrate.

3) The consistently cultured diatoms could be favored prey.

4) The consistently cultured diatoms are small and sticky, and can be easily missed during cleaning. Holzmann et al. (2006) noted finding diatoms clinging to the surface of foraminifers even after cleaning. Even after vigorous cleaning, it's impossible to see if small, sticky diatoms are in the cracks, crevices, or on the surface of the foraminifer, without the aid of Scanning Electon Microscope (SEM), which destroys the sample. Lee and Correia (2005)

cultured diatoms from several species of Foraminifera, and the *Calcarina* specimens yielded the most diverse assemblage of cultured diatoms, 14 species and varieties of diatoms were cultured from 30 host specimens. The calcarinid foraminifers are characterized by spines and numerous pits in their test that create many "hiding spots" for epiphytic diatoms, making them harder to clean than other, smoother diatom-bearing Foraminifera.

5) Less common diatom taxa could be easily missed (or grouped with more common taxa) when scanning through an SEM stub that might contain thousands of diatoms. One of the issues I encountered during my culture studies was trying to identify some species that had widely different morphologies or possible deformities, so I resorted to genus-level groupings and a convenient "other" category. However, there were likely more than 25 species in my roughly 900 samples. Additionally, I cultured some genera of diatoms (such as *Cyclotella* and *Cymatosira*) and non-diatom species not previously reported as endosymbionts. In previous culture isolation studies, "new species" or "new varieties" were added with every subsequent study (e.g., Lee et al., 1979a, 1980a, 1989, 1992, 1995a, Lee and Correia, 2005; see Table 1.1). Additionally, in data from past studies, different host species and different sites were pooled and compared to later studies, giving the impression of consistency when, in fact, new species were being added (e.g. Lee et al., 1989, 1992, 1995a). Lee et al. (1995a and others) discussed deformities and classified some specimens down to species level that did not appear to resemble the typical members of that species. There is the strong possibility that different species were being lumped into familiar species categories because of the imprecise nature of morphological identification.

Shifting Paradigms, the Scientific Process, and Closing Thoughts

The scientific process involves testing and retesting of hypotheses to support or reject those hypotheses. Nevertheless, the history of science provides numerous examples of valid observations that were formulated into hypotheses that were subsequently ignored or even suppressed for decades, generations, centuries, or even millennia. Aristotle, in the 3rd century BC, recognized the significance of fossil shells and their implications for the antiquity of the Earth; Leonardo da Vinci also recognized the difference between fossil and modern shells roughly 500 years ago; there are still millions of people, including some scientists, that deny the concept of Evolution and the antiquity of the Earth. Alfred Wegner recognized that South America and Africa seemed to fit together like puzzle pieces and in 1912 proposed that they had once been connected. Despite strong stratigraphic evidence in the similarity of rocks on both sides of the Atlantic Ocean, the theory of Plate Tectonics did not find acceptance until the 1960s. At the same time that Wegener as being ridiculed for his ideas, Ivan Wallin, a microbiologist, proposed that mitochondria in eukaryotic cells had originated as free-living bacteria, and was similarly universally dismissed. Yet by the 1970s, thanks to electron microscopy in particular, the hypothesis that eukaryotic cells originated from the symbioses of at least two kinds of prokaryotes and that algae and higher plants from at least three, began to be accepted.

While certainly not as important as Aristotle's hypothesis of the antiquity of the Earth, Darwin's theory of evolution, Wegener's continental drift, or Wallin's hypothesis regarding cell evolution, Leutenegger's (1983, 1984) hypothesis that the diatom symbionts in the *Amphistegina* spp. that she examined were species specific has largely been ignored over the past several decades. As a consequence, paleontologists and biologists studying the biology, ecology, paleoecology and evolution of the foraminifers that host algal endosymbionts have not benefited

from the understanding of the host-symbiont specificity and have erroneously assumed that the relationship, especially in diatoms, was only facultative for the symbiont.

With advancements in technology, the shift from using one or a few genes (phylogenetics) to using entire genomes or large portions of it (phylogenomics) may show that the symbionts of *Amphistegina* and other diatom-bearing foraminifers are more diverse than my results indicate. More advanced methods or further examination could also reveal biases in the DNA extraction, PCR, cloning, or sequencing methods.

Moreover, future studies may reveal that the diatom taxa that have commonly been found in culture studies are important food items of the *Amphistegina*, perhaps their growth is even promoted by the waste products of *Amphistegina*. Thus, they could still be important associates, even if they are clearly not the primary endosymbionts.

My findings support the original Leutenegger (1983, 1984) observations and are consistent with the findings of Holzmann et al. (2006) and Schmidt et al. (2015), that the diatom symbionts are specific to their host species. As a consequence, suites of new hypotheses can be proposed and tested regarding the primary endosymbionts and their roles in the biology, ecology and evolution of foraminifers with diatom symbionts. At the same time, the description and identification of new benthic diatom species, which emerged from the emphasis on culturing the foraminiferal associates, has implications for understanding the microhabitats that the foraminifers occupy and that they provide for other micro-organisms.

And while ideally the hypotheses and observations of some scientists should not be ridiculed, suppressed or simply ignored by other scientists, science is carried out by humans, and humans too often ridicule, suppress or simply ignore valid findings. One can hope that the findings of this dissertation research might influence other researchers to look more carefully at

the assumptions and conclusions upon which they are building their research. Unfortunately, much effort can be wasted when the research, upon which a prevailing hypothesis is based, has excluded or ignored the existence of contradictory observations.

REFERENCES

Baker, A. C. (2001). Ecosystems—Reef Corals Bleach to Survive Change. *Nature, 411*(6839), 765-766.

Baker, R. D., Hallock, P., Moses, E. F., Williams, D. E., & Ramirez, A. (2009). Larger Foraminifers of the Florida Reef Tract, USA: Distribution Patterns on Reef-Rubble Habitats. *Journal of Foraminiferal Research, 39*(4), 267-277.

Berthold, W. U. (1978). Ultrastructure Analysis of Endoplasmic Algae in *Amphistegina lessonii* d'Orbigny, Foraminifera (Protozoa) and their Systematic Position. *Archiv für Protistenkunde, 120*(1-2), 16-62.

Beszteri, B., Acs, E., Makk, J., Kovacs, G., Marialigeti, K., & Kiss, K. T. (2001). Phylogeny of Six Naviculoid Diatoms Based on 18S rDNA Sequences. *International Journal of Systematic and Evolutionary Microbiology, 51*(4), 1581-1586.

Brown, B. (1997). Coral Bleaching: Causes and Consequences. *Coral Reefs, 16*(1), S129-S138.

Buddemeier, R. W., & Fautin, D. G. (1993). Coral Bleaching as an Adaptive Mechanism. *Bioscience, 43*(5), 320-326.

Carr, L., & Mendelsohn, R. (2003). Valuing Coral Reefs: A Travel Cost Analysis of the Great Barrier Reef. *AMBIO: A Journal of the Human Environment*, 32(5), 353-357.

Cesar, H., Burke, L., & Pet-Soede, L. (2003). The Economics of Worldwide Coral Reef Degradation. Retrieved from http://eprints.uberibz.org/id/eprint/48.

Chai, J. Y., & Lee, J. J. (1999). Initial Recognition of Endosymbiotic Diatom Surface Antigens by the Larger Foraminifer *Amphistegina lobifera*. *Symbiosis, 26*(1), 39-53.

Chai, J. Y., & Lee, J. J. (2000). Recognition, Establishment and Maintenance of Diatom Endosymbiosis in Foraminifera. *Micropaleontology, 46*, 182-195.

Chesnick, J. M., Kooistra, W. H., Wellbrock, U., & Medlin, L. K. (1997). Ribosomal RNA Analysis Indicates a Benthic Pennate Diatom Ancestry for the Endosymbionts of the Dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrhophyta). *Journal of Eukaryotic Microbiology, 44*(4), 314-320.

Chesnick, J. M., Morden, C. W., & Schmieg, A. M. (1996). Identity of the Endosymbiont of *Peridinium foliaceum* (Pyrrophyta): Analysis of the rbcLs Operon. *Journal of Phycology, 32*(5), 850-857.

Costanza, R., d'Arge, R., de Groot, R., Faber, S., Grasso, M., Hannon, B., . . . & van den Belt, M. (1997). The Value of the World's Ecosystem Services and Natural Capital. *Nature. 387*: 253-260.

Diez, B., Pedros-Alio, C., Marsh, T. L., & Massana, R. (2001). Application of Denaturing Gradient Gel Electrophoresis (DGGE) to Study the Diversity of Marine Picoeukaryotic Assemblages and Comparison of DGGE with other Molecular Techniques. *Applied and Environmental Microbiology, 67*(7), 2942-2951.

du Buf, H., & Bayer, M. M. (2002). ADIAC Achievements and Future Work. In H. du Buf & M. M. Bayer (Eds.), *Automatic Diatom Identification, Series in Machine Perception and Artificial Intelligence* (Vol. 51, pp. 289-298). River Edge, NJ: World Scientific.

Edgar, R. C. (2004). MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Research, 32*(5), 1792-1797.

Ehrlich, P. R. (1975). The Population Biology of Coral Reef Fishes. *Annual Review of Ecology and Systematics*, 211-247.

Fisher, E. M., Fauth, J. E., Hallock, P., & Woodley, C. M. (2007). Lesion Regeneration Rates in Reef-Building Corals *Montastraea* spp. as Indicators of Colony Condition. *Marine Ecology-Progress Series, 339*, 61-71.

Gast, R. J., & Caron, D. A. (2001). Photosymbiotic Associations in Planktonic Foraminifera and Radiolaria. *Hydrobiologia, 461*, 1-7.

Gattuso, J.-P., Allemand, D., & Frankignoulle, M. (1999). Photosynthesis and Calcification at Cellular, Organismal and Community Levels in Coral Reefs: A Review on Interactions and Control by Carbonate Chemistry. *American Zoologist, 39*(1), 160-183.

Glynn, P. W. (1996). Coral Reef Bleaching: Facts, Hypotheses and Implications. *Global Change Biology, 2*(6), 495-509.

Goldstein, S. T., (1999). Foraminifera: A Biological Overview. In Sen Gupta, B.K. (Ed.) *Modern Foraminifera* (pp. 37-55). Norwell, MA: Kluwer Academic Publishers.

Guo, L., Sui, Z., Zhang, S., Ren, Y., & Liu, Y. (2015). Comparison of Potential Diatom 'Barcode' Genes (the 18S rRNA Gene and ITS, COI, rbcL) and their Effectiveness in Discriminating and Determining Species Taxonomy in the Bacillariophyta. *International Journal of Systematic and Evolutionary Microbiology, 65*(4), 1369-1380.

Hallock, P. (1981a). Production of Carbonate Sediments by Selected Large Benthic Foraminifera on 2 Pacific Coral Reefs. *Journal of Sedimentary Petrology, 51*(2), 467-474.

Hallock, P. (1981b). Algal Symbiosis: A Mathematical Analysis. *Marine Biology 62*:249-255.

Hallock, P. (1999). Symbiont-Bearing Foraminifera. In Sen Gupta, B.K. (Ed.) *Modern Foraminifera* (pp. 123-139). Norwell, MA: Kluwer Academic Publishers.

Hallock, P. (2000a). Larger Foraminifera as Indicators of Coral-Reef Vitality. In Martin, R.E., (Ed.) *Environmental Micropaleontology* (pp. 121-150). New York, NY: Springer US.

Hallock, P. (2000b). Symbiont-Bearing Foraminifera: Harbingers of Global Change? *Micropaleontology, 46*, 95-104.

Hallock, P. (2011). FORAM Index. In D. Hopley (Ed.), *Encyclopedia of Modern Coral Reefs: Structure, Form and Process* (pp. 415-416). Dordrecht, Netherlands: Springer Netherlands.

Hallock, P. (2012). The FORAM Index Revisited: Uses, Challenges, and Limitations. *Proceedings, 12th International Coral Reef Symposium, Cairns, Australia*. Retrieved from http://www.icrs2012.com/proceedings/manuscripts/ICRS2012_15F_2.pdf

Hallock, P., Forward, L. B., & Hansen, H. J. (1986). Influence of Environment on the Test Shape of *Amphistegina*. *Journal of Foraminiferal Research, 16*(3), 224-231.

Hallock, P., H. K. Talge, K. Smith and E. M. Cockey. (1993). Bleaching in a Reef-Dwelling Foraminifer, *Amphistegina gibbosa*. *Proceedings, 7th International Coral Reef Symposium, Guam, June 1992*, pp. 42-47.

Hallock, P., Talge, H. K., Cockey, E. M., & Muller, R. G. (1995). A New Disease in Reef-Dwelling Foraminifera—Implications for Coastal Sedimentation. *Journal of Foraminiferal Research, 25*(3), 280-286.

Hallock, P., Lidz, B. H., Cockey-Burkhard, E. M., & Donnelly, K. B. (2003). Foraminifera as Bioindicators in Coral Reef Assessment and Monitoring: The FORAM Index. *Environmental Monitoring and Assessment, 81*(1-3), 221-238.

Hallock, P., Sheps, K., Chapronière, G., & Howell, M. (2006a). Larger Benthic Foraminifers of the Marion Plateau, Northeastern Australia (ODP Leg 194): Comparison of Faunas from Bryozoan (Sites 1193 and 1194) and Red Algal (Sites 1196–1198) Dominated Carbonate Platforms. In *Proceeding of ODP, Scientific Results* (Vol. 194, pp. 1-31). Retrieved from http://www-odp.tamu.edu/publications/194_SR/009/009_.htm.

Hallock, P., D.E. Williams, S.K. Toler, E.M. Fisher, and H.K. Talge. (2006b). Bleaching in Reef-Dwelling Foraminifers: Implications for Reef Decline. *Proceedings, 10th International Coral Reef Symposium, Okinawa, Japan, June 2004*. pp. 729-737.

Harney, J. N., Hallock, P., & Talge, H. K. (1998). Observations on a Trimorphic Life Cycle in *Amphistegina gibbosa* Populations from the Florida Keys. *Journal of Foraminiferal Research, 28*(2), 141-147.

Hasle, G. R., & Syvertsen, E. E. (1997). Marine Diatoms. In C. R. Tomas (Ed.), *Identifying Marine Phytoplankton* (pp. 5-385). San Diego, CA: Academic Press.

Hoegh-Guldberg, O. (1999). Climate Change, Coral Bleaching and the Future of the World's Coral Reefs. *Marine and freshwater research, 50*(8), 839-866.

Holzmann, M., Berney, C., & Hohenegger, J. (2006). Molecular Identification of Diatom Endosymbionts in Nummulitid Foraminifera. *Symbiosis, 42*(2), 93-101.

Holzmann, M., & Pawlowski, J. (1996). Preservation of Foraminifera for DNA Extraction and PCR Amplification. *Journal of Foraminiferal Research, 26*(3), 264-267.

Hustedt, F. (1955). *Marine Littoral Diatoms of Beaufort, North Carolina*. Durham, NC: Seeman Printery.

Jackson, J. B. (1997). Reefs Since Columbus. *Coral Reefs, 16*(1), S23-S32.

Jalba, A. C., Wilkinson, M. H. F., Roerdink, J., Bayer, M. M., & Juggins, S. (2005). Automatic Diatom Identification Using Contour Analysis by Morphological Curvature Scale Spaces. *Machine Vision and Applications, 16*(4), 217-228.

Koestler, R. J., Lee, J. J., Reidy, J., Sheryll, R. P., & Xenophontos, X. (1985). Cytological Investigation of Digestion and Reestablishment of Symbiosis in the Larger Benthic Foraminifera *Amphistegina lessonii*. *Endocytobiosis and Cell Research, 2*(1), 21-54.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution, 33*(7): 1870-1874.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics, 5*, 150-163.

Kushmaro, A., Loya, Y., Fine, M., & Rosenberg, E. (1996). Bacterial Infection and Coral Bleaching. *Nature, 380*(6573), 396-396.

LaJeunesse, T. C., & Pinzon, J. H. (2007). Screening Intragenomic rDNA for Dominant Variants can Provide a Consistent Retrieval of Evolutionarily Persistent ITS (rDNA) Sequences. *Molecular Phylogenetics and Evolution, 45*(1), 417-422.

Langer, M. R., Silk, M. T., & Lipps, J. H. (1997). Global Ocean Carbonate and Carbon Dioxide Production; the Role of Reef Foraminifera. *Journal of Foraminiferal Research, 27*(4), 271-277.

Larsen, A. R. (1978). Phylogenetic and Paleobiogeographical Trends in the Foraminiferal Genus *Amphistegina*. *Revista Española de Micropaleontología, 10*(2), 217-243.

Lee, J. J. (1983). Perspective on Algal Endosymbionts in Larger Foraminifera. *International Review of Cytology, Supplement 14*, 49-77.

Lee, J. J. (1992). Isolation of Endosymbiotic Algae from Larger Foraminifera. *Protocols in Protozoology*, A5.1-A5.4.
Lee, J. J. (1993). On a Piece of Chalk—Updated. *Journal of Eukaryotic Microbiology, 40*(4), 395-410.
Lee, J. J. (1995). Living Sands—The Symbiosis of Protists and Algae can Provide Good Models for the Study of Host Symbiont Interactions. *Bioscience, 45*(4), 252-261.
Lee, J. J. (1998). "Living Sands"—Larger Foraminifera and their Endosymbiotic Algae. *Symbiosis, 25*(1-3), 71-100.
Lee, J. J. (2002). Living Sands: Symbiosis Between Foraminifera and Algae. In J. Seckbach (Ed.), *Symbiosis: Mechanisms and Model Systems* (pp. 489-506). Dordrecht, Netherlands: Springer Netherlands.
Lee, J. J. (2006). Algal Symbiosis in Larger Foraminifera. *Symbiosis, 42*(2), 63-75.
Lee, J. J. (2011a). Fueled by Symbiosis, Foraminifera have Evolved to be Giant Complex Protists. In J. Seckbach & Z. Dubinsky (Eds.), *All Flesh Is Grass: Plant-Animal Interrelationships* (pp. 427-452). Dordrecht, Netherlands: Springer Netherlands.
Lee, J. J. (2011b). Diatoms as Endosymbionts. In J. Seckbach & P. Kociolek (Eds.), *The Diatom World* (pp. 437-464). Dordrecht, Netherlands: Springer Netherlands.
Lee, J. J., & Anderson, O. R. (1991). Symbiosis in Foraminifera. In J.J. Lee & O.R. Anderson (Eds.), *Biology of Foraminifera.* (pp. 157-220) San Diego, CA: Academic Press.
Lee, J. J., & Corliss, J. O. (1985). Symposium on "Symbiosis in Protozoa": Introductory Remarks. *Journal of protozoology*, *32*(3), 371-372.
Lee, J. J., & Correia, M. (2005). Endosymbiotic Diatoms from Previously Unsampled Habitats. *Symbiosis, 38*(3), 251-260.
Lee, J. J., & Ellis, R. (1982). A Comparative Study of Photo Adaptation in 4 Diatoms Isolated as Endosymbionts from Larger Foraminifera. *Marine Biology (Berlin), 68*(2), 193-198.
Lee, J. J., & Hallock, P. (1987). Algal Symbiosis as the Driving Force in the Evolution of Larger Foraminifera. *Annals of the New York Academy of Sciences, 503*, 330-347.
Lee, J. J., & McEnery, M. E. (1983). Symbiosis in Foraminifera. In L.J. Goff (Ed.), *Algal Symbiosis: A Continuum of Interaction Strategies*, (pp. 37-66). New York, NY: Cambridge University Press.
Lee, J. J., & Reimer, C. W. (1982). Isolation and Identification of Endosymbiotic Diatoms From Larger Foraminifera of the Great Barrier Reef, Australia, Makapuu Tide Pool, Oahu, Hawaii, and the Gulf of Elat, Israel, with a Description of Three New Species *Amphora roettgerii*, *Navicula hanseniana* and *Nitzschia frustulum* var. symbiotica. In D. G. Mann (Ed.), *Proceedings 7th International Diatom Symposium* (pp. 327-343). Koenigstein, Germany: Otto Koeltz.
Lee, J. J., & Reimer, C. W. (1990). Unusual Endosymbiotic Diato-ms Isolated from Larger Foraminifera From Semitropical Pacific Habitats. *Journal of Phycology, 26*(2 SUPPL), 6-6.
Lee, J. J., Müller-Merz, E., Hagen, J., McEnery, M., Mastropa. C, & Garrison, J. R. (1974). Symbiosis in Benthic Foraminifera (Abstract). *Journal of Protozoology, 21*(3), 437-437.
Lee, J. J., McEnery, M. E., Kahn, E., & Shuster, F. (1977). Symbiosis and Evolution of Larger Foraminifera. *Journal of Paleontology, 51*(2), 17-17.
Lee, J. J., McEnery, M., Rottger, R., & Reimer, C. (1979a). Isolation, Culture, and Identification of Endosymbiotic Diatoms from *Heterostegina depressa* and *Amphistegina lessonii* (Larger Foraminifera) from an Hawaiian Tide Pool. *Journal of Phycology, 15*, 18-18.

Lee, J. J., McEnery, M. E., Shilo, M., & Reiss, Z. (1979b). Isolation and Cultivation of Diatom Symbionts from Larger Foraminifera (Protozoa). *Nature, 280*(5717), 57-58.
Lee, J. J., McEnery, M. E., Kahn, E. G., & Schuster, F. L. (1979c). Symbiosis and the Evolution of Larger Foraminifera. *Micropaleontology, 25*(2), 118.
Lee, J. J., Reimer, C. W., & McEnery, M. E. (1980a). Identification of Diatoms Isolated as Endosymbionts from Larger Foraminifera from the Gulf-of-Eilat (Red-Sea) and the Description of 2 New Species, *Fragilaria shiloi* sp. nov. and *Navicula reissii* sp. nov. *Botanica Marina, 23*(1), 41-48.
Lee, J. J., McEnery, M. E., Rottger, R., & Reimer, C. W. (1980b). The Isolation, Culture and Identification of Endosymbiotic Diatoms from *Heterostegina depressa* d'Orbigny and *Amphistegina lessonii* d'Orbigny (Larger Foraminifera) from Hawaii. *Botanica Marina, 23*(5), 297-302.
Lee, J. J., McEnery, M. E., Lee, M. J., Reidy, J. J., Garrison, J. R., & Röttger, R. (1980c). Algal Symbionts in Larger Foraminifera. In W. Schwemmler & H Schenk (Eds.) *Endocytobiology: Endosymbiosis and Cell Biology.* (pp. 113-124) Berlin, Germany: Walter de Gruyter.
Lee, J. J., McEnery, M. E., & Garrison, J. R. (1980d). Experimental Studies of Larger Foraminifera and their Symbionts from the Gulf of Elat on the Red Sea. *Journal of Foraminiferal Research, 10*(1), 31-47.
Lee, J. J., McEnery, M. E., Koestler, R., & Lee, M. J. (1982). Experimental Studies of Symbiont Preference in Diatom-Bearing Species of Larger Foraminifera from the Red-Sea. *Journal of Protozoology, 29*(3), 493-493.
Lee, J. J., Saks, N. M., Kapiotou, F., Wilen, S. H., & Shilo, M. (1984). Effects of Host-Cell Extracts on Cultures of Endosymbiotic Diatoms from Larger Foraminifera. *Marine Biology, 82*(2), 113-120.
Lee, J. J., Erez, J., McEnery, M. E., Lagziel, A., & Xenophontos, X. (1986). Experiments on Persistence of Endosymbiotic Diatoms in the Larger Foraminifer *Amphistegina lessonii*. *Symbiosis, 1*(3), 211-226.
Lee, J. J., Chan, Y., & Lagziel, A. (1988a). An Immunofluorescence Approach Toward the Identification of Endosymbiotic Diatoms in Several Species of Larger Foraminifera. In C.M. Yentsch, F.C. Mague, & P.K. Horan, (Eds.) *Immunochemical Approaches to Coastal, Estuarine and Oceanographic Questions* (pp. 230-241). New York, NY: Springer US.
Lee, J. J., Erez, J., Ter Kuile, B., Lagziel, A., & Burgos, S. (1988b). Feeding Rates of Two Species of Larger Foraminifera *Amphistegina lobifera* and *Amphisorus hemprichii*, from the Gulf of Eilat (red Sea). *Symbiosis, 5*(1-2), 61-102.
Lee, J. J., McEnery, M. E., Kuile, B. T., Erez, J., Röttger, R., Rockwell, R. F., ... & Lagziel, A. (1989). Identification and Distribution of Endosymbiotic Diatoms in Larger Foraminifera. *Micropaleontology, 35*(4), 353-366.
Lee, J. J., Faber, W. W., Nathason, B., & Rottger, R. (1991a). Endosymbiotic Diatoms From Larger Foraminifera Collected in Pacific Habitats. *Journal of Phycology, 27*(3 SUPPL), 43-43.
Lee, J. J., Sang, K., Terkuile, B., Strauss, E., Lee, P. J., & Faber, W. W. (1991b). Nutritional and Related Experiments on Laboratory Maintenance of 3 Species of Symbiont-Bearing, Large Foraminifera. *Marine Biology, 109*(3), 417-425.

Lee, J. J., Faber, W. W., & Lee, R. E. (1991c). Antigranulocytes Reticulopodal Digestion—A Possible Preadaptation to Benthic Foraminiferal Symbiosis. *Symbiosis, 10*(1-3), 47-61.
Lee, J. J., Faber, W. W., Jr., Nathanson, B., Rottger, R., Nishihira, M., & Kruger, R. (1993). Endosymbiotic Diatoms From Larger Foraminifera Collected in Pacific Habitats. *Symbiosis, 14*(1-3), 265-281.
Lee, J. J., Morales, J., Symons, A., & Hallock, P. (1995a). Diatom Symbionts in Larger Foraminifera from Caribbean Hosts. *Marine Micropaleontology, 26*(1-4), 99-105.
Lee, J. J., Wray, C. G., & Lawrence, C. (1995b). Could Foraminiferal Zooxanthellae be Derived from Environmental Pools Contributed to by Different Coelenterate Hosts. *Acta Protozoologica, 34*(2), 75-85.
Lee, J. J., Reimer, C. W., Correia, M., & Morales, J. (2000). A Revised Description of the *Nitzschia frustulum* var. symbiotica Lee and Reimer Emend. Complex, the Most Common of the Endosymbiotic Diatoms in Larger Foraminifera. *Micropaleontology, 46*, 170-181.
Lee, J. J., Cervasco, M. H., Morales, J., Billik, M., Fine, M., & Levy, O. (2010). Symbiosis Drove Cellular Evolution. *Symbiosis, 51*(1), 13-25.
Lee, M. J., Ellis, R., & Lee, J. J. (1982). A Comparative-Study of Photo-Adaptation in 4 Diatoms Isolated as Endosymbionts from Larger Foraminifera. *Marine Biology, 68*(2), 193-197.
Leutenegger, S. (1977). Symbiosis Between Larger Foraminifera and Unicellular Algae in the Gulf of Elat, Red Sea. *Utrecht Micropaleontological Bulletins*, 241-244.
Leutenegger, S. (1983). Specific Host-Symbiont Relationship in Larger Foraminifera. *Micropaleontology, 29*(2), 111-125.
Leutenegger, S. (1984). Symbiosis in Benthic Foraminifera—Specificity and Host Adaptations. *Journal of Foraminiferal Research, 14*(1), 16-35.
Lough, J. (2000). 1997-98: Unprecedented Thermal Stress to Coral Reefs? *Geophysical Research Letters, 27*(23), 3901-3904.
Mann, D. G. (1999). The Species Concept in Diatoms. *Phycologia, 38*(6), 437-495.
Martin, R. E. (Ed.). (2000). Environmental micropaleontology: the application of microfossils to environmental geology (Vol. 15). New York, NY, Springer US.
McCoy, A. M. (2004). *Symbiosis Between the Invasive Marine Acoel Convoluta Convoluta and Diatom Endosymbionts.* (Doctoral dissertation). Retrieved from http://id.lib.harvard.edu/aleph/010233012/catalog
McEnery, M. E., & Lee, J. J. (1981). Cytological and Fine Structural Studies of Three Species of Symbiont-Bearing Larger Foraminifera from the Red Sea. *Micropaleontology, 27*(1), 71-83.
Medlin, L. K. (2014). Evolution of the Diatoms: VIII. Re-Examination of the SSU-rRNA Gene Using Multiple Outgroups and a Cladistic Analysis of Valve Features. *Journal of Biodiversity, Bioprospecting and Development*. Retrieved from http://www.omicsgroup.org/journals/evolution-of-the-diatoms-viii-reexamination-of-the-ssurrna-gene-using-multiple-outgroups-and-a-cladistic-analysis-of-valve-features-2376-0214.1000129.pdf.
Medlin, L., Elwood, H. J., Stickel, S., & Sogin, M. L. (1988). The Characterization of Enzymatically Amplified Eukaryotic 16S-like rRNA-Coding Regions. *Gene, 71*(2), 491-499.
Muller, P. H. (1974). Sediment Production and Population Biology of the Benthic Foraminifer *Amphistegina madagascariensis*. *Limnology and Oceanography, 19*(5), 802-809.

Muscatine, L., & Porter, J. W. (1977). Reef Corals: Mutualistic Symbioses Adapted to Nutrient-Poor Environments. *Bioscience, 27*(7), 454-460.

Nayak, B. S. (2009). Microbial Population Analysis in Leachate From Simulated Solid Waste Bioreactors and Evaluation of Genetic Relationships and Prevalence of Vancomycin Resistance Among Environmental *Enterococci*. (Doctoral Dissertation). Retrieved from http://scholarcommons.usf.edu/etd/3910.

Pandolfi, J. M., Jackson, J. B. X. C., Baron, N., Bradbury, R. H., Guzman, H. M., Hughes, T. P., ... & Sala, E. (2005). Are US coral reefs on the slippery slope to slime?. *Science, 307*(5716), 1725-1726.

Paul, J. H. (2001). Gene Expression by mRNA Analysis. *Methods in Microbiology, 30*, 395-408.

Paul, J. H., Alfreider, A., & Wawrik, B. (2000). Micro-and Macrodiversity in rbcL Sequences in Ambient Phytoplankton Populations from the Southeastern Gulf of Mexico. *Marine Ecology-Progress Series, 198*, 9-18.

Pawlowski, J., Holzmann, M., Fahrni, J. F., & Hallock, P. (2001a). Molecular Identification of Algal Endosymbionts in Large Miliolid Foraminifera: 1. Chlorophytes. *Journal of Eukaryotic Microbiology, 48*(3), 362-367.

Pawlowski, J., Holzmann, M., Fahrni, J. F., Pochon, X., & Lee, J. J. (2001b). Molecular Identification of Algal Endosymbionts in Large Miliolid Foraminifera: 2. Dinoflagellates. *Journal of Eukaryotic Microbiology, 48*(3), 368-373.

Pichler, T., Amend, J. P., Garey, J., Hallock, P., Hsia, N. P., Karlen, D. J., . . . Price, R. E. (2006). A Natural Laboratory to Study Arsenic Geobiocomplexity. *Eos, Transactions American Geophysical Union, 87*(23), 221-225.

Pomar, L., & Hallock, P. (2008). Carbonate Factories: A Conundrum in Sedimentary Geology. *Earth-Science Reviews, 87*(3), 134-169.

Pruesse, E., Peplies, J., & Glockner, F. O. (2012). SINA: Accurate High-Throughput Multiple Sequence Alignment of Ribosomal RNA Genes. *Bioinformatics, 28*(14), 1823-1829.

Richardson, L. L., Goldberg, W. M., Kuta, K. G., Aronson, R. B., Smith, G. W., Ritchie, K. B., . . . Miller, S. L. (1998). Florida's Mystery Coral-Killer Identified. *Nature, 392*(6676), 557-558.

Round, F. E., & Crawford, R. M. (1989). Phylum Bacillariophyta. In L. Margulis, J. O. Corliss, M. Melkonian, & D. J. Chapman (Eds.), *Handbook of Protoctista* (pp. 574-596). Boston, MA: Barlett.

Round, F. E., Crawford, R. M., & Mann, D. G. (1990). *The Diatoms: Biology & Morphology of the Genera*. New York, NY: Cambridge University Press.

Round, F. E., Hallsteinsen, H., & Paasche, E. (1999). On a Previously Controversial "Fragilarioid" Diatom Now Placed in a New Genus *Nanofrustulum*. *Diatom Research, 14*(2), 343-356.

Rowan, R. (1998). Diversity and Ecology of Zooxanthellae on Coral Reefs. *Journal of Phycology, 34*(3), 407-417.

Rozen, S., & Skaletsky, H. (1999). Primer3 on the WWW for General Users and for Biologist Programmers. In S. Misener & S. Krawetz (Eds.), *Bioinformatics Methods and Protocols* (Vol. 132, pp. 365-386). Totowa, NJ: Humana Press.

Saitou, N., & Imanishi, T. (1989). Relative Efficiencies of the Fitch-Margoliash, Maximum-Parsimony, Maximum-Likelihood, Minimum-Evolution, and Neighbor-Joining Methods of Phylogenetic Tree Construction in Obtaining the Correct Tree. *Molecular Biology and Evolution, 6*(5), 514-525.

Santavy, D. L., & Peters, E. C. (1997). Microbial Pests: Coral Disease in the Western Atlantic. *Proceedings, 8th International Coral Reef Symposium, Panama, June 1996*. 1, 607-612.
Schmidt, C., Heinz, P., Kucera, M., & Uthicke, S. (2011). Temperature-Induced Stress Leads to Bleaching in Larger Benthic Foraminifera Hosting Endosymbiotic Diatoms. *Limnology and Oceanography, 56*(5), 1587-1602.
Schmidt, C., Morard, R., Almogi-Labin, A., Weinmann, A. E., Titelboim, D., Abramovich, S., & Kucera, M. (2015). Recent Invasion of the Symbiont-Bearing Foraminifera *Pararotalia* into the Eastern Mediterranean Facilitated by the Ongoing Warming Trend. *PLoS One, 10*(8), e0132917.
Schueth, J. D., & Frank, T. D. (2008). Reef Foraminifera as Bioindicators of Coral Reef Health: Low Isles Reef, Northern Great Barrier Reef, Australia. *Journal of Foraminiferal Research, 38*(1), 11-22.
Sen Gupta, B. K. (1999). *Modern Foraminifera*. Dordrecht: Kluwer Academic Publishers.
Sitnikova, T. (1996). Bootstrap Method of Interior-Branch Test for Phylogenetic Trees. *Molecular Biology and Evolution, 13*(4), 605-611.
Sitnikova, T., Rzhetsky, A., & Nei, M. (1995). Interior-Branched and Bootstrap Tests of Phylogenetic Trees. *Molecular Biology and Evolution, 12*(2), 319-333.
Smith, C. J., Collins, L. S., & Hayek, L.-A. C. (2013). Biogeographic Effects of the Closing Central American Seaway on Benthic Foraminifera of Venezuela. *Bulletin of Marine Science, 89*(4), 921-936.
Stoeck, T., Hayward, B., Taylor, G. T., Varela, R., & Epstein, S. S. (2006). A Multiple PCR-Primer Approach to Access the Microeukaryotic Diversity in Environmental Samples. *Protist, 157*(1), 31-43.
Talge, H., Williams, D., Hallock, P., & Harney, J. (1997). Symbiont Loss in Reef Foraminifera: Consequences for Affected Populations. *Proceedings, 8th International Coral Reef Symposium, Panama, June 1996*, 1:589-594.
Talge, H. K., & Hallock, P. (2003). Ultrastructural Responses in Field-Bleached and Experimentally Stressed *Amphistegina gibbosa* (Class Foraminifera). *Journal of Eukaryotic Microbiology, 50*(5), 324-333.
Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution, 24*(8), 1596-1599.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). Mega6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution, 30*(12), 2725-2729.
Tanaka, T., Kawasaki, K., Daimon, S., Kitagawa, W., Yamamoto, K., Tamaki, H., ... & Kamagata, Y. (2014). A Hidden Pitfall in the Preparation of Agar Media Undermines Microorganism Cultivability. *Applied and Environmental Microbiology, 80*(24), 7659-7666.
Theriot, E. C., Ashworth, M., Ruck, E., Nakov, T., & Jansen, R. K. (2010). A Preliminary Multigene Phylogeny of the Diatoms (Bacillariophyta): Challenges for Future Research. *Plant Ecology and Evolution, 143*(3), 278-296.

Toler, S. K., & Hallock, P. (1998). Shell Malformation in Stressed *Amphistegina* Populations: Relation to Biomineralization and Paleoenvironmental Potential. *Marine Micropaleontology, 34*(1-2), 107-115.

Uthicke, S., Thompson, A., & Schaffelke, B. (2010). Effectiveness of Benthic Foraminiferal and Coral Assemblages as Water Quality Indicators on Inshore Reefs of the Great Barrier Reef, Australia. *Coral Reefs, 29*(1), 209-225.

Veron, J. E. N. (1995). *Corals in Space and Time: The Biogeography and Evolution of the Scleractinia*. Ithaca, NY: Cornell University Press.

Veron, J. E. N. (2000). *Corals of the World, Vol. 1–3*. Townsville, Australia: Australian Institute of Marine Science.

Vickerman, K. (1992). The Diversity and Ecological Significance of Protozoa. *Biodiversity and Conservation, 1*(4), 334-341.

Walker, R., Hallock, P., Torres, J.J., Vargo, G.A. (2011) Photosynthesis and Respiration in Five Species of Benthic Foraminifera that Host Algal Endosymbionts. *Journal of Foraminiferal Research. 41*(4):314-325.

Wang, Y., Tian, R. M., Gao, Z. M., Bougouffa, S., & Qian, P. Y. (2014). Optimal Eukaryotic 18S and Universal 16S/18S Ribosomal RNA Primers and their Application in a Study of Symbiosis. *PLoS One, 9*(3), e90053.

Wawrik, B., Paul, J. H., Campbell, L., Griffin, D., Houchin, L., Fuentes-Ortega, A., & Muller-Karger, F. E. (2003). Vertical Structure of the Phytoplankton Community Associated with a Coastal Plume in the Gulf of Mexico. *Marine Ecology-Progress Series, 251*, 87.

Williams, D. E. (2003). Symbiont-Bearing Foraminifera of Navassa Island. In M. W. Miller (Ed.), *Status of Reefs of Navassa Island. National Oceanic and Atmospheric Administration Technical Memorandum NMFS-SEFSC-501* (pp. 84-89). Retrieved from http://docs.lib.noaa.gov/noaa_documents/NMFS/SEFSC/TM_NMFS_SEFSC/NMFS_SEFSC_TM_501.pdf

Williams, D. E., & Hallock, P. (2004). Bleaching in *Amphistegina gibbosa* d'Orbigny (Class Foraminifera): Observations from Laboratory Experiments using Visible and Ultraviolet Light. *Marine Biology, 145*(4), 641-649.

Williams, D. E., Hallock, P., Talge, H. K., Harney, J. N., & McRae, G. (1997). Responses of *Amphistegina gibbosa* Populations in the Florida Keys (USA) to a Multi-Year Stress Event (1991-1996). *Journal of Foraminiferal Research, 27*(4), 264-269.

Zechman, F. W., Zimmer, E. A., & Theriot, E. C. (1994). Use of Ribosomal DNA Internal Transcribed Spacers for Phylogenetic Studies in Diatoms. *Journal of Phycology, 30*(3), 507-512.

APPENDIX

List and Description of Attached Supplemental Files:

1) The key to the abbreviated sequence names that were used in the phylogenetic trees and alignments, which were not listed in Table 3.1 and a summary of the BLAST search results. The file was saved as a document (.doc) file. File Name: SeqBLASTname.doc

2) The alignment of the entire *rbcL* data set plus the reference sequences used. The alignment was saved in FASTA format. File Name: AlignedrbcL.fasta

3) The alignment of the entire *18S* data set plus the reference sequences used. The alignment was saved in FASTA format. File Name: Aligned18S.fasta

4) The uncompressed NJ phylogentic tree of the entire *rbcL* data set and reference sequences. The tree file was saved in Newick format. File Name: rbcLNJTree.nwk

5) The uncompressed ML phylogentic tree of the entire *rbcL* data set and reference sequences. The tree file was saved in Newick format. File Name: rbcLMLTree.nwk

6) The uncompressed MP phylogentic tree of the entire *rbcL* data set and reference sequences. The tree file was saved in Newick format. File Name: rbcLMPTree.nwk

7) The uncompressed ME phylogentic tree of the entire *rbcL* data set and reference sequences. The tree file was saved in Newick format. File Name: rbcLMETree.nwk

8) The uncompressed NJ phylogentic tree of the entire *18S* data set and reference sequences. The tree file was saved in Newick format. File Name: 18SNJTree.nwk

9) The uncompressed ML phylogentic tree of the entire *18S* data set and reference sequences. The tree file was saved in Newick format. File Name: 18SMLTree.nwk

10) The uncompressed MP phylogentic tree of the entire *18S* data set and reference sequences. The tree file was saved in Newick format. File Name: 18SMPTree.nwk

11) The uncompressed ME phylogentic tree of the entire *18S* data set and reference sequences. The tree file was saved in Newick format. File Name: 18SMETree.nwk

ABOUT THE AUTHOR

Kwasi H. Barnes is husband to a beautiful, intelligent, and loving wife and father of two wonderful daughters. He was born and raised on the island of St. Croix in the United States Virgin Islands by his maternal grandmother. Throughout his childhood, he spent countless hours exploring, enjoying, and learning about the natural environment of his tropical island home by doing things like hiking, snorkeling, fishing, swimming, and camping. He still enjoys doing those things today, especially with his family. He is particularly fascinated with learning about different species and their connections to each other. During his childhood explorations, he noticed a steady decline of coral reefs, mangrove forests, and their inhabitants and wanted to do something about it. This inspired him to pursue his degrees in marine biology and mathematics at the University of the Virgin Islands, volunteer for and coordinate things like beach clean ups and mangrove plantings, and go to different schools to teach kids about their environment and things they can do to keep it healthy. Throughout his undergraduate, graduate, professional, and personal endeavors, he has combined his love for science, mathematics, and education!