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The Ecology and Evolution of Soritid Foraminifera with *Symbiodinium* Dinoflagellates

by

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Committee in charge:

Professor Jere H. Lipps, Chair

Professor Ellen Simms

Professor George Roderick

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ABSTRACT

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Soritid foraminifera host dinoflagellate symbionts of the genus *Symbiodinium*, the same algae that power the formation and persistence of coral reefs through mutualism with cnidarians, molluscs, sponges, and other reef-dwelling hosts. This dissertation examines the interactions between these foraminiferal hosts and their symbionts, placing those interactions in the context of the overall reef photosymbiotic system. Given the finding of multiple symbiont types within an individual, the general phenomenon of multiple *Symbiodinium* infections is examined in light of extant theory on the evolution of mutualism.

The first chapter reviews the scientific literature that reports the incidence of mixed *Symbiodinium* infections. This trait has a wide phylogenetic distribution across major host groups. A similarly wide distribution of this trait is also found across the phyletic diversity of scleractinian corals. Extant theory suggests that a mixed population of symbionts can be disadvantageous to the host, and that hosts should evolve mechanisms to control mixing of their symbionts. Stability of *Symbiodinium*-host mutualisms is maintained though any of three model mechanisms: partner fidelity feedback, cooperator association, and partner choice. Which operates depends on the particular biological processes that mediate the interaction.

The second chapter examines the distribution of multiple symbiont types within individual foraminifer, reporting the finding that multiple types of *Symbiodinium* are distributed differentially across the radius of the foraminifer. Multiple hypotheses could explain this phenomenon, including: processing of the symbionts as they move into the host, partitioning of symbiont functional roles, or differential competition of symbionts within a heterogeneous internal host environment.

The third chapter explores symbiont acquisition by soritid foraminifera. I report that soritid foraminifera typically do not acquire new symbiont types as adults. Symbionts move from internal chambers to the newly formed outermost chambers. Foraminifera transmit their symbionts vertically through rounds of asexual reproduction and horizontally through rounds of sexual reproduction, and thus may optimize these different symbiont acquisition strategies for different environmental conditions.

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CHAPTER ONE

Framework for Study

Algal endosymbiosis is a critical process in the evolution of plastids, which are the result of a single primary and multiple secondary and tertiary endosymbiotic events (Keeling 2004). By studying the ecology and evolution of modern photosymbiotic systems, we can more fully understand the selective pressures that contributed to the transformation of photoendosymbionts into fully integrated plastids. This dissertation was originally motivated by a drive to document the ecological processes in a unicellular host that may help determine the trajectory of this transformation. This interest has lead, in what is hopefully a not entirely unrelated arc, to a study that addresses questions of coral reef ecology.

Foraminifera, a group of single-celled testate marine protists, provide a fascinating comparative framework for the study of algal endosymbiosis because they host such a broad array of symbionts, including red algae, green algae, diatoms, and dinoflagellates (Leutenegger 1984). Photoendosymbiosis is a polyphyletic trait in foraminifera, having independently evolved multiple times. The Soritacea are a monophyletic group of particular interest because they evolved from an asymbiotic lifestyle into symbiosis with rhodophytes, chlorophytes and most recently dinophytes, in a stepwise manner, each acquisition of a new symbiont type followed by phyletic diversification (Richardson 2001).

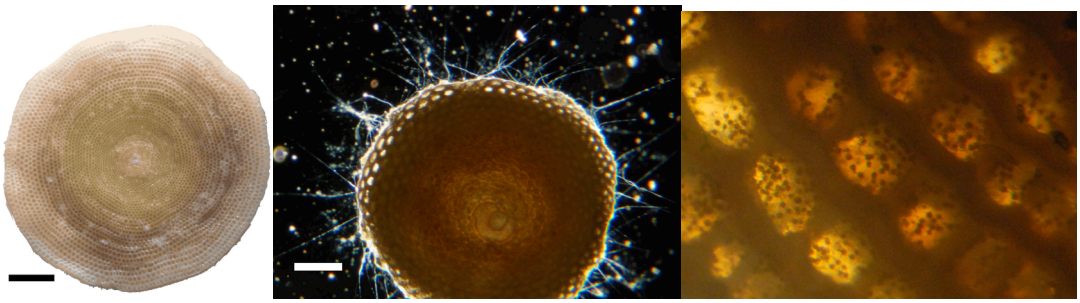
Foraminifera in the subfamily Soritinae Ehernberg 1839, referred to as soritids, host dinoflagellates of the genus *Symbiodinium*. They have a calcareous disk-shaped test up to 3 cm in diameter (Figure 1). On the margins of the disk are apertures through which the foraminifera interact with their environment using rhizopodia, which are anastomosing filose pseudopodial networks. The subfamily Soritinae is comprised of three extant genera (Loeblich and Tappan 1988): *Amphisorus* Ehrenberg 1839, *Marginopora* Quoy and Gaimard 1830, and *Sorites* Eherberg 1839. *Sorites* has a circumtropical distribution and the genera *Amphisorus* and *Marginopora* are limited to the Indo-Pacific (Langer and Hottinger 2000). They live loosely attached to their substrate, typically algal turf, coral rubble, macroalgae, or seagrass.

The symbiotic dinoflagellate *Symbiodinium* is found in a broad diversity of other hosts, including corals, giant clams, sponges, sea slugs, anemones, jellies, the giant ciliate *Maristentor dinoferus* Lobban 2002. Though relatively morphologically homogenous, molecular markers have revealed a remarkably broad and deep genetic diversity in this genus, with the broadest levels of divergence referred to as clades lettered A–H (Coffroth and Santos 2005). A strict one-to-one specificity of host to symbiont (Figure 2) does not exist. Phenotypic diversity in the genus is not well understood (Stat et al. 2008).

Symbiodinium is of broad interest because of its role in energetically supporting the growth of coral reefs. Over the last few decades this interest has increased because of the central role the algae plays in coral bleaching, a breakdown in the mutualism driven by increased sea water temperatures. Rising sea surface temperatures, driven by anthropogenic climate change, endanger coral reef ecosystems worldwide because of bleaching (Hoegh-Guldberg 1999). The ability of the host to acclimate to environmental change by switching symbiont types is a contentious issue among researchers who study *Symbiodinium.*

Soritid foraminifera are rather inconspicuous, yet abundant, inhabitants of the coral reef. They host many different clades of *Symbiodinium,* and certain lettered clades of *Symbiodinium* were first discovered in foraminifera only to later be discovered in corals (Pochon and Pawlowski 2006). Thus foraminifera, along with other non-coral hosts, may act as an important alternative reservoir of symbiont diversity. This dissertation aims to clarify the role of these protists in the coral reef ecosystem by studying the relationship between soritid foraminifera and their *Symbiodinium* symbionts. This work uncovers the diversity of *Symbiondinium* symbionts

in individual foraminifera, examines symbiont acquisition, and explores the implications of mixed infections on the stability of *Symbiodinium*-host mutualism.



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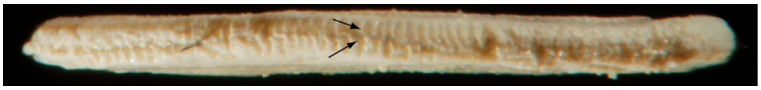

D.

Figure 1.

A. Dried *Amphisorus hemprichii* test. Note the brownish coloration that results from the symbiotic dinoflagellates. Scale = 1mm.

B. Living *Amphisorus hemprichii* individual. Note rhizopodia and dense "bulls-eye" distribution of brown dinoflagellates inside test. Scale = 200µm.

C. *Symbiodinium* dinoflagellates in *Marginopora vertebralis* chambers. The symbionts are ~10µm in diameter.

D. Edge-on view of *Amphisorus hemprichii,* with double row of apertures. Arrows point to individual apertures.

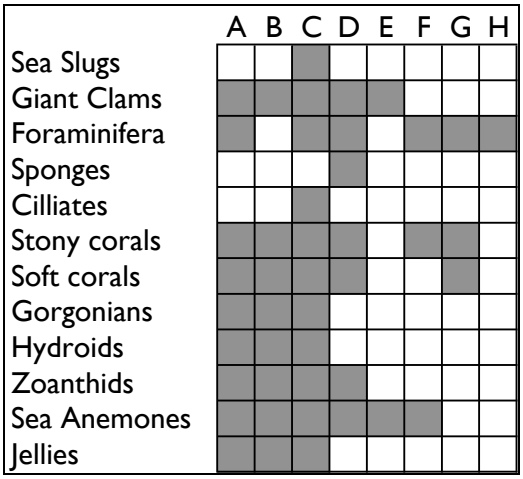

Figure 2.

Distribution of different lettered clades of *Symbiodinium* dinoflagellates among different groups of hosts. Most clades are broadly distributed across multiple unrelated groups of hosts. Data from Stat et al. (S2006).

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CHAPTER TWO

Mixed infections and the stability of *Symbiodinium*-host mutualism

ABSTRACT

Mutualisms involving *Symbiodinium* dinoflagellates allow the growth and persistence of coral reefs. The stability of this mutualism is threatened by environmental change such as rising sea surface temperatures. Swapping symbiont type is a hypothesized mechanism by which reef hosts acclimate to environmental change, since different symbiont types may function better in different environments. The ability to host multiple types of *Symbiodinium* is a critical prerequisite for swapping. Mixed infection has been reported in a phylogenetically broad diversity of hosts. However, an individual host typically has one dominant symbiont type with any additional types found in low abundance. Potential fitness costs associated with hosting multiple types demand an examination of the mechanisms that allow for the evolutionary stability of *Symbiodinium*-host interactions.

When fitness costs exist for each of the partners, a range of ecological forces can stabilize interspecific mutualism. We describe two axes of : partner fidelity feedback, cooperator association, and partner choice. Using this general model, the many diverse interactions that occur between *Symbiodinium* and its hosts can be categorized. Vertical transmission stabilizes mutualism by partner fidelity feedback, symbiont recognition stabilizes mutualism by cooperator association, and regulation of symbiont populations stabilizes mutualism by partner choice. Adaptive bleaching and symbiont shuffling operate through partner choice.

INTRODUCTION

Coral reefs are inherently valuable for their high biodiversity (Wilson 2003), and they also provide goods and services such as food, shoreline protection, recreation, live organisms for the reef tank trade, building materials, and a record of past climate (Moberg and Folke 1999). The biogenic deposition of carbonate that forms coral reefs is driven by symbiosis between dinoflagellate algae of the genus *Symbiodinium* and a wide diversity of hosts including scleractinian corals, foraminifera, giant clams, and sponges. When the mutualism fails, as in mass coral bleaching, severe biotic shifts and changes in reef trophic structure may occur, which in turn threaten reef biodiversity (Hoegh-Guldberg et al. 2007).

Flexibility of this symbiosis may have a major role in determining resilience of coral reefs in the face of environmental changes such as sea surface warming. Several models have been proposed, including the Adaptive Bleaching Hypothesis and the Symbiont Shuffling hypothesis, which seek to understand the ability of the host to adjust to environmental change by switching symbiont type (Buddemeier and Fautin 1993, Baker 2001, 2003). Flexibility in symbiont-host associations is a prerequisite for both of these models but empirical support for such flexibility is equivocal. Some hosts are flexible in their association with particular symbiont lineages and others are not (Baker 2001, Goulet 2006, Thornhill et al. 2006b, Baker and Romanski 2007, Stat et al. 2009a). Understanding host ability to switch symbionts under thermal stress is important for predicting response to rising seawater temperatures, a critical issue for reef conservation.

Mixed infections are one indication of the potential for an individual to be flexible in its symbiont associations. From some of the earliest molecular genetic studies on *Symbiodinium* came the discovery of the presence of two genotypes of symbiont within individual *Pocillopora meandrina* colonies (Rowan and Powers 1991). However, little of the subsequent work describing *Symbiodinium* genetic diversity within hosts has focused on the detection of mixed infections and the idea that the majority of coral species are able to host multiple types of *Symbiodinium* continues to be contentious (Goulet 2006, Baker and Romanski 2007, Goulet 2007). The first objective of this paper is to review the evidence for mixed *Symbiodinium* infections and place that evidence into the context of host phyletic diversity.

Theory postulates that natural selection should drive hosts to control mixing of their symbiont lineages; if a tradeoff exists between how competitive a symbiont is and how well it cooperates with the host, hosts that maintain multiple symbiont lineages will bear a cost caused by competition among the symbionts (Frank 1996). However, mixed infections may be common (Schlick-Steiner et al. 2008). At the intersection of these new findings and the theory with which they apparently conflict are rich new avenues of research.

Certain host/microbe systems have evolved to control mixing of their symbionts, sanction cheaters, or reward cooperativeness, yielding a complex landscape of symbiotic interactions. For example, fungus-growing ants and the fungal mutualists themselves both contribute to maintaining host-symbiont fidelity and thus control symbiont mixing, yet an ephemeral window exists during the life cycle of a colony where horizontal transmission of fungus can sometimes occur, explaining a lack of cocladogenesis in this system with vertical transmission (Poulsen and Boomsma 2005, Poulsen et al. 2009). The legume/rhizobia mutualism is also very complex: uncooperative rhizobia are widespread in environmental populations and their host legumes sanction these less cooperative strains, preferentially nodulating more cooperative ones, exerting

partner choice both before and after nodulation (Sachs and Simms 2008, Heath and Tiffin 2009). Less beneficial types of symbiotic soil fungi, arbuscular mycorrhizae, tend to proliferate in well-mixed fungal communities, implying a tradeoff between cooperation and competition (Bever 2002); plant hosts exert parner choice, allocating more resources to more beneficial types of fungi, demonstrating the tension between natural selection for less beneficial strains and host selection for more beneficial ones (James et al. 2009).

Hosts of *Symbiodinium* are also likely to encounter varying levels of cooperation. Some species of dinoflagellate are parasitic and some produce allelopathic compounds (Loeblich 1982, Arzul et al. 1999). Virulent strains of *Symbiodinium* can evolve which readily infect, more rapidly proliferate, and reduce the fitness of the host by not cooperating (Sachs and Wilcox 2006). Clearly there is potential for intra-host competition between *Symbiodinium* lineages and cheating by withholding benefits from the host. If a tradeoff between cooperativeness and increased competitiveness among *Symbiodinium* lineages occurs, fitness costs may affect hosts that maintain mixed infections and thus select for host control of symbiont mixing.

Hosts to *Symbiodinium* have a very broad taxonomic diversity, deep evolutionary histories (*e.g.,* Scleractinia, ~237Ma, (Stanley Jr and Fautin 2001)), and varied symbiont transmission strategies (van Oppen 2004, Stat et al. 2008a, van Oppen et al. 2009). Thus hosts are likely to have evolved diverse strategies for stabilizing the mutualism. The second aim of this paper is to review current research on *Symbiodinium*/host interactions, identify strategies that hosts may use to stabilize the mutualism, and categorize these strategies into a flexible theoretical framework. This framework will allow comparison of these strategies with those from other systems and will help bring to light new testable hypotheses.

MIXED *SYMBIODINIUM* INFECTIONS

Specificity is a critical trait for the establishment of all mutualisms. This trait varies among different hosts of *Symbiodinium* and these varying degrees of specificity fit into three main categories:

Simultaneous mixed infection

- *Within-colony simultaneous polymorphism:* An individual *Montastrea* colony has different *Symbiodinium* types in the polyps on top (high irradiance) and sides (low irradiance) (Rowan et al. 1997).
- *Within cell simultaneous polymorphism*: Mixed communities of *Symbiodinium* occur within individual *Amphisorus* foraminifera (Fay et al. 2009).

Polymorphic symbiosis

- *Temporal heterogeneity within an individual:* The symbiont population in an individual coral colony shifts with environmental change (Thornhill et al. 2006b) or ontogeny (Coffroth et al. 2001, Abrego et al. 2009b).
- *Biogeographic heterogeneity*: Within a coral species symbiont populations vary geographically (Santos et al. 2003, Howells et al. 2009).
- *Ecological zonation*: Within a coral species symbiont populations vary with depth (Rowan and Knowlton 1995, Sampayo et al. 2007) or reef habitat (Oliver and Palumbi 2009).

Strict specificity

- In some corals, multiple closely related species share specificity for a particular genotype of *Symbiodinium* across a wide range of light irradiance levels (Diekmann et al. 2002).

Detecting mixed infection

Multiple *Symbiodinium* infections have not frequently been reported, but this may be an artifact of the methodological limitations discussed below. Some studies that have explicitly examined mixed infection show that low-frequency types may be quite common and are difficult detect without real-time PCR or cloning (Carlos et al. 2000, Apprill and Gates 2007, Mieog et al. 2007, Fay et al. 2009). Community-wide surveys show that most individual host colonies have one dominant type in their tissues, though mixes of symbiont types in an individual are not unusual (LaJeunesse 2002, LaJeunesse et al. 2004, Stat et al. 2009b). One study rigorously quantifing relative genotype frequencies within individuals showed that the majority of individual samples (79%) did not have symbiont mixes. However, a majority of the coral taxa examined (83%) did have at least one case of mixed infection (Correa et al. 2009b). This general qualitative pattern, with most individual hosts having a single symbiont but with a few individuals with mixed infection, is reflected in a review of the literature discussed in the next section.

With the application of molecular genetic techniques, our understanding of the diversity of *Symbiodinium* has changed; what was previously considered a handful of different species is now classified into many physiologically distinct genotypes. Some molecular markers are useful for identifying well-established, lettered (A - H) clades; others are more useful for identifying much finer-scale genetic variants (reviewed in Coffroth and Santos 2005). No agreement has been reached about which marker best represents functionally distinct lineages or taxonomic species. However, it is clear that reproductively isolated lineages of *Symbiodinium* exist within the lettered clades, as evidenced by congruent gene trees from unlinked loci and evidence that variation in rapidly evolving molecular markers correlates with ecological traits (Pochon et al. 2006, Sampayo et al. 2009).

Many different methods have been used to characterize genetically *Symbiodinium* populations. The method used determines the degree to which mixed infections can be recognized and whether the relative frequencies of different genotypes can be quantified. The most frequently used tools, Denaturing Gel Gradient Electrophoresis (DGGE), Retriction Fragment Length Polymorphism (RFLP) and direct sequencing, are not sensitive to low background levels of additional symbiont genotypes (Thornhill et al. 2006b). Other tools such as cloning, FISH, and real-time qPCR explicitly aim to identify and quantify mixed *Symbiodinium* communities (Apprill and Gates 2007, Loram et al. 2007a, Mieog et al. 2007, Correa et al. 2008, Mieog et al. 2009), but these methods suffer their own drawbacks. Both FISH and qPCR can fail to detect types that are not accounted for in probe/primer design, so these methods can only be used with previously well-characterized *Symbiodinium* communities; furthermore, they are limited by the number of different fluorophores that can be detected simultaneously. Even when cloning and sequencing rDNA ITS-2 (the most well-characterized fine-scale genetic marker) PCR products, it can be difficult to distinguish between intragenomic variants and functionally distinct lineages (Thornhill et al. 2007, Sampayo et al. 2009), though analytic methods may help overcome these limitations (Hunter et al. 2007, Correa and Baker 2009). Cloning is inappropriate for quantifying relative genotype frequencies because of several factors, including

non-linear PCR amplification, widely varying copy numbers of rRNA genes (a thirty-fold difference between lineages in one study by Loram et al. (2007a)), and uncertainty in cloning efficiency of different PCR products.

Whether the presence of low-level background symbiont types in a host is ecologically significant for that host is unknown at present. As more loci are characterized, new genotyping tools such as microarrays become available, and broader communities are sampled, the frequency and significance of mixed infection will be clarified. This paper examines the distribution of multiple infections in hosts across the evolutionarily deepest levels of host divergence and then proceeds to discuss its significance for stability of the mutualism.

Taxonomic and phyletic distribution of mixed infection

Studies of *Symbiodinium* diversity find that hosts with mixed symbiont infection occur across a very broad taxonomic range (Table 1). Three major groups, the foraminifera, mollusca, and cnidaria, all yield examples of simultaneous mixed *Symbiodinium* infections. Other groups that host symbiotic dinoflagellates, such as sponges, flatworms, radiolaria and ciliates, are not well sampled; whether they are able to host multiple types is yet to be determined.

Since scleractinian corals are major reef builders, highly diverse, and vulnerable to bleaching due to increased sea surface temperatures, they have been extensively sampled for *Symbiodinium*. Examples of simultaneous mixed infection occur across a very broad phylogenetic distribution of scleractinia (Figure 3), including most clades of zooxanthellate scleractinia.

Three zooxanthellate scleractinian clades lack evidence of simultaneous mixed infection. Among these are two clades that do show evidence of polymorphic symbiosis: Poritidae 1 (*Alveopora,* a monogeneric clade in the Kerr tree; *Alveopora japonica* hosts both clades C and F at one site (Rodriguez-Lanetty et al. 2000)) and Astrocoeniidae 2 (*Stephanocoenia,* which appears as a monogeneric clade in the Kerr tree, shifts symbiont type when transplanted (Baker 2001)). The third clade, Siderastreidae 3, is represented in the Kerr tree by a single species, *Pseudosiderastrea tamayi*. Notably, among the studies we could find, natural populations of *Symbiodinium* in this species had been examined only once (Chen et al. 2005b).

The literature suggests that most individuals host a single dominant *Symbiodinium* type. However, certain lineages of Scleractinia, such as *Acropora, Montastrea,* and *Pocillopora,* are particularly prone to hosting multiple types at once (Fig. 3). In the case of *Montastrea,* symbiont polymorphism appears to allow optimization of symbiont type to light irradiance levels (Rowan et al. 1997). Some *Acropora* species seem to be relatively labile in their *Symbiodinium* associations, as they can swap symbiont types when transplanted (Berkelmans and van Oppen 2006). In contrast is *Porites*, a widely distributed and abundant coral genus, extensively sampled, for which we found only one report of a mixed infection (LaJeunesse 2002).

Whether a host has mixed infections and whether it can actively change its dominant symbiont type are separate questions. The answer to each depends on how a host becomes infected and the mechanisms it uses to maintain the mutualism. Infection composition may also be determined as much by the symbionts themselves as by the hosts. If the environment or ontogentic stage of the host changes, it could result in a shift in the dominant competitor among symbiont lineages in a mixed infection, independent of any host-driven mechanism. New infection is dependent on the symbiont types available in the environment, which in turn depends on free-living *Symbiodinium* distributions. Free-living populations of *Symbiodinium* are diverse

and have a very different distribution of genotypes from the community found in hosts on the nearby reef (Pochon et al. 2010).

Mixed infection may be observed when one symbiont type is replacing another in response to environmental change. When Caribbean coral symbiont type swapped following tempurature changes, such transient mixed communities were directly observed (Thornhill et al. 2006). *Symbiodinium* type can also change within an individual host subjected to light irradiance changes (Rowan et al. 1997, Baker 2001, Toller et al. 2001a).

Transient mixed infections can also be ontogenetic in origin: when some octocoral juveniles with horizontal transmission first acquire symbionts, they contain multiple types, but eventually equilibrate on one (Coffroth et al. 2001). Many corals easily adopt heterologous symbionts as larvae (Harii et al. 2009). In larvae of two *Acropora* species, a short window of opportunity exists for acquiring symbionts from the environment; while in this window, they take up multiple types, but eventually equilibrate on a single type. Moreover, this equilibrium type is distinct from the type hosted by adults, indicating the potential for yet another switch in symbiont type later in ontogeny (Little et al. 2004). Such windows of infectivity may have evolved to limit the risk of infection by virulent symbiont types, as predicted by theory on the evolution of virulence, which postulates a trade off between virulence and parasite reproductive ability (Messenger et al. 1999).

Variations in host environment can drive spatial differences in symbiont type. In some host species, light irradience gradients are coincident with gradients of symbiont type (Rowan and Knowlton 1995, Sampayo et al. 2007). Such light irradiance-symbiont type gradients also exist within individual coral colonies, and shift when colonies are toppled (Rowan et al. 1997). What controls such a response is unclear. The shift could be driven by a process under host control, such as an ability to assess and respond to the performance of its symbionts. Alternately, it could result from differential competition between symbiont lineages that dominate in different zones within the host, as determined by external factors such as light and nutrients or internal factors such as cytoplasmic or tissue variability. Interestingly, disease has not been found to significantly alter the distribution of *Symbiodinium* types within affected host tissues (Correa et al. 2009a).

If different symbiont lineages play functionally distinct roles, physiological niche partitioning of the internal host environment by the symbionts is also possible. Microbial symbiont populations composed of multiple lineages that occupy different roles are illustrated by numerous examples from insects. Diverse lineages of bacterial symbionts found in some insects (sharpshooters and aphids) have remarkable complimentary metabolic roles, their genomes reduced so that their biosynthetic pathways are interdependent (Oliver et al. 2006, McCutcheon and Moran 2007, McCutcheon et al. 2009). Termite hindgut symbionts are a diverse and metabolically integrated mixed community of prokaryotes and protists (Wier et al. 2002, Warnecke et al. 2007). Different lineages of *Symbiodinium* are distinct in their production of microsporine-like amino acids (UV-protection compounds), release of fixed carbon, and response to different environmental factors such as light irradiance levels and heat stress (Iglesias-Prieto and Trench 1997, Banaszak et al. 2000, Loram et al. 2007b, Stat et al. 2008b, Takahashi et al. 2009). Persistently coexisting lineages of *Symbiodinium* might be fulfilling separate ecological roles for the host or each other. On the other hand, theory predicts that niche conservatism may make this unlikely: in the absence of any selective force that promotes physiological diversification of . Examination of the physiological differences between lineages of *Symbiodinium* should proceed with these hypotheses in mind.

The balance of fitness costs and benefits for each of the partners determines the stability of a mutualism. Costs and benefits shift with changing environmental conditions (Bronstein 1994). Coral bleaching, a breakdown in mutualism, can be caused by shifts in temperature, oxygen, and salinity (Muller-Parker and D'Elia 1997). Fitness costs and benefits also shift among different host-symbiont pairings; some partners are more cooperative than others. By definition, cheater lineages reap benefits from a partner without reciprocating. They are selectively favored as they suffer lower fitness costs than do more cooperative lineages. Selection at the level of the individual should thus make the evolution and persistence of cooperation unlikely (Hamilton 1964, Williams 1966). Yet, interspecific mutualism is common in natural systems (Moran 2006).

Many models have been proposed to explain how cooperation can persist via natural selection in the face of this dilemma. These models fall into three main categories: Shared Genes, Directed Reciprocation, and Byproducts (Hamilton 1964, Trivers 1971, Connor 1995, for a review of this framework see Sachs et al. 2004). Directed Reciprocation is characterized by costly cooperation that then reaps benefits from a partner that reciprocates. This model fits *Symbiodnium*-host interactions since both partners are likely to experience fitness costs (reviewed in (Muller-Parker and D'Elia 1997): costs for the host may include exposure to high UV light levels, high oxygen tension, vulnerability to stresses that affect algae, and investment in controlling algal populations; costs for the algae may include a restricted growth rate, limited dispersal, and loss of photosynthate to the host. Little work has been done to quantify the fitness effects of these potential costs, highlighting the need for further research in this area. The assumption that there are real fitness costs for both partners is supported by two pieces of evidence: a shift in environmental parameters can cause mutualism breakdown and the existence of cheater lineages of *Symbiodinium* (Muller-Parker and D'Elia 1997, Sachs and Wilcox 2006, Stat et al. 2008b). Costs for both partners support a fit for Directed Reciprocation. The other two models do not fit: Shared Genes is not applicable because the alga is not related to the host; Byproducts is not applicable if costs exist for both partners. These costs necessitate a stabilization mechanism.

Directed Reciprocation (Trivers 1971) has dominated thought about interspecific cooperation for several decades, particularly when put in the context of game theory as the Prisoner's Dilemma (Axelrod and Hamilton 1981) and its variations (reviewed by Doebeli and Hauert 2005). The forces acting in Directed Reciprocation models are typically categorized into two components, partner fidelity feedback and partner choice (Bull and Rice 1991). Partner fidelity feedback aligns the fitness interests of partners through repeated interaction, as with vertical transmission of symbionts. Partner choice reinforces positive interactions through active response to cooperativeness, such as sanctions and rewards in legume-*Rhizobium* interactions (Simms and Taylor 2002) and image scoring in cleaner fish (Bshary and Grutter 2006). A third mechanism, cooperator association, was conceived for a quantitative model developed by Foster and Wenseleers (2006). Cooperator association accounts for situations when cooperativeness-linked genotypes of mutualists associate across generations, such as the effect of spatial structure previously described in certain Prisoner's Dilemma models (Doebeli and Knowlton 1998). A particularly attractive aspect to Foster and Wenseleer's (Foster and Wenseleers 2006) model is its implementation of varying degrees of byproduct benefits; the lower the overall fitness costs,

the easier the mutualism is to maintain. Thus in one mathematical model they effectively account for any combination of the mechanisms that are thought to maintain interspecific mutualism. Partner fidelity feedback, cooperator association, and partner choice each has a role in stabilizing *Symbiodinium*-host mutualisms.

Mechanisms that stabilize interspecific mutualism Directed reciprocation stabilization mechanisms to mutualism as falling along two axes. One axis represents degree of repeated association with the same lineage of symbiont, at one end strict partner fidelity (one-to-one host-symbiont relationship) resulting from strict vertical transmission, the other extreme being random association of symbiont with host. Along the second axis is the degree to which partner choice is exerted at one end of the gradient being a mechanism that includes both a direct assessment of cooperativeness and a coordinated response either through rewards or sanctions and at the other extreme no ability to respond to cooperativeness.

PARTNER FIDELITY FEEDBACK (WILLIAMS 1966, BULL AND RICE 1991)

Partner fidelity feedback operates when the two partners' fates are coupled over time, *i.e.,* the two partners' fitness interests are aligned by continued interaction. Among hosts to *Symbiodinium* continued interaction between individual partners is maintained by vertical transmission (Figure 4).

Strict vertical transmission of *Symbiodinium* through rounds of sexual reproduction only occurs in about 15% of corals, cases where symbionts are passed from the adult to either the egg in spawning corals or the larvae in brooding corals (Babcock and Heyward 1986, van Oppen et al. 2009). Many corals can also vertically transmit their symbionts through asexual reproduction via colony fragmentation and budding (Highsmith 1982). A population of *Fungia scutaria* corals transplanted over three decades ago to Jamaica maintains a *Symbiodinium* population unrelated to any others found in the Caribbean Sea, most likely a result of vertical transmission through host budding (LaJeunesse et al. 2005).

Fidelity arising from vertical transmission can extend into an evolutionary timescale; the reproductively isolated brooding coral *Madracis mirabilis* contains a specific unique *Symbiodinium* type distinct from the type hosted by its *Madracis* congeners, which interbreed (Diekmann et al. 2003). Such host fidelity indicates that new infections are rare and that some corals function largely as closed systems. However, despite this fidelity between certain *Symbiodinium* types and their vertically transmitting hosts, no deep phylogenetic congruence has been discovered between vertically transmitting hosts and their symbiont lineages. Thus there must be infrequent swapping of symbionts through hybridization or new infection (Barneah et al. 2004).

Given that coral reefs are subject to varying frequencies and intensity of disturbance and that some coral colonies can be long-lived (e.g., *Porites* colonies more than 600 years old, (Potts et al. 1985)), strictly maintaining a single genotype of symbiont through vertical transmission may confer a disadvantage in that it restricts ability to acclimate to change. Symbiont type is important to how a holobiont responds to change; *Stylophora pistillata* corals with certain genotypes of *Symbiodinium* are more susceptible to bleaching than those containing other genotypes (Sampayo et al. 2008). If partner fidelity feedback through vertical transmission is the only mechanism in place for stabilizing the mutualism, there may be a tradeoff between the advantages conferred by fidelity and the pressures of a changing environment. Furthermore, genetic models have shown that the accumulation of mutation (similar in effect to Muller's

Ratchet) in the confined symbiont population may cause decreased fitness in both host and symbiont with increasing frequency of vertical transmission (O'Fallon and Hansen 2009).

COOPERATOR ASSOCIATION (FRANK 1994, FOSTER AND WENSELEERS 2006)

Cooperator association stabilizes mutualism when there is a correlation between partner genotypes. This is described as "between species relatedness" by (Frank 1994). Such a correlation reinforces selection for cooperativeness in both partners, a mechanism similar to partner fidelity feedback except that it does not require strict repeated association between individuals, only an association between partner genotypes. This category of mutualism stabilization mechanisms was originally conceived to describe how spatial associations reinforce cooperative traits (Foster and Wenseleers 2006). Here we extend cooperator association also to include associations that arise from genotype recognition mechanisms between partners (Figure 4).

Sexual reproduction and recombination among *Symbiodinium* lineages is not well understood. Since gene trees from unlinked loci are congruent at the "lettered clade" level, different clades represent isolated lineages (Pochon et al. 2006). Ecological evidence strongly suggests lineages are isolated at a much finer scale than this; rRNA-ITS2 phylotypes, of which there are probably hundreds, are hypothesized to be "species," and show distinct ecological traits (LaJeunesse 2001, Santos et al. 2004, Sampayo et al. 2009). All hosts to *Symbiodinium* are assumed to show at least some degree of recognition for symbiont type, that is, they always host *Symbiodinium* dinoflagellates instead of other unicellular algae. Thus specificity is a matter of degree. Some hosts may be highly specific for one particular genotype and others more flexible.

In horizontally transmitting hosts, specificity via recognition is inferred from co-occurrence of specific host-symbiont pairs across wide geographic ranges and habitat types and fine-scale genetic differentiation of symbiont type among closely related hosts (LaJeunesse 2002, Santos et al. 2004). Thus recognition can establish an association between symbiont and host lineages, and may help explain the observation that mode of symbiont transmission (vertical vs. horizontal) does not predict symbiont diversity in corals (van Oppen 2004). Recognition has been observed during onset of symbiosis in some corals and temperate anemones (Rodriguez-Lanetty et al. 2003, Rodriguez-Lanetty et al. 2004). Some horizontally transmitting corals also exhibit strict fidelity through environmental change; *Briareum asbestinum* only hosts two subtypes of clade B and through sublethal bleaching exhibited no change in symbiont type (Hannes et al. 2009).

So that the host can distinguish between symbiont and non-symbiont, cell-cell interactions through specific binding proteins are thought to mediate symbiont acquisition. Both pre-phagocytic and post-phagocytic processes are involved in symbiont specificity in corals (Rodriguez-Lanetty et al. 2006). In particular, glycan/lectin cell surface interactions may be important to symbiont recognition and specificity (Wood-Charlson et al. 2006). Mannose-binding lectins, critical to non-adaptive immunity in other invertebrates, have been found in *Acropora millepora* corals. These lectins bind to *Symbiodinium* dinoflagellates and are highly diverse, which is particularly interesting because it may allow for genetic variation or plasticity in the recognition of different *Symbiodinium* lineages (Kvennefors et al. 2008). Glycoconjugate exudates differ between different species of *Symbiodinium* and may also play a critical role in host/symbiont specificity (Markell and Wood-Charlson 2010). In order for cooperator association to be successful at stabilizing mutualism, cooperative species must have different cell

surface markers than less cooperative ones. The model developed by (Foster and Wenseleers 2006) suggests that cooperator association is not as powerful at stabilizing mutualism as Partner Fidelity or partner choice. Recognition acts without regard to a direct assessment of cooperativeness, which instead is a hallmark of Partner Choice.

PARTNER CHOICE (BULL AND RICE 1991)

Partner choice stabilizes mutualism when an organism is able to assess the cooperativeness of its partner and then reward cooperative partners or impose sanctions on uncooperative partners. For a host, partner choice does not strictly depend on the nature of the behavioral, cellular or molecular mechanism underlying symbiont regulation, but rather on the host's ability to both detect and respond to cooperativeness, thus promoting association with more cooperative types. Reef hosts which are flexible in their association with *Symbiodinium* are more likely to stabilize the mutualism via partner choice, because when new symbiont lineages are introduced into the partnership from the environment, infection with parasitic, virulent or cheating lineages becomes possible.

If the symbiont zonation seen in individual *Montastrea* colonies (Rowan et al. 1997) results from host regulation rather than competition between symbiont lineages, then the coral is directly optimizing its symbiont populations, exerting partner choice. Similarly, any host species that contains different symbiont types across different habitats may be using partner choice to stabilize the mutualism, but this must be confirmed by further examination of the mechanism underlying the pattern; competition between symbiont types, environmental availability of the symbionts, and intraspecific host variation in specificity for symbiont type could also explain these patterns. Distribution by depth of different *Symbiodinium* types in conspecific hosts is well documented (Rowan and Knowlton 1995, Rowan et al. 1997, Sampayo et al. 2007). Numerous examples of other habitat-distinct distributions of *Symbiodinium* populations are known, including Palauan reefs versus an adjacent marine lake (Fabricius et al. 2004), *Acropora millepora* across different reef habitats on the Great Barrier Reef (van Oppen et al. 2001), and forereef versus lagoonal habitats in Ofu, American Samoa (Oliver and Palumbi 2009).

Host-driven swapping of symbiont type in response to environmental change represents a special case of partner choice. A heterogeneous mix of symbiont types may offer the host a more flexible response to stress (Rowan 1998). According to the adaptive bleaching and symbiont shuffling hypotheses, following adverse environmental change, the dominant symbiont type is expelled during bleaching. Then a new or previously low-background-level symbiont type which is a better fit with the host to the changed environment multiplies to become the new dominant type (Buddemeier and Fautin 1993, Kinzie et al. 2001, Baker 2003, Baker et al. 2004, Fautin and Buddemeier 2004). The verification of these hypotheses is contentious because of the critical importance of being able to predict coral reef response in the face of global climate change (Hoegh-Guldberg et al. 2002). Seeing coral bleaching through the lens of extant ecological theory may help clarify this issue. Moreover, it may help bring the focus back to the basic biology of the mutualism and facilitate communication between disciplines (Edmunds and Gates 2003).

Several observations support the adaptive bleaching / symbiont shuffling hypothesis. Sublethal bleaching is common and *Symbiodinium* can persist at low levels through bleaching events (Gates 1990). A shift in dominance of symbiont communities can occur in response to environmental change (Thornhill et al. 2006). Corals can acquire new symbiont types after

bleaching (Lewis and Coffroth 2004). Also, persistent, low background level, stress-tolerant symbiont types are found in some corals (Mieog et al. 2007). Transplanted *Acropora millepora* corals shuffled their symbiont types (Berkelmans and van Oppen 2006). However, some corals have been shown to not change their symbionts through sublethal bleaching (Hannes et al. 2009), and whether the majority of coral species are able to host multiple types remains unknown (Goulet 2006, Baker and Romanski 2007). Furthermore, in order to show that this is an evolved host adaptation, a host mechanism must be shown that actively controls symbiont populations in response to stress.

In anthozoans *Symbiodinium* is harbored in endodermal cells (Glider et al. 1980), so for the host to actively respond to symbiont cooperativeness, intracellular regulation of symbionts is necessary. Different mechanisms may be responsible for the regulation of *Symbiodinium* in corals, including apoptosis, host cell detachment, and *in situ* degradation of symbionts (Gates et al. 1992) (Figure 4). Evidence for programmed cell death in symbiont-bearing host cells alone does not provide direct evidence for partner choice because it indicates nothing about assessment of cooperativeness, though it does provide a putative mechanism whereby a host rejects algae that are uncooperative. Reactive oxygen species and nitric oxide are generated in the holobiont under heat stress and can induce programmed cell death in the host; the associated signaling pathways are thus a mechanism whereby the system may integrate and respond to feedback about its performance in the current environment (see review by Weis 2008). Control of the interaction is probably a product of input from both partners (Weis 2008), emphasizing how choice may be driven by both partners. Symbiont cell division rate drives symbiont expulsion rate in corals (Baghdasarian and Muscatine 2000), an especially compelling case for partner choice in the host, since the mechanism operates irrespective of symbiont genotype. Symbiont cell division rate may be a proxy for cooperativeness, as algae that devote more photosynthate resources to cell division may be devoting less to the host. If cell detachment or programmed cell death are the mechanisms that hosts use to regulate less cooperative symbionts, then partner choice comes at a material cost to the host. The frequency of environmental stress should influence this cost; the real effect of environmental change on the balance of fitness costs and benefits in the interaction is not well known, but the disruption in that balance is the hypothesized cause of coral bleaching (Muller-Parker and D'Elia 1997).

Can natural selection on individual hosts evolve adaptive bleaching or symbiont shuffling, given that there are potential costs to being able to host multiple types? The stabilization of the mutualism through partner choice gives an evolutionary pathway whereby the mutualism can be stabilized irrespective of mixed infection. A feedback mechanism that responds to cooperativeness provides the additional benefit of being able to respond to shifting fitness costs and benefits in a changing environment.

Any of the three strategies, partner fidelity feedback, cooperator association and partner choice, may stabilize *Symbiodinium*/host mutualism. These different mechanisms are not mutually exclusive and may operate simultaneously in a given species. Different strategies may be advantageous under different circumstances. Which mechanism is dominant may change during different life history stages; many corals reproduce both sexually and asexually, and exhibit horizontal transmission in one mode and vertical transmission in another. The interplay between mode of host reproduction and mode of symbiont transmission helps determine the evolutionary trajectory of a holobiont (Day et al. 2008). Generalizations are difficult to make about the complex landscape of diverse interactions between *Symbiodinium* dinoflagellates and

their many hosts. However, a general model for the evolutionary stability of mutualism allows categorization of these interactions, which in turn can illuminate new research directions.

Phylum	Class	Order	Genus (ref.)
Cnidaria	Anthozoa	Actinaria	*Anthopleura (8, 10)*
			Condylactis (7, 16)
		Alcyonacea	*Briareum (5)*
			Eunicea (7)
			Plexaura (4, 7, 13)
			Pseudopterogorgia (13, 14)
		Zoantharia	*Palythoa (6)*
			Zoanthus (7, 9)
	Hydrozoa	Anthomedusae	*Millepora (7)*
	Scyphozoa	Rhizostomae	*Cassiopeia (7)*
Mollusca	Bivalvia	Veneroidia	*Corculum (2)*
			Hippopus (1, 2)
			Tridacna (1, 2)
	Gastropoda	Nudibranchia	*Pteraeolidia (11)*
Rhizaria	Foraminifera	Globiginerida	*Orbulina (15)*
			Globigerinoides (15)
		Miliolida	*Amphisorus (3, 12)*
			Sorites (12)

Table 1. Evidence of mixed infection in non-scleractinian hosts. Hosts from a broad taxonomic range, including most groups that have been extensively sampled, have documented occurrence of mixed infection of symbiotic dinoflagellates. Reference key: 1. (Belda-Baillie et al. 1999), 2. (Carlos et al. 2000), 3. (Fay et al. 2009), 4. (Goulet and Coffroth 2003), 5. (Hannes et al. 2009), 6. (Kemp et al. 2006), 7. (LaJeunesse 2002), 8. (LaJeunesse and Trench 2000), 9. (LaJeunesse et al. 2008), 10. (Lewis and Muller-Parker 2004), 11. (Loh et al. 2006), 12. (Pochon et al. 2007), 13. (Santos and Coffroth 2003), 14. (Santos et al. 2003), 15. (Shaked and de Vargas 2006), 16. (Venn et al. 2008)

Figure 3.

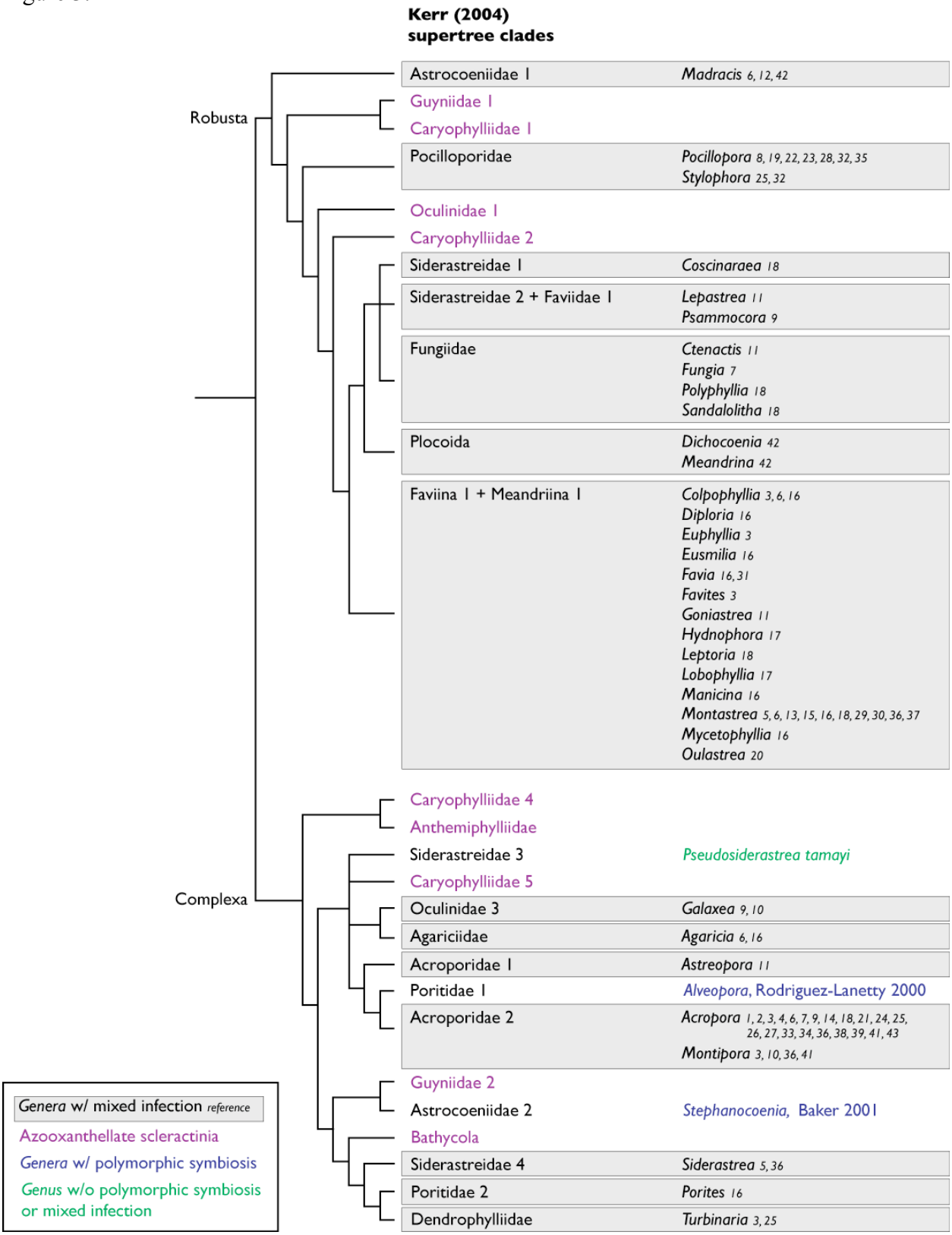
Kerr (2004)
supertree clades
Robusta
Complexa
Astrocoeniidae 1
Madracis 6, 12, 42
Guyniidae 1
Caryophylliidae 1
Pocilloporidae
Pocillopora 8, 19, 22, 23, 28, 32, 35
Stylophora 25, 32
Oculinidae 1
Caryophylliidae 2
Siderastreidae 1
Coscinaraea 18
Siderastreidae 2 + Faviidae 1
Lepastrea 11
Psammocora 9
Fungiidae
Ctenactis 11
Fungia 7
Polyphyllia 18
Sandalolitha 18
Plocoida
Dichocoenia 42
Meandrina 42
Faviina 1 + Meandriina 1
Colpophyllia 3, 6, 16
Diploria 16
Euphyllia 3
Eusmilia 16
Favia 16, 31
Favites 3
Goniastrea 11
Hydnophora 17
Leptoria 18
Lobophyllia 17
Manicina 16
Montastrea 5, 6, 13, 15, 16, 18, 29, 30, 36, 37
Mycetophyllia 16
Oulastrea 20
Caryophylliidae 4
Anthemiphylliidae
Siderastreidae 3
Pseudosiderastrea tamayi
Caryophylliidae 5
Oculinidae 3
Galaxea 9, 10
Agariciidae
Agaricia 6, 16
Acroporidae 1
Astreopora 11
Poritidae 1
Alveopora, Rodriguez-Lanetty 2000
Acroporidae 2
Acropora 1, 2, 3, 4, 6, 7, 9, 14, 18, 21, 24, 25, 26, 27, 33, 34, 36, 38, 39, 41, 43
Montipora 3, 10, 36, 41
Guyniidae 2
Astrocoeniidae 2
Stephanocoenia, Baker 2001
Bathycola
Siderastreidae 4
Siderastrea 5, 36
Poritidae 2
Porites 16
Dendrophylliidae
Turbinaria 3, 25
Genera w/ mixed infection reference
Azooxanthellate scleractinia
Genera w/ polymorphic symbiosis
Genus w/o polymorphic symbiosis or mixed infection

Figure 3.
Cladogram showing relationships between major scleractinian coral clades adapted from supertree of Kerr (Kerr 2005). Genera with mixed infection are broadly distributed across the phylogeny, and most of those without evidence for mixed infection are azooxanthellate clades. Two zooxanthellate clades without evidence for mixed infection do have evidence for polymorphic symbiosis, and the remaining zooxanthellate clade without evidence for mixed infection represents a poorly sampled monospecific clade in the Kerr tree.

Reference key: 1. (Abrego et al. 2009a), 2. (Berkelmans and van Oppen 2006), 3. (Chen et al. 2005a), 4. (Chen et al. 2005b), 5. (Correa et al. 2009b), 6. (Correa et al. 2009a), 7. (Crabbe and Carlin 2009), 8. (Darius et al. 1998), 9. (Dong et al. 2009), 10. (Dong et al. 2008), 11. (Fabricius et al. 2004), 12. (Frade et al. 2008), 13. (Garren et al. 2006), 14. (Huang et al. 2006), 15. (Kemp et al. 2008), 16. (LaJeunesse 2002), 17. (LaJeunesse et al. 2003), 18. (LaJeunesse et al. 2004), 19. (LaJeunesse et al. 2008), 20. (Lien et al. 2007), 21. (Little et al. 2004), 22. (Magalon et al. 2006), 23. (Magalon et al. 2007), 24. (McClanahan et al. 2005), 25. (Mieog et al. 2007), 26. (Mieog et al. 2009), 27. (Oliver and Palumbi 2009), 28. (Rowan and Powers 1991), 29. (Rowan and Knowlton 1995), 30. (Rowan et al. 1997), 31. (Savage et al. 2002), 32. (Sebastian et al. 2009), 33. (Stat et al. 2008a), 34. (Stat et al. 2009a), 35. (Stat et al. 2009b), 36. (Thornhill et al. 2006a), 37. (Toller et al. 2001b), 38. (Ulstrup and van Oppen 2003), 39. (Ulstrup et al. 2007), 40. (van Oppen et al. 2001), 41. (van Oppen 2004), 42. (Venn et al. 2009), 43. (Visram and Douglas 2006)

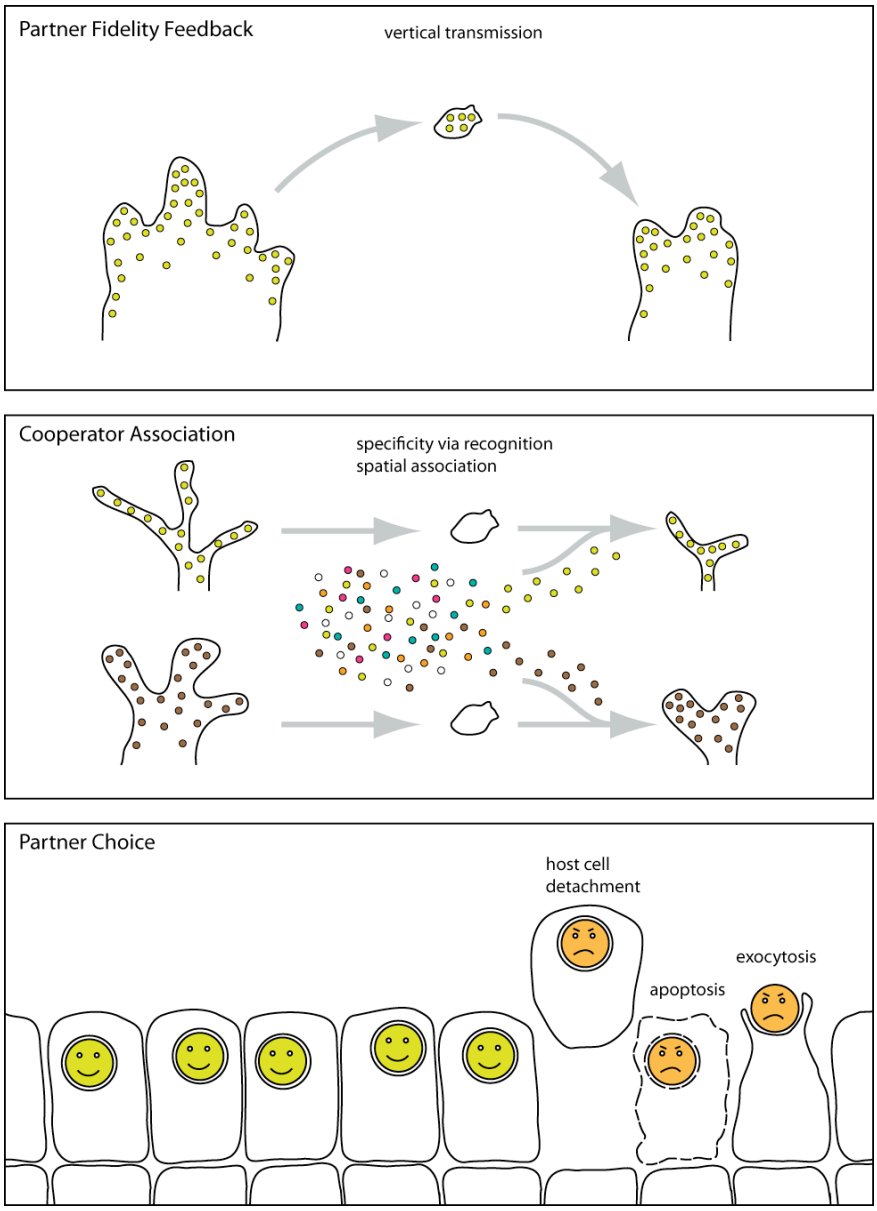

Figure 4.
Diagram of the three mechanisms that stabilize *Symbiodinium*-host interaction.

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CHAPTER THREE

The distribution of *Symbiodinium* diversity within individual host foraminifera

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ABSTRACT

While one-to-one specificity between reef-dwelling hosts and symbiotic dinoflagellates of the genus *Symbiodinium* may occur, detailed examination of some hosts reveals that they contain multiple symbiont types. Individuals of the foraminifer *Amphisorus hemprichii* living in Papua New Guinea contained mixed communities of *Symbiodinium* dominated by symbiont types in clades C and F. Moreover, the types showed a distinct pattern in their distribution across the radius of the foraminifer, with clade F *Symbiodinium* more prevalent in the center of the host cell. The mixed community of symbionts and their pattern of distribution within the foraminifer is likely the result of processes happening both inside the foraminifer and in its external environment. Persistent mixed symbiont communities in foraminifera may be stabilized through benefits conferred by maintaining multiple symbiont lineages for symbiont shuffling. Alternatively they may be stabilized through a heterogeneous internal host environment, partitioning of symbiont functional roles or limitation of symbiont reproduction by the host. Six factors generally determine the presence of any particular symbiont type within a foraminifer: mode of transmission, availability from the environment, recognition by the host, regulation by the host, competition between lineages, and fitness of the holobiont.

INTRODUCTION

The formation and persistence of modern coral reefs depends largely on organisms that host dinoflagellate algal symbionts of the genus *Symbiodinium*. *Symbiodinium* displays wide genetic diversity, both within its many hosts and across multiple spatial scales (Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). This diversity groups into eight clades, lettered A through H, and within each of these clades further genetic diversity represents ecologically distinct lineages of *Symbiodinium,* hereafter referred to as "types" (reviewed in Coffroth and Santos 2005). Phenotypic differences exist between different clades, such as susceptibility to bleaching or physiological variation under different light and temperature conditions (Kinzie and Chee 1979; Rowan et al. 1997; Rowan 2004). Different *Symbiodinium* types within a clade are also ecologically distinct and are differentially distributed over factors such as biogeography, habitat, host type, and host ontogeny (LaJeunesse et al. 2004 ; Rodriguez-Lanetty et al. 2004 ; Sampayo et al. 2007). Temporary shifts in symbiont type following environmental perturbations are also known (Thornhill et al. 2006). However, many of the biological factors that influence the composition of *Symbiodinium* lineages within an individual host remain to be discovered.

Characterization of the diversity of *Symbiodinium* has frequently assumed that an individual host contains only a single physiologically or ecologically important symbiont lineage. Some methods used to genetically identify *Symbiodinium* types, such as direct sequencing and Denaturing Gradient Gel Elecrophoresis (DGGE), can fail to recognize low levels of alternate *Symbiodinium* genotypes in an individual sample (Apprill and Gates 2007). In studies on corals using methods that are explicitly designed to identify mixed genotypes, such as FISH and real-time Q-PCR, a mix of symbionts is commonly found (Loram et al. 2007). Intragenomic variation at the ribosomal rRNA locus potentially confounds the interpretation of multiple rRNA haplotypes (especially in the ITS regions) as multiple independent lineages, or types, of *Symbiodinium* (Thornhill et al. 2007). Fortunately the extent of this intragenomic variation does not appear to obscure the signal from sequence differences seen between the different clades (Sampayo et al. 2009).

Mixed symbiont communities do appear to be common in some corals (Baker and Romanski 2007). In one study, four species of coral that previously were thought to possess only a single clade were shown to harbor multiple clades of *Symbiodinium* in nearly 80% of the individuals sampled (Mieog et al. 2007). Non-coral host individuals may also commonly host multiple clades; for example, individuals of certain species of tridacnid clams harbor multiple symbiont clades (Carlos et al. 2000).

An important component of reef communities, symbiont-bearing foraminifera produce on average nearly 5% of the carbonate deposited on coral reefs, and up to 25% on some reefs (Langer et al. 1997). They host a more genetically diverse assortment of symbiont types than most coral species (Pochon et al. 2007), supporting a hypothesis that they may be a reservoir for *Symbiodinium* diversity in the reef community. While most *Symbiodinium* hosts such as corals, clams, and sponges feed primarily from the seawater flowing over a reef, foraminifera directly feed from the surface upon which they live (see video in electronic supplementary material) and thus may directly interact with the benthic *Symbiodinium* community.

Symbionts of soritid foraminifera were recognized early on as cytologically similar to the zooxanthellae of corals and clams (Doyle and Doyle 1940). The first published sequences from

Symbiodinium found in foraminifera showed that the symbionts are genetically similar to those found in corals (Langer and Lipps 1995). Since then, Xavier Pochon and his colleagues have described in detail the genetic diversity of *Symbiodinium* found in these foraminifera (reviewed in Pochon and Pawlowski 2006), examining factors such as host specificity (Pochon et al. 2001; Garcia-Cuetos et al. 2005), biogeographic distribution (Pochon et al. 2004), and local ecology (Pochon et al. 2007). Analysis of *Symbiodinium* from foraminifera using DGGE indicated that 15% of samples from Guam had mixed symbiont types (Pochon et al. 2007), but an explicit study of symbiont heterogeneity in foraminifera has, until now, not been done. Thus the first aim of this study was to examine more closely the symbiont composition within individual foraminifera.

The second aim of this study was to see whether the symbiont composition is distributed evenly within an individual foraminifer. The cytoplasm of a soritid foraminifer is not a homogenous mix of its contents. The test is divided into chambers, and the apertures between the chambers allow the foraminifer to partition its cytoplasm into different zones (Figure 5, adapted from Muller-Merz and Lee 1976). Algal symbionts are found throughout the host. Foraminiferal nuclei are concentrated in the central zone and the zone along the edge has many food vacuoles. The symbionts are most densely packed in the intermediate zone, which appears as a darker circle, giving the discoidal foraminifer a bulls-eye appearance. These three zones suggested a sampling strategy to see if different symbiont types occur in different parts of the foraminifer.

METHODS

Field site and collection

In August of 2005, on SCUBA, *Amphisorus hemprichii* foraminifera were hand-collected into Ziploc bags from the forereef on the Pacific Ocean side of Nusalik Island, near Kavieng, New Ireland Province, Papua New Guinea. The foraminifera were collected in two areas approximately 1 km apart (2°34'26"S, 150°46'26"E, and 2°34'58"S, 150°46'15"E). Within each area three samples were collected, one each at 20m, 12m, and 6m deep. Ten minutes at each depth was spent collecting as many soritid foraminifera as possible, typically more than thirty individuals. All *A. hemprichii* foraminifera collected were 3mm to 6mm in diameter. In the laboratory, sixteen individuals from each sample were brushed clean in filtered seawater then broken in half. One half was placed into tubes with RNAlater nucleic acid stabilization reagent (Qiagen), the other half dried for morphological identification.

Extraction and PCR

In the laboratory, from each foraminifer three samples of approximately 1mm^3 were taken, one from each zone: inner, intermediate, and outer. Extracts were made from each sample using a guanidinium-based protocol, (adapted from Sambrook et al. 1989). Three foraminifera from each depth in the two sampling areas were examined; nine foraminifera total were examined, six from one area and three from the other.

Dinoflagellate nuclear DNA from the rRNA locus (ITS1 - 5.8S - ITS2 - partial 28S) was PCR amplified using an MJ PTC-200 thermocycler with the program (94ºC 3:00 min., 64ºC 1:30 min., 35 x (72ºC 2:00 min., 94ºC 0:45 min., 64ºC 0:45 min.), 72ºC 5:00 min.) using primers

S_DINO and L_O (Pochon et al. 2001) and the enzyme AmpliTaq Gold (Applied Biosystems) with manufacturer's recommended reagent concentrations.

Cloning and sequencing

PCR products (N=27) were cloned using a TOPO TA cloning kit (Invitrogen K250020). Twenty-four colonies from each reaction were picked and cultured in 4mL of LB+Kanamycin overnight, centrifuged, and plasmid was extracted from pelleted bacteria using the phenol-chloroform protocol (Sambrook et al. 1989). Extracted plasmid was quantitated and checked for correct size insert on a 0.8% agarose gel/TBE. From eight clones per reaction, plasmid with correct size insert was sequenced on an ABI 3730 capillary sequencer using S_DINO as a primer. In total, 199 clones were sequenced since some of the cloning reactions resulted in fewer than eight clones.

Analysis

The resulting clone sequences were first examined using MEGA-BLAST (http://www.ncbi.nlm.nih.gov/blast). Some (5/199, <3%) of the sequences were discarded because they were identified as pseudogenes based on large deletions in rRNA-coding regions (Thornhill et al. 2007; Scott Santos pers. comm.). All of the remaining clone sequences were aligned with MUSCLE (Edgar 2004) and checked by eye. Since phylogenetic inference software for DNA sequences can only handle gaps as either a fifth character state or missing data, gaps and their associated poorly aligned sequence segments were then removed using GBLOCKS (Castresana 2000). The resulting 194 aligned clone sequences were analyzed with TCS (Clement et al. 2000) using a 95% statistical parsimony criterion.

The original sequences of the resulting five ancestral haplotypes (representing the clusters) were then aligned (using MUSCLE + GBLOCKS) to representative sequences from the literature (see Table 2 for GenBank accession numbers). A phylogeny was inferred from this new alignment using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), substitution model GTR + I + gamma as determined by ModelTest (Posada and Crandall 1998), for 1.2×10^6 generations, discarding 2×10^5 generations as burn-in.

To identify and name the ancestral haplotypes, their ITS2 sequences were aligned (using MUSCLE) to those sequences found in Pochon et al. 2007, wherein the most fine scale diversity of ITS2 types to date has been described. Pairwise distance to the closest match sequence was calculated to quantify homology.

The individual haplotype clusters were interpreted as distinct types of *Symbiodinium*. Symbiont type versus depth, area, and intracellular host zone were visualized using JMP 7.0 software (SAS Institute, Inc.). The null hypothesis that symbiont type and host zone are independent variables was tested using the Pearson's chi-square statistic.

RESULTS

Clustering of haplotypes and identity of clusters

The clone sequences were divided by TCS into five different clusters (Figure 6). These clusters were separated by a greater than eleven base-pair difference, the 95% parsimony criterion for this data set. No cycles were seen in the networks, which would be evidence for either potential recombination events or chimeras resulting from cloning.

TCS inferred an ancestral haplotype for each cluster (boxed in Figure 6), which was used to identify and name the cluster. Aligned to previously published sequences found in GenBank (Figure 7), phylogenetic analysis of the ancestral haplotype sequences for each of these networks (clones 168, 281, 207, 415, and 238) showed that they come from three clades (see Coffroth and Santos 2005), C, F, and H.

The full sequence of clone 168 closely matches two sequences from Genbank, both identified as from clade C, one obtained from the giant ciliate *Maristentor* sp. (AJ278598, Lobban et al. 2002) with 99.2% sequence identity and another from a foraminifer *Marginopora vertebralis* (AJ311941, Pochon et al. 2001) with 99.3% sequence identity). The ITS2 sequence of clone 168 most closely matches an ITS2 sequence from C1 (AM748551) with 99.6% sequence identity; and is named C1.168. The ITS2 sequence from clone 415 most closely matches F3.1 (AM748565), with 99.4% sequence identity; it is named F3.1.415. The ITS2 sequences from clones 281 and 207 are similar to but relatively divergent from F3.1 (AM748565), with 95% and 93% sequence identity, respectively; by extending the Pochon et al. 2007 classification of types in sub-clade F3 and creating two new sub-clades, they are named F3.5.281 and F3.6.207, respectively.

Symbiont heterogeneity and patterns in distribution

All the foraminifera studied contained a mixed community of symbiont types (Figure 8a). Each individual hosted at least two clades and one hosted three. The majority of the symbionts found in these foraminifera were of two main types, C1.168 and F3.5.281 (as described by the clusters). Clade H *Symbiodinium* was found only in the outer and intermediate chambers.

When data from all the foraminifera in this study were combined, a significant (χ^2=34.969, p<0.0001) pattern was evident across the radius of a foraminifer. Type F3.5.281 was slightly more prevalent than C1.168 in the inner chambers of the foraminifer, but on the edge, C1.168 was dominant. The three other types made up a minor part of the total community of *Symbiodinium*. There was no gradient in *Symbiodinium* type by depth or significant difference (χ^2=2.414, p=0.2991) between the two geographic areas.

DISCUSSION

Symbiont diversity within individual foraminifera

Nine conspecific foraminiferan individuals from a single population in Papua New Guinea showed a great diversity of symbiont haplotypes: 97 different unique haplotypes (ITS1-5.8S-ITS2 rDNA) from within three different clades (Figure 6). In an earlier study of 1,010 different individual foraminifera from Guam, a high diversity of *Symbiodinium* ITS2 types from DGGE bands was found, with 61 different types from five different clades (Pochon et al. 2007). Though diversity of cloned PCR products and diversity of DGGE bands are not directly comparable, populations of foraminifera from Guam and PNG both harbor a diverse array of symbionts.

The rRNA locus in *Symbiodinium* is by far the best represented in the literature to date. However, it has drawbacks as a molecular marker, most particularly its considerable intragenomic variability. In a study of intragenomic variation at the ITS1 – 5.8S – ITS2 rDNA locus, many of the variants deviated from the dominant haplotype by a single base pair, with others diverging by multiple base-pair substitutions (Thornhill et al. 2007). The pattern is similar

to that seen in this data set (Figure 6); the clones have many single base pair differences, likely an artifact of either intragenomic variation or PCR mutations. Since ecologically distinct ITS2 haplotypes can be separated by only a few base-pair changes (LaJeunesse et al. 2004; Sampayo et al. 2009), grouping diverse *Symbiodinium* ITS-region haplotypes into statistical parsimony networks using TCS is a method that conservatively forms groups that are ecologically distinct (Rodriguez-Lanetty 2003; Rodriguez-Lanetty et al. 2006b; Pochon et al. 2007, Correa and Baker 2009).

All of the individual foraminifera studied contained *Symbiodinium* of more than one clade (Figure 8a). The presence of up to three different clades of *Symbiodinium* within such a tiny host seems remarkable, more so considering that foraminifera are single-celled organisms. Rather than being a phenomenon localized to Micronesia (see Pochon et al. 2007), populations of foraminifera throughout the western Pacific, if not the globe, likely maintain genetically diverse assemblages of *Symbiodinium*, implicating foraminifera as important reservoirs of symbiont diversity in coral reef ecosystems.

The ability to pair with multiple *Symbiodinium* types may be normal for hosts with horizontal transmission of their symbionts (Baker and Romanski 2007). The data reported here support this hypothesis. Ecological theory suggests that competition between multiple symbiont lineages destabilizes mutualism by selecting for more virulent, less cooperative strains; this is disadvantageous to the host (Frank 1996). The adaptive bleaching hypothesis and symbiont shuffling together (Buddemeier and Fautin 1993; Baker 2003; Fautin and Buddemeier 2004) provide a possible explanatory counterbalancing benefit to this cost. A heterogeneous mix of symbiont types may offer the host a more flexible response to stress (Rowan 1998). In this model, following adverse environmental change, the dominant type is expelled during bleaching, and a low-level background symbiont type, multiplies to become the new dominant type because it is more advantageous in the new environment. However, in trying to explain the persistence of mixed symbiont communities within an individual host, alternative hypotheses such as a heterogeneous internal host environment, distinct functional roles for the different symbiont lineages (symbiont niche partitioning), or limitation of symbiont reproduction by the host should also be considered.

Structure in symbiont distribution from outer to inner chambers

The symbionts in these soritid foraminifera show a shift in distribution of *Symbiodinium* type from the outer chambers to the inner chambers (Figure 8b). Because the copy number at the nuclear rRNA locus in *Symbiodinium* spans a wide range and because PCR does not amplify DNA in a linear fashion throughout the reaction, these results are not an absolute quantitative measure of the proportions of these symbiont types (Apprill and Gates 2007; Loram et al. 2007; Thornhill et al. 2007). Rather, the data represent a relative measure of symbiont distribution. Several alternative hypotheses can account for what might cause this pattern.

These foraminifera actively feed, and thus may acquire new symbionts from their environment (Lee and Anderson 1991). If a free-living, changing, mixed community of *Symbiodinium* is present in the environment, the pattern of symbiont distribution in the foraminifer could represent sampling by the host through time. The types near the edge could be those most recently encountered in the environment. Those in the center could have been acquired from an earlier time, which have since moved inward. Similarly, the pattern could represent the symbiont mix that was present in the environment when each successive row of chambers formed.

This pattern might also result if the foraminifer were sorting, processing, or otherwise regulating the symbionts as they move inward. The community of symbionts on the edge of the foraminifer may represent the environmental assemblage and those in the center the enriched type(s). Clade C and H *Symbiodinium,* the lineages that dominate at the edge of the foraminifer, were found free-living in samples of Pacific Ocean seawater (Manning and Gates 2008). Clade F symbionts, which appear to be specialists to foraminifera (Pochon and Pawlowski 2006), are in greater proportion in the innermost chambers. These observations suggest an enrichment mechanism.

A third mechanism that may explain this pattern is competition or self-sorting of the symbionts within the heterogeneous environment of the host. Different parts of the foraminifer may provide a better habitat for different lineages of symbiont, which either migrate to or compete for them. Since the different zones of these foraminifera are distinct in terms of their cellular contents (McEnery and Lee 1981), this is also a reasonable hypothesis.

Multiple distantly related *Symbiodinium* lineages exist within an individual foraminifer and show a distinct concentric pattern of distribution. These facts raise new questions about the basic biology of soritid foraminifera and how they relate to the overall reef community. These findings highlight the fact that the host itself is an environment, and that the relationship between symbiont and host is subject to multiple ecological forces.

Factors that determine the symbiont assemblage found in foraminifera

Three factors have been suggested to explain the symbiont specificity seen in soritid foraminifera (Garcia-Cuetos et al. 2005): recognition of the symbiont by the host, vertical transmission of the symbiont, and localized coevolution of the holobiont. Here this model is built upon and broadened by identifying six factors that determine which symbionts are found in a foraminiferan host:

Mode of symbiont transmission

The mode of symbiont transmission in soritid foraminifera is dependent upon the life cycle of the host. Soritid foraminifera have a paratrimorphic life cycle, with both sexual and asexual reproductive phases (Kloos and Macgillavry 1978; Zohary et al. 1980; Fujita et al. 2000). In this type of life cycle, a lineage can go through multiple rounds of asexual reproduction. Symbionts are transmitted vertically, from mother to daughter cells, until eventually the host lineage undergoes meiosis to form haploid individuals (gamonts). When these gamonts reach maturity, they produce gametes. The gametes are too small to contain or otherwise transmit symbionts, so this newly diploid zygote (agamont) must adopt symbionts anew from the environment (Lee and Anderson 1991).

The paratrimorphic life cycle offers foraminifera a potential benefit in its flexibility. Vertical transmission can be beneficial because it maintains fidelity with a well-suited symbiont, aligning the interests of the partners (Herre et al. 1999). Horizontal transmission allows a shift to a new symbiont pool, advantageous during times of environmental change (Douglas 1998; Rowan 1998). Thus a paratrimorphic life cycle allows a strategy where both of these forces can act within a single system.

Symbiont availability from the environment

Soritid foraminifera have a dynamic relationship with their benthic environment, transporting materials to and from their cell body with rhizopodia. Living foraminifera typically collect benthic microorganisms and detritus around their margins. Whether or not adult foraminifera can acquire new symbionts from their environment is unknown, though zygotes certainly must. Thus the habitat preferences of different types of *Symbiodinium* may help determine which types are found in foraminifera. Free-living planktic and benthic strains of *Symbiodinium* have been cultured and identified (Coffroth et al. 2006), and research increasingly focuses on directly characterizing free-living populations of *Symbiodinium*, especially their relationship to populations *in hospite* (Manning and Gates 2008). Yet much remains to be learned about the biogeography and autecology of the alga in its free-living state.

Recognition of symbionts by the host

In systems with horizontal transmission, the host must encounter free-living symbionts at the boundary between host and external environment. Recognition represents a gateway where only certain genotypes of symbiont (which presumably express idiosyncratic cell surface molecules) avoid digestion by the host. In coral hosts, initial steps have been taken towards understanding the molecular underpinnings that determine recognition of *Symbiodinium* (Reynolds et al. 2000; Yuyama et al. 2005; Rodriguez-Lanetty et al. 2006a; Deboer et al. 2007). In foraminifera, molecular factors have been discovered that are important in diatom symbiont recognition (Chai and Lee 1999), but the antibodies used in these experiments do not bind to *Symbiodinium* cells (Lee and Reyes 2006); no other studies have further addressed this question for soritid foraminifera. Hypotheses that explain the distribution of *Symbiodinium* in foraminifera must take into account the potential for recognition, especially given the evidence for symbiont specificity in foraminifera (Garcia-Cuetos et al. 2005; Pochon and Pawlowski 2006). Since the clades C, F, and H together are monophyletic, the data presented here cannot reject the hypothesis that these symbionts share some common attribute that allows their recognition by foraminifera.

Regulation by the host: the internal environment

After a symbiont enters the host, the host must have some way to regulate the symbiont population. An array of regulation mechanisms have been proposed and studied in corals, operating either by controlling reproduction rates of, selectively destroying, or expelling unwanted symbionts (Gates et al. 1992; Falkowski et al. 1993; Baghdasarian and Muscatine 2000; Dunn et al. 2002, Dunn and Weis 2009). Such post-phagocytic winnowing mechanisms may be responsible for the pattern of the distribution of symbiont types found in the foraminifera in this study. Symbiont type distribution is influenced by location within the host cell (Figure 8b), which suggests intracellular regulation.

Symbiont competition within the host

Whenever mixed symbiont types occur within a host, potential conflict arises between the interests of the host and the interests of the competing symbionts (Frank 1996). Competition and virulence can be important factors in determining which symbiont type(s) will ultimately be found in a host (Sachs and Wilcox 2006). Certain types may compete more successfully within a

particular host or region within a host. If foraminifera do not selectively regulate symbiont populations, then competitive interactions between symbiont types may help explain their distribution within their internal environment.

Holobiont fitness

A particular host-symbiont pairing is most successful when the fitness interests of the partners are aligned (Herre et al. 1999; Sachs et al. 2004). Holobiont fitness by definition is an increase in the abundance of a particular host/symbiont pair (Zilber-Rosenberg and Rosenberg 2008). It is possible that in some environments a holobiont consisting of a host with multiple symbiont types is more fit than one with a single type. Since all of the foraminifera examined in this study contained multiple symbiont types, perhaps foraminifera with more types have a fitness advantage over those with only one symbiont type in this particular reef environment.

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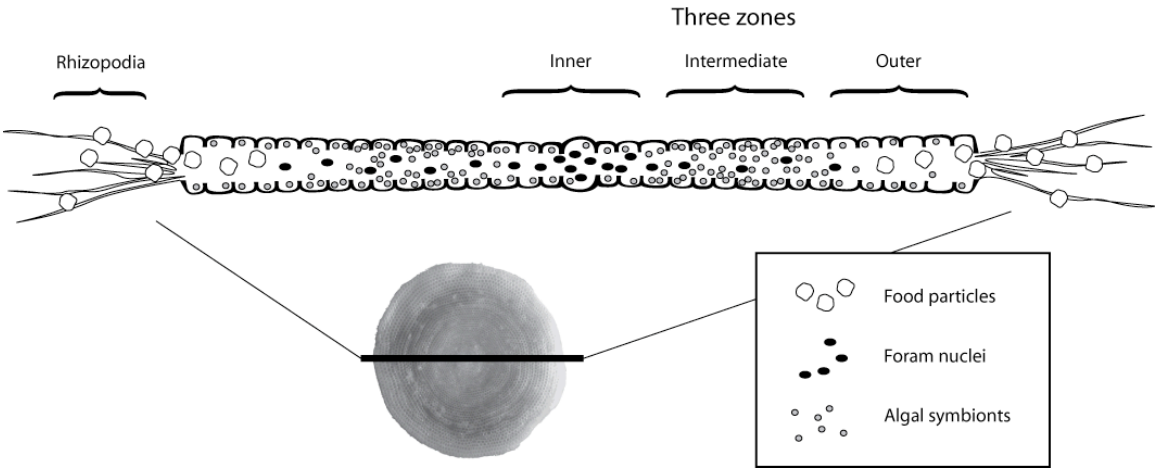

Figure 5. A cross-section diagram of a soritid foraminifer, adapted from Muller-Merz and Lee 1976. The test can be divided into three zones: 1. the inner zone, with some symbionts but mostly foraminiferal nuclei; 2. the intermediate zone, with some foram nuclei but mostly symbionts; and 3. the outer zone, with some symbionts but also food particles being digested.

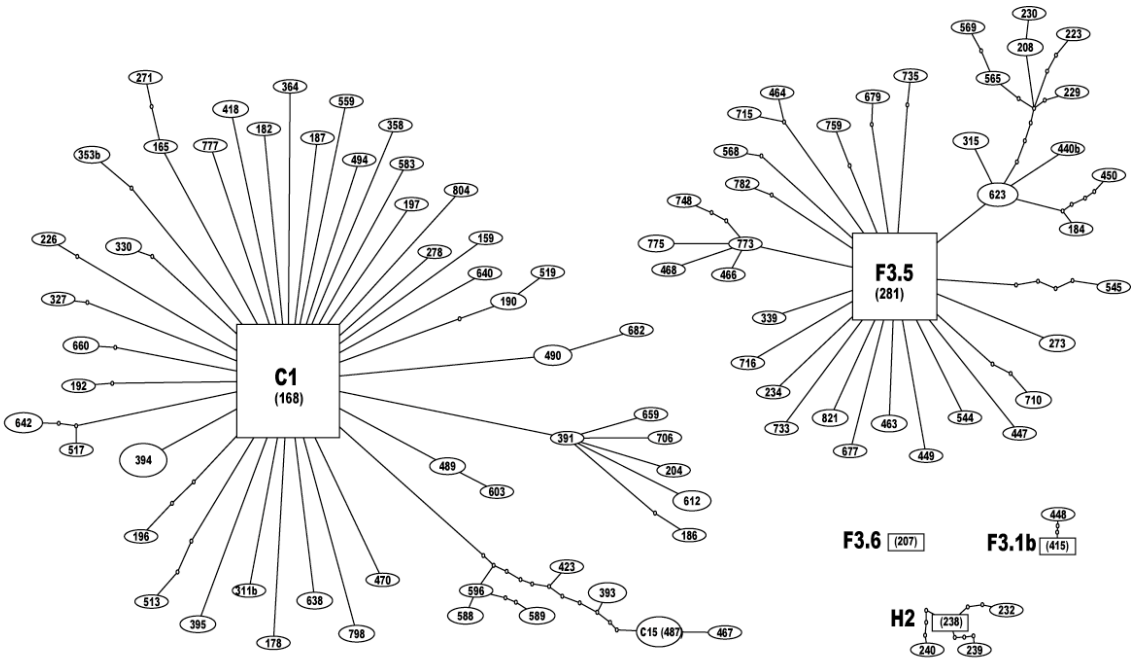

Figure 6. Unrooted statistical parsimony networks of all clones obtained from the nine foraminifera. Ancestral haplotypes are represented by a box, all others by ovals. The size of the box/oval is proportional to the number of clones with that haplotype. Numbers correspond to clone numbers from Table 2. *Symbiodinium* type designations follow that of Pochon et al. (2007) except "F3.5" and "F3.6," which are our own designations.

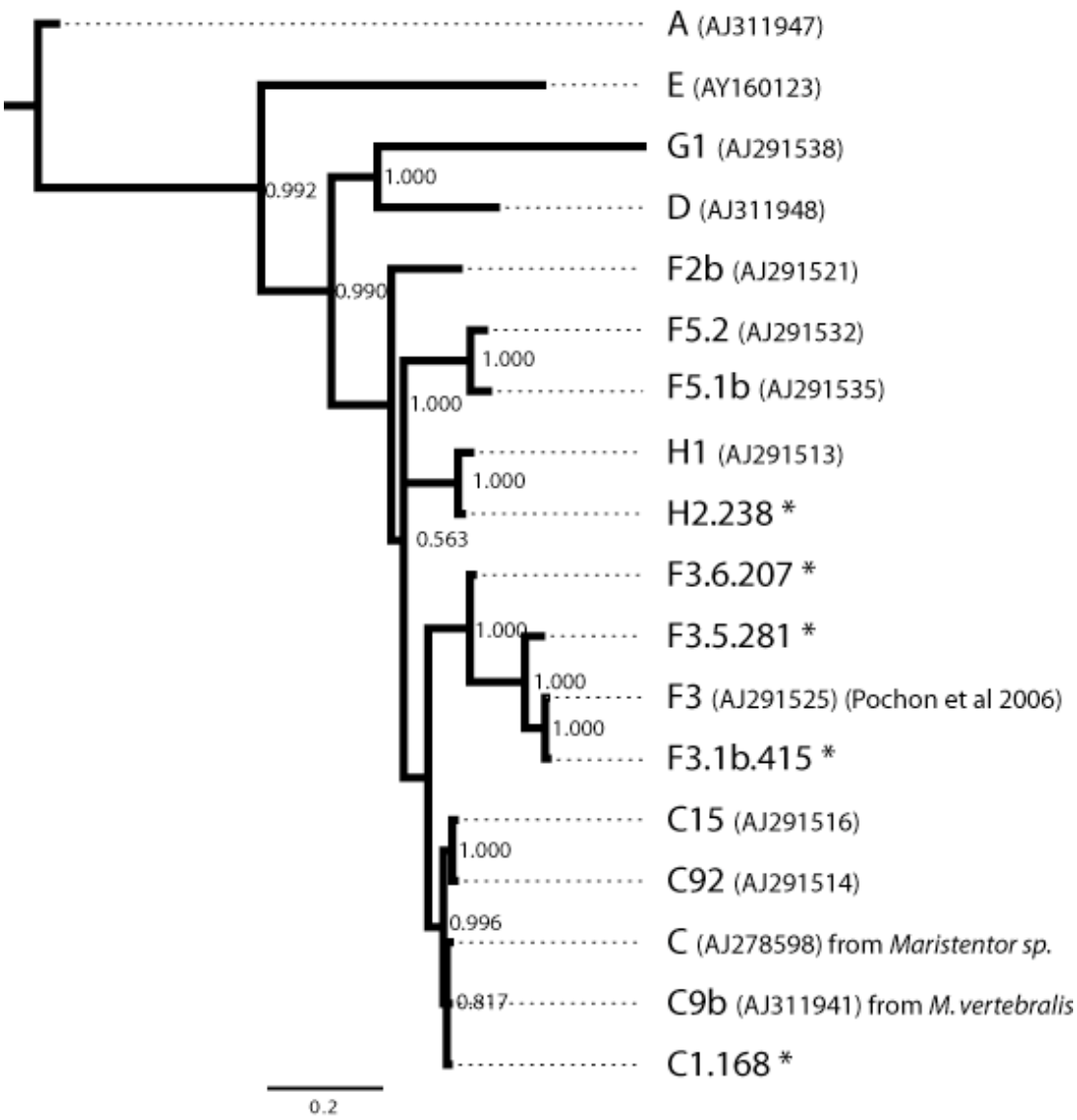

Figure 7. Ancestral cluster haplotypes, indicated with an asterisk, placed in context of previously identified *Symbiodinium* haplotypes using Bayesian inference of phylogeny. Branch support indicates Bayesian posterior probabilities; nodes with < 0.75 support collapsed. The sequence data is from the rRNA locus: ITS1, 5.8S, ITS2, and partial LSU.

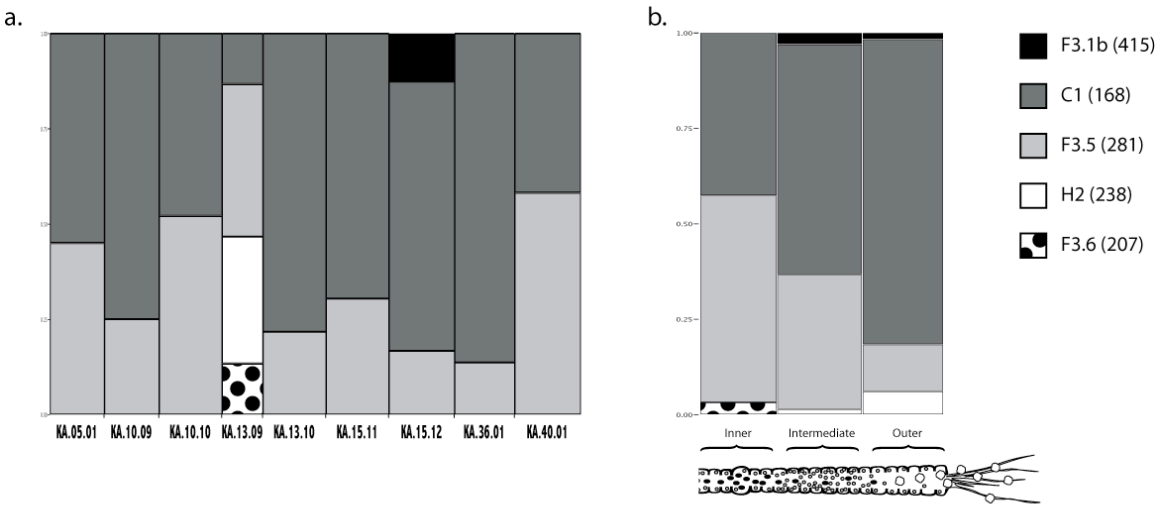

Figure 8. Mosaic plots of symbiont genotype distribution. Each vertical bar is proportional to the total number of clones recovered from each haplotype cluster for each sample. a. Combined data for each foraminifer. ($24 \geq n \geq 20$) b. Combined data for the three host "zones." Clade F types are enriched toward the center. (n=61 for inner, 68 for intermediate, 65 for outer, $\chi^2=34.969$, $p<0.0001$)

Clone ID	GenBank Accession	Cluster ID	# of clones
159	EU785998	C1	1
165	EU786001	C1	1
168	EU786002	C1	28
178	EU786004	C1	1
182	EU786007	C1	1
184	EU786008	F3.5	1
186	EU786009	C1	1
187	EU786010	C1	1
190	EU786011	C1	2
192	EU786012	C1	1
196	EU786014	C1	1
197	EU786015	C1	1
204	EU786017	C1	1
207	EU786018	F3.6	2
208	EU786019	F3.5	2
223	EU786022	F3.5	1
226	EU786023	C1	1
229	EU786024	F3.5	1
230	EU786025	F3.5	1
232	EU786026	H2	1
234	EU786027	F3.5	1
238	EU786028	H2	2
239	EU786029	H2	1
240	EU786030	H2	1
271	EU786032	C1	1
273	EU786033	C1	1
278	EU786034	C1	1
281	EU786036	F3.5	20
311	EU786038	C1	1
315	EU786039	C1	2
327	EU786040	C1	1
330	EU786041	C1	2
339	EU786045	F3.5	1
353	EU786046	C1	2
358	EU786047	C1	1
364	EU786050	C1	1
391	EU786053	C1	1
393	EU786054	C1	3
394	EU786055	C1	7
395	EU786056	C1	2
415	EU786061	F3.1B	2
418	EU786063	C1	2
423	EU786067	C1	1
440	EU786068	F3.5	1
447	EU786070	F3.5	1
448	EU786071	F3.1B	1
449	EU786072	F3.5	1
450	EU786073	F3.5	1
463	EU786075	C1	2
464	EU786076	F3.5	1
466	EU786078	F3.5	1
467	EU786079	C1	1
468	EU786080	F3.5	1
470	EU786082	C1	1
487	EU786083	C1	6
489	EU786085	C1	1
490	EU786086	C1	4
494	EU786088	C1	1
513	EU786090	C1	1
517	EU786093	C1	1
519	EU786094	C1	1
544	EU786100	F3.5	1
545	EU786101	F3.5	1
559	EU786102	C1	1
565	EU786105	F3.5	1
568	EU786107	F3.5	1
569	EU786108	F3.5	1
583	EU786110	C1	1
588	EU786111	C1	2
589	EU786112	C1	2
596	EU786115	C1	1
603	EU786117	C1	1
612	EU828666	C1	3
623	EU828667	C1	4
821	EU828668	C1	2
638	EU828669	C1	2
640	EU828670	C1	1
642	EU828671	C1	3
659	EU828672	C1	1
660	EU828673	C1	2
677	EU828674	F3.5	1
679	EU828675	F3.5	1
682	EU828676	C1	1
706	EU828677	C1	1
710	EU828678	C1	2
715	EU828679	F3.5	1
716	EU828680	F3.5	1
733	EU828681	F3.5	1
735	EU828682	F3.5	1
748	EU828683	F3.5	1
759	EU828684	F3.5	1
773	EU828685	F3.5	1
775	EU828686	C1	2
777	EU828687	C1	1
782	EU828688	F3.5	1
798	EU828689	C1	2
804	EU828690	C1	1

Table 2. List of unique rRNA ITS2 haplotypes found in the 9 foraminifera; Clone ID corresponds to the ID numbers in Figure 6, Cluster ID represents the ancestral sequence identity of the TCS cluster that contains the haplotype, and number of clones indicates how many clones of that unique haplotype were discovered.

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CHAPTER FOUR

Soritid foraminifera do not acquire new *Symbiodinium* dinoflagellates during growth

ABSTRACT

A host's strategy for stabilizing mutualism depends on its mode of symbiont acquisition. Soritid foraminifera host *Symbiodinium* dinoflagellates, which are also found as symbionts in corals and many other reef-dwelling hosts. These foraminifera have a paratrimorphic life cycle that alternates between sexual and asexual reproduction. They transmit their symbionts horizontally through rounds of sexual reproduction and vertically through rounds of asexual reproduction. Experiments exposing soritid foraminifera to exogenous *Symbiodinium* strains shows that they do not Foraminifera quarantine their newly formed chambers from environmental algal populations, populate new chambers using symbionts from their inner chambers, and thus do not acquire new symbionts from their environment as they grow.

INTRODUCTION

To stabilize interspecific mutualism, a host must maintain infection with cooperative symbiont lineages and prevent infection with cheater symbiont lineages (Sachs et al. 2004). A host can maintain control of its infection in three ways: symbiont recognition, assessment of cooperativeness or vertical transmission (see Chapter One). The mode of symbiont acquisition, *i.e.*, horizontal vs. vertical transmission, helps determines how the host maintains control over infection with new, potentially virulent, symbiont lineages.

The life history of a host helps determines its mode of symbiont transmission. Soritid foraminifera that host *Symbiodinium* dinoflagellates have a paratrimorphic life cycle (Figure 9), which alternates between sexual and asexual reproduction, with an indefinite number of asexual reproduction (schizogony) events (Leutenegger 1977, Kloos and Macgillavry 1978, Zohary et al. 1980, Fujita et al. 2000). Though the exact details of the reproductive cycle vary between different species, they all transmit their symbionts vertically through asexual rounds of reproduction when the daughter cells are formed inside the test of the mother cell. This asexual mode is the dominant form of reproduction in these foraminifera. Through the less frequent rounds of sexual reproduction they must acquire their symbionts horizontally since the foraminiferal gametes are smaller than the symbionts themselves. Thus foraminifera can alternate between vertical and horizontal transmission of their symbionts as they alternate between reproductive strategies. Such a combined strategy, with dominant asexual reproduction and vertical transmission, may account for the "limited specificity" seen between these foraminifera and the genotype of their *Symbiodinium* symbionts (Garcia-Cuetos et al. 2005). This hypothesis is especially compelling since the genus with the highest degree of specific identity between different genotypes of host and symbiont, *Sorites*, rarely undergoes sexual reproduction, if at all (Kloos 1984, Garcia-Cuetos et al. 2005).

Soritid foraminifera are relatively well-studied hosts in terms of *Symbiodinium* diversity and distribution. Their basic biology is less well understood partially because, despite efforts, they have never been cultured through multiple rounds of reproduction (Lee and Anderson 1991, Lee et al. 1991). Symbionts must be newly acquired by foraminifera when they go through rounds of sexual reproduction and they must inherit symbionts from the mother cell during rounds of asexual reproduction, but whether they can acquire new symbionts from their environment as adults is not known. This study aims to clarify this question.

Soritid foraminifera have an active relationship with their external environment. They eat detritus, including unicellular algae, and get some of their nutrients from algal food (Lee et al. 1991). *Symbiodinium* is found free living in benthic reef environments (Hirose et al. 2008, Littman et al. 2008). Soritid foraminifera collect *Symbiodinium* dinoflagellates near their margin when cultured together (see video, http://www.springerlink.com/content/g78l6061337321q5/MediaObjects/338_2009_511_MOESM1_ESM.mp4 previously published in (Fay et al. 2009)). To investigate symbiont acquisition in adult soritid foraminifera, this study directly examined the movement of symbionts into newly formed chambers using time-lapse photomicrography. The presence of exogenous algae in foraminifera exposed to cultured algae was further tested using molecular markers.

METHODS

Incubation of foraminifera with Symbiodinium *cultures*

Soritid foraminifera collected April 2007 from Qalawi, Red Sea, Egypt were maintained in dishes of sterile filtered artificial seawater (FASW) changed monthly. After more than a year, megalospheric daughter cells, the product of asexual reproduction, appeared in the dish. These new juveniles were picked out and brushed five times successively in FASW to remove external debris. Forty-two live individuals were isolated, one per well of a six-well dish (Falcon 353046). Each dish with six foraminifera were exposed to *Symbiodinium* dinoflagellates from a different culture: P.div 44a, Cx, and Mf 08.3Td, obtained from the culture collection of Mary Alice Coffroth, and Mv, A003, and 13, obtained from the collection of Scott Santos. Dinoflagellate cultures were maintained in f/2 medium made from filtered Pacific Ocean seawater collected from Bodega Bay, CA, prepared with ProCulture algal culture formula additive from Kent Marine Inc., Marietta, GA. A seventh six-well dish, without added *Symbiodinium* cultures, was used as a negative control.

Measurement of test growth

Each foram was photographed at three-day intervals on a Nikon Diaphot inverted phase-contrast microscope using a Nikon D80 camera. To measure foraminiferal test size, image sets from twenty-four day intervals were examined using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA). The outline of each foraminiferan was traced using the lasso tool and the area pixel count was recorded. The pixel count was converted to 2D area using a stage micrometer standard; 425 pixels per 100µm, 180,625 pixels per 100µm^2.

Symbiodinium identification using molecular markers

Symbiodinium type was identified from cultures and within individual foraminifera using direct sequencing. DNA was extracted using the Guanidinium protocol (Fay et al. 2009). Aliquots from *Symbiodinium* cultures, 2mL each, were centrifuged at 2.5krpm for 5 min., resuspended and extracted in 250µL of Guanidinium extraction buffer. Two forams from each treatment were selected and carefully brushed in three washes of FASW before being crushed and extracted in 100µL Guanidinium extraction buffer. Extracts were used as template for PCR amplication of the dinoflagellate rRNA 18S SSU gene using primers S_DINO and L_O (Pochon et al. 2001). Dinoflagellate nuclear DNA from the rRNA locus (ITS1 - 5.8S - ITS2 - partial 28S) was PCR amplified using an PTC-200 thermocycler (MJ Research, Waltham, MA) with the program (94ºC 3:00 min., 64ºC 1:30 min., 35 x [72ºC 2:00 min., 94ºC 0:45 min., 64ºC 0:45 min.], 72ºC 5:00 min.) using primers S_DINO and L_O (Pochon et al. 2001) and the enzyme AmpliTaq Gold (Applied Biosystems Inc., Foster City, CA) with manufacturer's recommended reagent concentrations.

Resulting sequences were then aligned aligned with MUSCLE (Edgar 2004) and checked by eye. A phylogeny was inferred from this alignment using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), substitution model GTR + I + gamma as determined by ModelTest (Posada and Crandall 1998), for 2x10^6 generations, discarding 2x10^5 generations as burn-in.

Time-lapse photomicrography

Time-lapse videos of soritid foraminifera exposed to *Symbiodinium* dinoflagellates were made using a Nikon Diaphot inverted phase-contrast microscope. A Nikon D80 camera and Mac iBook G4 were used to capture images at 3s intervals using gphoto2 (open source, available from

http://www.gphoto.org/) software, and images were compiled into video using iStopMotion software (Boinx Software Ltd., Germany).

RESULTS

Growth

There was no significant difference in the growth of the foraminifera between the control treatment and those exposed to different dinoflagellate cultures ($F=1.345$, $p=0.28$; Figure 10). There was an average of 120% increase in 2D surface area over the 100-day trial.

Formation of new chambers

Soritid foraminifera move their symbionts into newly formed chambers from inner chambers (Kloos 1984). We have documented this with time-lapse videography. The video can be found as part of the supplementary materials. We observed the formation of outer protective envelopes in all of the foraminifera that were growing new chambers. The OPEs can also be seen in the time-lapse video.

Cultured exogenous Symbiodinium *cells are not acquired as symbionts*

Though all of the foraminifera increased in size during the experiment, none of the foraminifera examined acquired exogenous dinoflagellates (Figure 11). All but one had the same symbiont type, different from any of the *Symbiodinium* cultures.

DISCUSSION

Foraminifera likely have a mechanism for limiting mixing of symbionts from their environment, given that the capacity to maintain fidelity with a symbiont type helps stabilize mutualism (see Chapter One and Frank 1996). Partner Fidelity Feedback is a model whereby successful partnerships propagate themselves more successfully because of the coupled fitness of the partners; fidelity aligns their fitness interests, stabilizing the mutualism (Bull and Rice 1991, Sachs et al. 2004). Maintaining fidelity with a particular symbiont type depends upon preventing infection with new virulent cheater strains from the environment. A potential disadvantage to such fidelity is a restricted ability to recombine with new symbiont types under changing environmental conditions.

Our results show that no individuals became infected with cultured *Symbiodinium* that they had been exposed to. When forming new chambers they exclude exogenous material, including algae. Symbionts were observed moving directly from older inner chambers into newly formed outer chambers. Though these findings suggest that adult foraminifera do not acquire new symbionts from their environment, we cannot exclude the possibility that they do adopt exogenous algae as symbionts under conditions different than those used in this experiment.

New chamber formation in foraminifera involves the creation of a temporary space that excludes external material, alternately called a protective cyst (Meyers 1935, Loeblich and Tappan 1964), outer protective envelope (OPE) (Bé et al. 1979), or growing cyst (Kloos 1984). Foraminifera form an OPE around their test when forming a new chamber. The OPE does not form the surface upon which new chambers form, but rather a chamber within which new chambers grow (Bé et al. 1979). While the primary function of the OPE is unknown, it likely

maintains the proper chemical environment for test wall deposition. Since the OPE excludes external material, it may have the additional benefit of maintaining fidelity with the existing symbiont population during growth. While this does not demonstrate that the OPE's main function is exclusion of exogenous algae to prevent infection, it does suggest a potential simple mechanism to maintain the current complement of symbionts as the foraminifer grows.

Since these foraminifera have the ability to go through multiple rounds of asexual reproduction, and perhaps only acquire new symbiont types during infrequent rounds of sexual reproduction, such a system potentially gives them the ability to tune their symbiont acquisition strategy by adopting different reproductive strategies depending on environmental factors or symbiont cooperativeness. An organism's reproductive strategy can vary with varying environmental factors (Bell 1982). Some foraminifera use asexual reproduction to increase their population size but switch to sexual reproduction under averse environmental conditions (Erskian and Lipps 1987). Reproductive strategy can evolve in response to selective pressures imposed by symbionts, reinforcing a prediction of the Red Queen hypothesis, whereby positive symbiotic interactions reinforce asexual reproduction and negative interactions reinforce sexual reproduction (Bell 1982, Lively 2009).

With foraminifera, recombination of host-symbiont pairing is coupled with a recombination of the foraminiferal genetic material through sex. To recombine alleles and symbiont type provides additional opportunities and risks. The potential advantages of holobiont recombination are analogous to the potential advantages conferred from recombination of alleles. Symbiont recombination promotes variation by formation of novel holobiont types, like the formation of novel allele combinations. Also symbionts, as a small captive population, can accumulate deleterious mutations (O'Fallon and Hansen 2009). Disadvantages are likewise analogous; loss of fidelity is analogous to the breakup of advantageous allele combinations.

With the ability to undergo repeated rounds of asexual reproduction between infrequent rounds of sexual reproduction, foraminifera have decoupled reproduction from necessarily recombining both their symbionts and their genetic material. They can maintain fidelity with their particular symbiont when the mutualism is successful. The "twofold cost of sex" does not apply if the organism is not restricted to sexual reproduction (Maynard Smith 1978). Nor are the costs of Muller's Ratchet borne if the organism is not limited to asexual reproduction (Felsenstein 1974).

Similar predictions may bear on corals that show horizontal transmission of symbionts over rounds of sexual reproduction and can also reproduce vegetatively, transmitting their symbionts vertically. Coral colonies show indeterminate growth and many can reproduce vegetatively (Highsmith 1982). The interplay between symbiont transmission strategy and host reproductive mode is fertile ground for future research on the evolutionary ecology of *Symbiodinium*-host systems. This emphasizes the need to more carefully study the effect of life history evolution on symbiont acquisition strategy.

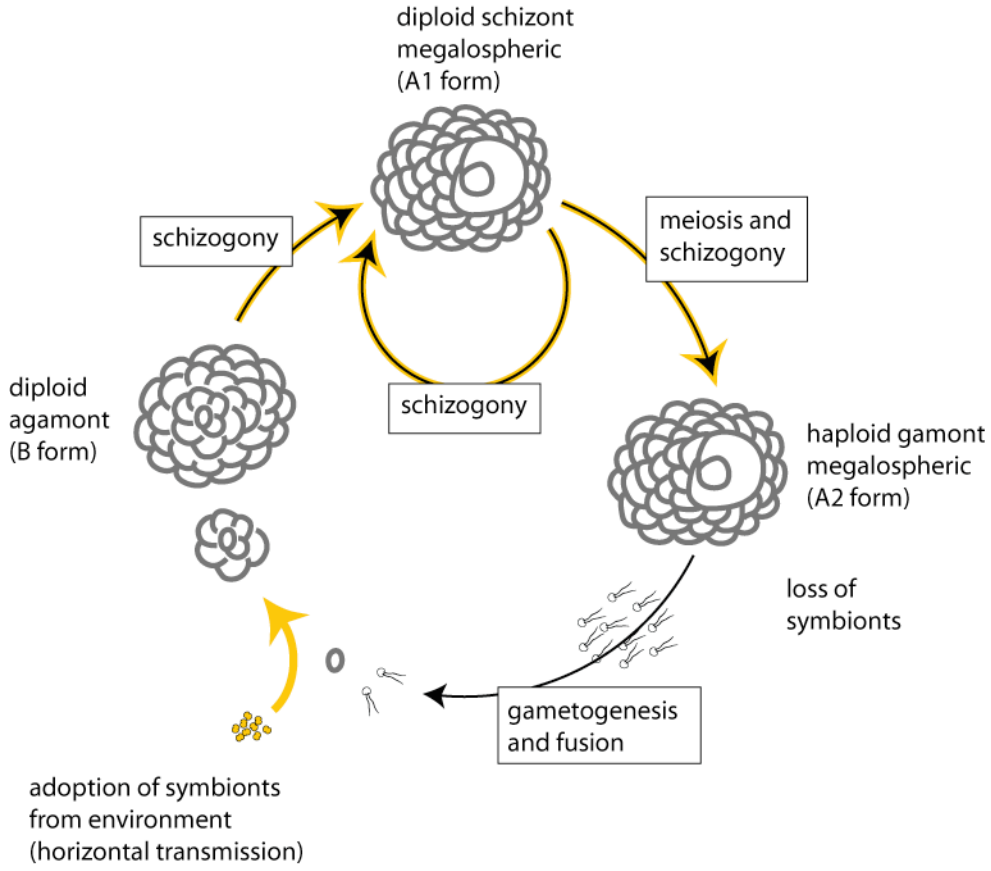

Figure 9. Paratrimorphic life cycle in symbiont-bearing larger foraminifera which shows symbiont transmission strategy. Symbionts are acquired from the environment, i.e., horizontally, after gamete fusion. Through rounds of asexual reproduction (schizogony), symbionts are transmitted from mother to daughter cell, i.e., vertically. Adapted from (Hottinger 1983) and (Leutenegger 1977).

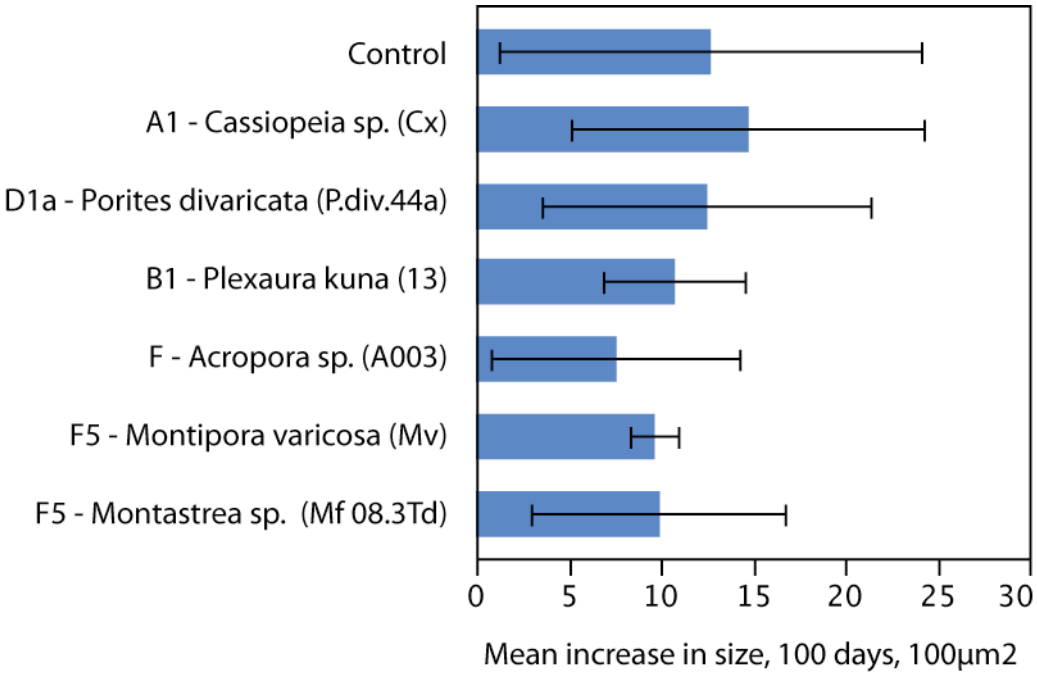

Figure 10.
Growth of foraminifera exposed to different *Symbiodinium* types, over 100 days, measured in 100µm^2. Error bars show a 95% confidence interval of the mean.

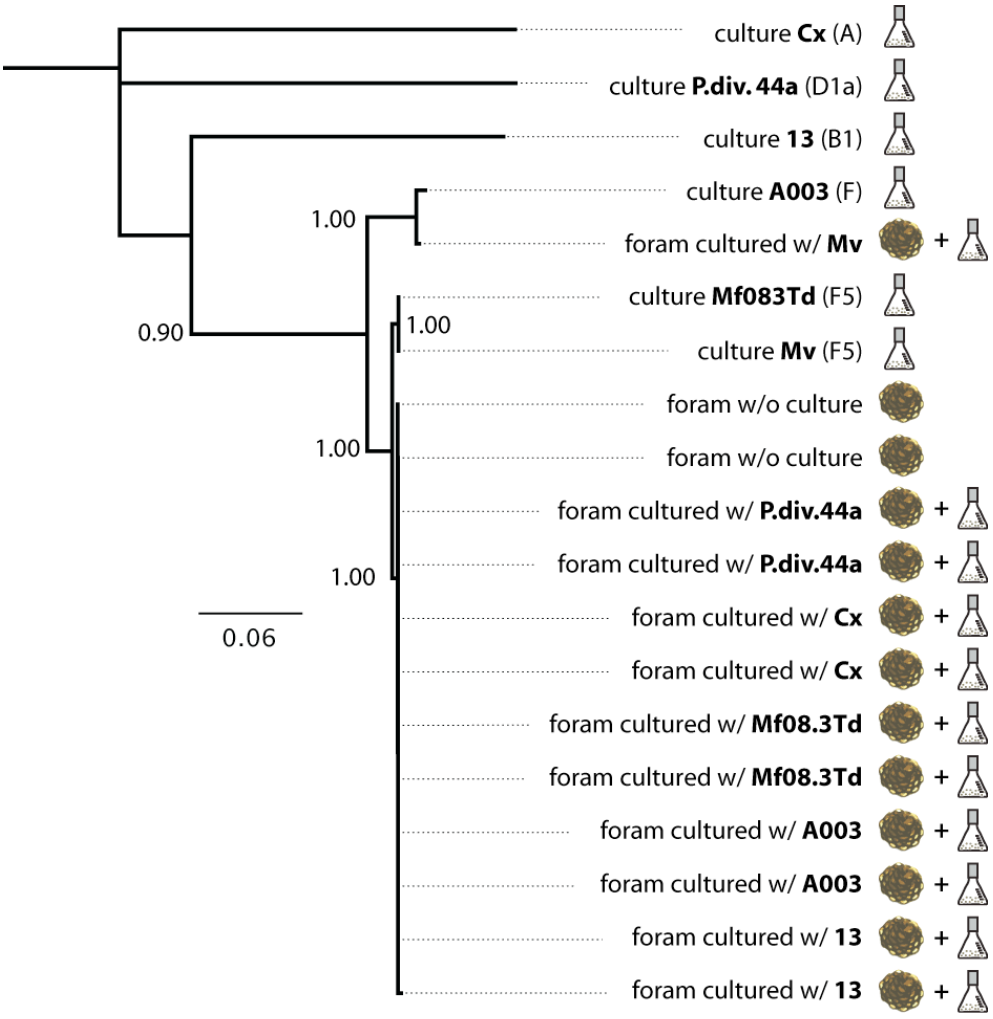

Figure 11.
This phylogram shows the identity of *Symbiodinium* ITS1-5.8S-ITS2-partialLSU DNA sequences amplified from host foraminifera and cultures. All foraminifera studied contained the same genotype of *Symbiodinium* except for one. None of the foraminifera showed evidence of containing algae from the culture they were exposed to. Clade designations in parentheses are derived from sequence identity with sequences from (Pochon et al. 2007) and (Thornhill et al. 2007). Scale bar shows number of substitutions per site.

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