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THE ROLE OF INTRASPECIFIC DIVERSITY IN CORAL-ALGAL SYMBIOSIS ECOLOGY AND EVOLUTION

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ABSTRACT

Coral-algal mutualisms, the foundation of reef ecosystems, can break down during periods of thermal stress. The extent to which such partnerships may acclimate or evolve to survive a changing climate is poorly understood. Though evolutionary responses are driven by the natural selection of functional variation among individuals within species, such variation has been difficult to quantify in coral systems. Here, I use high-resolution molecular techniques to differentiate individuals, populations, and species within coral symbioses and subsequently quantify the ecological and evolutionary relevance of intraspecific variation. In the first chapter, I review the technological advances that have made such work possible, and describe preliminary data from several researchers indicating that intraspecific variation may be extensive among coral hosts and symbionts. In the second chapter, I test the value of manipulating coral-algal partnerships during host larval development for restoration purposes, finding little influence of symbiont identity on growth rates during early ontogeny. In the third chapter, I formally describe several new species of *Symbiodinium*, the morphologically cryptic dinoflagellate endosymbionts that associate with cnidarians, and draw attention to the ecological diversity that can be found even among closely-related groups. In the fourth chapter, I characterize molecular variation among individuals of the coral *Acropora palmata* that affects the photochemistry of a clonal symbiont responding to thermal stress, emphasizing that fine scale partner interactions can yield functional variation among coral holobionts with potential evolutionary consequences. In the fifth chapter, I compare gene content and steady-state expression among several closely related *Symbiodinium* within the Clade B lineage, identifying phylogenetic and ecological transcriptional signatures among species. This work represents a few first steps into the complex and exciting territory of fine scale variation among marine mutualisms.

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Chapter 1

THE EXTENDED PHENOTYPES OF MARINE SYMBIOSES: ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES OF INTRASPECIFIC GENETIC DIVERSITY IN CORAL-ALGAL ASSOCIATIONS.

Abstract

Reef-building corals owe much of their success to a symbiosis with dinoflagellate microalgae in the genus *Symbiodinium*. In this association, the performance of each organism is tied to that of its partner, and together the partners form a holobiont that can be subject to selection. Climate change affects coral reefs, which are declining globally as a result. Yet the extent to which coral holobionts will be able to acclimate or evolve to handle climate change and other stressors remains unclear. Selection acts on individuals and evidence from terrestrial systems demonstrates that intraspecific genetic diversity plays a significant role in symbiosis ecology and evolution. However, we have a limited understanding of the effects of such diversity in corals. As molecular methods have advanced, so too has our recognition of the taxonomic and functional diversity of holobiont partners. Resolving the major components of the holobiont to the level of the individual will help us assess the importance of intraspecific diversity and partner interactions in coral-algal symbioses. Here, we hypothesize that unique combinations of coral and algal individuals yield functional diversity that affects not only the ecology and evolution of the coral holobiont, but associated communities as well. Our synthesis is derived from reviewing existing evidence and presenting novel data. By incorporating the effects of holobiont extended phenotypes into predictive models, we may refine our understanding of the evolutionary trajectory of corals and reef communities responding to climate change.

Introduction

Fundamentally, evolution by way of natural selection acts on functional variation among individuals within a species (Fisher 1930). When the success of two (or more) organisms are linked, such as among mutualistic symbiotic partners, variation within one species interacts with the variation in the other, as well as with the environment (Thompson 2005; Warren and Bradford 2014), potentially driving direct and indirect evolutionary interactions (Wootton 1994; Rowntree et al. 2014). Thus, the adaptive capacity of symbiotic organisms may be underestimated when intraspecific variation is not accounted for. The increasing scale of reef degradation has called into question the ability of coral-algal symbioses to acclimate or evolve to deal with a changing world (Lasker and Coffroth 1999; Glynn et al. 2001; Hoegh-Guldberg et al. 2002; Reshef et al. 2006; Brown and Cossins 2011; Barshis et al. 2013). Acclimation occurs over the course of an organism's lifetime, while evolution takes place over generations; the time frame for both processes can overlap when evolution is particularly rapid (Hairston et al. 2005). Despite the fact that host and symbiont genomes are often decoupled each generation, coevolution clearly occurs (Thornhill et al. 2014). Current forecasts of reef perseverance do not explicitly incorporate the effects of intraspecific diversity driving coevolution among coral-algal partners because such effects have rarely been assessed.

Classically, biodiversity has been measured at the species level, and such diversity has generally had positive effects on higher-order community diversity, function, and resilience (Balvanera et al. 2006). Modern molecular techniques are revolutionizing species delineation in coral holobionts. Using genetic and complementary phenetic evidence, many traditional host species designations and higher-order relationships are being reevaluated (Fukami et al. 2004; Fukami et al. 2008; Huang et al. 2011; Pinzon and LaJeunesse 2011; Budd et al. 2012, Keshavmurthy et al. 2013). Microalgae (including *Symbiodinium*) are likewise receiving

renewed taxonomic attention emphasizing molecular data (LaJeunesse et al. 2012; Jeong et al. 2014; LaJeunesse et al. 2014; Leliaert et al. In press).

More recently, intraspecific diversity has been revealed to be just as important (in some cases, more important) than interspecific diversity in explaining variation in associated community traits (Hughes et al. 2008). For example, the diversity, richness, and abundance of arthropods on trees are better explained by the number of *Populus* genotypes than tree species diversity (Shuster et al. 2006; Whitham et al. 2006). However, similar investigation is lacking for corals and their microalgae. Few studies have addressed whether genotype diversity of a coral species affects the diversity of its symbiont community or other associated invertebrates and vertebrates. This is partly because the resolution of species (let alone individuals) in the coral holobiont has been contentious (Stat et al. 2012). Within a given coral species, morphologically distinct colonies can be genetically identical owing to phenotypic plasticity among asexual fragments (Highsmith 1982; Todd 2008), while genetically disparate colonies may share striking resemblance (*e.g.* Pinzon and LaJeunesse 2011). All *Symbiodinium* species and cell lines look superficially similar even under high magnification (LaJeunesse 2001). Without high-resolution genetic markers, intraspecific effects on the ecology and evolution of coral-algal symbioses have been difficult to quantify accurately.

Population genetic microsatellite markers are increasingly used to study both scleractinian hard corals (Lopez et al. 1999; Maier et al. 2001; Magalon et al. 2004; Severance et al. 2004; Baums et al. 2005a; Underwood et al. 2006; Mangubhai et al. 2007; van Oppen et al. 2007; Isomura and Hidaka 2008; Starger et al. 2008; Andras and Rypien 2009; Baums et al. 2009; Wang et al. 2009; Concepcion et al. 2010; Polato et al. 2010; Banguera-Hinestroza et al. 2013; Chen et al. 2013; Davies et al. 2013) and *Symbiodinium* (Santos and Coffroth 2003; Magalon et al. 2004; Pettay and LaJeunesse 2007; Bay et al. 2009; Howells et al. 2009; Kirk et al. 2009; Pettay and LaJeunesse 2009; Andras et al. 2011; Pinzon et al. 2011; Wham et al. 2011; Wham et

al. 2013). Armed with such markers, it is now possible to sample a single coral colony and determine not only its host and symbiont species compositions, but also to resolve unique multi-locus genotypes (i.e. individuals) within each species. However, only rarely have both host and symbiont genotype composition been analyzed in concert (Andras et al. 2011; Pettay et al. 2011; Andras et al. 2013; Pettay and LaJeunesse 2013; Thornhill et al. 2013; Baums et al. 2014, Prada et al. 2014b). So far this has only been done in a general population survey context, with most evidence suggesting that the genetic structuring of the host and the symbiont are not the same (*e.g.* Baums et al. 2014). No studies have manipulated host-symbiont pairings to examine genotype-level interspecific interactions while unambiguously resolving both partners. Such work is routine in the study of terrestrial mutualisms, but represents a new frontier in the marine realm.

Researchers now stand poised to answer previously intractable questions about the nature of coral-algal symbioses. In this review, we argue that intraspecific diversity is an important component shaping interspecific interactions within a holobiont, and that such interactions may influence the evolutionary trajectory of reef ecosystems faced with a changing climate. We have four major goals: (i) to briefly review the role of intraspecific diversity in other systems, (ii) to describe what we currently know about intraspecific diversity in coral hosts and algal symbionts, (iii) to present preliminary data illustrating the potential extent of functional intraspecific diversity in coral-algal systems, and (iv) to identify research questions and methodologies that will shed further light on this understudied component of marine microbial symbiosis ecology. We posit two central, testable hypotheses: (i) genotypic interactions between coral hosts and algal symbionts influence functional diversity and therefore evolutionary capacity in coral holobionts, and (ii) intraspecific diversity among corals affects reef community function. Dawkins (1982) introduced the concept of ‘extended phenotypes’ to incorporate the indirect effects of genes on the environment independent of the individual bodies in which they reside. In this framework,

unique combinations of coral and *Symbiodinium* individuals might be thought of as holobionts with unique extended phenotypes that may shape reef community dynamics.

Significance of intraspecific functional diversity in other systems

The importance of genotypic diversity (i.e. the number of distinct multi-locus genotypes) among symbiotic partners in affecting the performance of the symbiosis is apparent in terrestrial systems, where genotype level resolution has been used in manipulative experiments for years. An illustrative example is the association between plants and arbuscular mycorrhizal fungi (AMFs). These fungi penetrate vascular plant roots, transmitting nutrients from the surrounding soil to the host. AMFs are obligate symbionts—they cannot survive without a host plant. Numerous studies have recorded symbiont genotype effects on host performance (and vice versa; reviewed by Johnson et al. 2012). For instance, Koch et al. (2006) inoculated clonal carrot roots with genetically distinct AMFs belonging to the single species *Glomus intraradices*; host root growth varied with symbiont genotype. Munkvold et al. (2004) monitored host and symbiont growth among holobionts composed of distinct genotype pairings; growth varied depending on intraspecific partner combinations. Scheublin et al. (2007) found that intraspecific symbiont identity affected the outcome of competitive interactions between the host and other plant species. Similar effects are found in other systems. Among genetically identical host clones of pea aphids, pathogen resistance was conferred to different degrees by distinct strains of a facultative bacterial symbiont species (Lukasik et al. 2013b). Conversely, host pathogen resistance and fecundity varied among host genotypes associating with a clonal symbiont (Lukasik et al. 2013a). These examples highlight that intraspecific diversity among holobiont partners can be high and drive complex interactive effects that mediate holobiont fitness in multiple ways. The same is likely true in coral-algal systems.

The effects of host-symbiont pairings are reflected not only in growth, competitive interactions, pathogen resistance, and fitness, but also in gene expression patterns. Heath et al.

(2012) explored the molecular underpinnings of partner interactions by partitioning genetic variation in plant and AMF transcriptomes into additive and interactive effects. The authors found that interactions between plant and AMF genotypes drove symbiont gene expression changes and transitioned host transcription from a nuclear dominated profile (i.e. basic housekeeping) to a plasmid dominated profile (i.e. nitrogen fixation). These polymorphisms altered access to nitrogen fixation, the chief benefit of symbiosis to the plant and a determinant of host reproductive fitness. When the fitness of one species is influenced by the genotype of its symbiotic partner, coevolution is possible (Thompson 2005; Wade 2007). Fitness and expression differences among distinct holobionts exemplify natural variation available to coevolutionary selection (Heath et al. 2012). Evolutionary innovation can arise from transcriptional variation in response to short-and long-term stress (Lopez-Maury et al. 2008), and such variation has been described in marine organisms responding to selective pressures associated with climate change, including temperature (e.g. DeSalvo et al. 2010; Barshis et al. 2013; Polato et al. 2013) and acidification (Pespeni et al. 2013). In the coral-algal system, genetically determined expression differences among holobionts responding to stress might be subject to natural selection and lead to adaptation.

Increasingly, diversity below the species level is recognized to be an important force shaping community dynamics, particularly among ecosystem engineers (Whitham et al. 2006; Bolnick et al. 2011). In pea aphid studies, symbiont genotype affected the extent of pathogen sporulation in dead hosts, which likely altered community dynamics by limiting or expanding the exposure of other aphids to the fungus (Lukasik et al. 2013a; Lukasik et al. 2013b). In the Pacific Northwest, locally-derived leaf litter from red alder trees (*Alnus rubra*) decomposed more rapidly than litter derived from trees at other riparian zones, indicating intraspecific variants might drive community-level changes to ecosystem flux (Jackrel and Wootton 2013). In poplar trees (*Populus* sp.), plant genotype was shown to explain three times as much variation in

associated arthropod communities as species-level differences (Shuster et al. 2006). Similarly, soil microbial community composition was driven largely by intraspecific genotype (Schweitzer et al. 2008). For the marine eelgrass (*Zostera marina*), genotypically diverse beds were more resistant to disturbance by grazing geese, as were their associated invertebrate fauna (Hughes and Stachowicz 2004). Intraspecific diversity improved not only seagrass biomass and density but also epifaunal abundance over the course of a warm water temperature anomaly (Reusch et al. 2005). Thus, genotypic diversity in seagrasses has both first-order effects on species resistance and/or resilience as well as second-order effects on ecosystem function. Corals are also marine ecosystem engineers; similar second-order effects may have a profound influence on reef function.

In summary, results from terrestrial studies suggest by extension that intraspecific variation among coral holobionts has the potential to scale up to influence the diversity, resilience, and function of entire reef ecosystem, including associated microbes, alga, invertebrates, and vertebrates. The critical first step in all future studies of intraspecific diversity will be establishing the individual identities of each coral colony and *Symbiodinium* strain under investigation.

Defining coral-algal diversity

The coral holobiont is composed of more than just the host and *Symbiodinium*. Within host tissues, additional symbionts may include apicomplexa (Toller et al. 2002; Kirk et al. 2013a; Kirk et al. 2013b), nitrogen-fixing cyanobacteria (Lesser et al. 2004), other bacteria (Rohwer et al. 2002), viruses (Wilson et al. 2005), archaea (Kellogg 2004; Wegley et al. 2004), and cell-associated microbial aggregates (Work and Aeby 2014), not to forget organisms found in the host skeletal structure such as endolithic algae (Odum and Odum 1955; Shashar and Stambler 1992) and fungi (Le Campion-Alsumard et al. 1995; Bentis et al. 2000). The partner for which the most

data are available and for which the role in the symbiosis is most clearly understood is *Symbiodinium*; we therefore use the term 'symbiont' to refer only to *Symbiodinium* in this review.

When it was first described, taxonomic diversity among *Symbiodinium* was assumed to be low (Freudenthal 1962; Taylor 1984). Over time, it was recognized that the genus included many different species based on various morphological, physiological, and early genetic data (Schoenberg and Trench 1980a; b; c). Molecular diversity in the group achieved more recognition when *Symbiodinium* were divided into low-resolution clades based on rDNA (Rowan and Powers 1992), and some corals were found to associate with members of different symbiont clades simultaneously (Rowan et al. 1997). At the time, it was acknowledged that the genetic distances between clades were similar to those observed among different genera and even families of dinoflagellates—an observation borne out by more recent molecular analyses (Stern et al. 2010; Ladner et al. 2012). Higher resolution was achieved by dividing *Symbiodinium* into subcladal 'types' using hypervariable regions of nuclear and chloroplast rDNA markers (LaJeunesse 2001; 2002; Santos et al. 2003a). Now, a suite of hierarchical molecular markers and population genetic data are being used to define precise species boundaries and refine *Symbiodinium* taxonomy (LaJeunesse et al. 2012; Jeong et al. 2014; LaJeunesse et al. 2014). Though it has yet to be physically observed, overwhelming molecular evidence indicates that *Symbiodinium* engage in sex at some frequency in the wild, either within the coral habitat or in the external environment (Baillie et al. 2000; LaJeunesse 2001; Santos et al. 2004; Sampayo et al. 2009; Pettay et al. 2011; Chi et al. 2014; Baums et al. 2014; Thorhnill et al. 2014). Sympatric symbionts found in distinct colonies of the same host species in the same environments exhibit diagnostic microsatellite allele frequencies, revealing genetic recombination within but not between groups (LaJeunesse et al. 2014). This satisfies the biological species concept, demonstrating that molecular data can be used to consistently delimit species boundaries in *Symbiodinium*—a necessity for investigating intraspecific diversity.

Similar molecular data have been used to resolve coral host species, which feature the added complication of introgressive hybridization among closely related taxa (Ladner and Palumb, 2012). Often, current taxonomic designations based on morphological characteristics are at odds with genetic evidence. For example, the entity designated *Stylophora pistillata* was recently determined to be composed of at least four species based on cytochrome oxidase I sequencing (Keshavmurthy et al. 2013), while multiple markers suggest that three of the Caribbean poritid morphospecies (*Porites divaricata, P. furcata, and P. porites*) should be collapsed into one entity (Prada et al. 2014a). Even within a single genus, molecular data indicate some lineages should be lumped while others should be split (Pinzon et al. 2013). Unlike *Symbiodinium*, it will be easier to combine data from experimental crosses, morphological assessments, and genetic sequencing to resolve coral species (Budd et al. 2010; 2012). Proper species identification is critical when designing experiments to understand coral evolution. Failure to recognize that colonies belong to distinct species when collecting population genetic data can produce misleading signatures of structure and hybridization (Combosch et al. 2008; Combosch and Vollmer 2011). Failure to recognize cryptic species can also mask important differences in ecological interactions and population dynamics (Boulay et al. 2014). Once coral species boundaries are established, it then becomes possible to assess functional diversity among individuals within species.

Biologically, the notion of an individual is difficult to define in corals. On one level, there is the smallest physical unit representing the organism's genome (the polyp). On another, there are units of contiguous tissue that connect multiple clonal polyps (the colony). In macro-scale contexts, these colonies are the ecologically significant units on a reef. Sometimes, physically separated colonies are clones (i.e. share the same genome), whereas others are genetically distinct. Throughout this review, when attributed to a given organism, we use the term 'genotype' to refer to the concept of genome identity within a species (that is, genetically

distinct individuals). All coral colonies that share an identical genome together comprise a 'genet,' with each member colony referred to as a 'ramet.' Coral genotypic diversity thus refers to the number of distinct genets on a reef. *Symbiodinium* are also capable of both clonal and sexual propagation, but their unicellular nature requires that we use different terminology than corals. A single *Symbiodinium* cell contains one genome and functions independently of all others cells. When residing within host cells, *Symbiodinium* typically reproduce asexually and generate homogenous populations of cells derived from a single ancestor. We use the term 'strain' to refer to this physical collection of clonal symbiont cells hosted within a coral colony. In contrast, sexual reproduction leads to new strains. Multiple *Symbiodinium* strains may be present within the habitat provided by a single coral colony, and multiple strains from either a single or many species may be present.

It has become clear that in many coral-algal symbioses, individual host colonies are dominated by a single symbiont species (that is, >99% of the symbiont cells in host tissue belong to a single species). In the Caribbean and Eastern Pacific, where most high-resolution assessments have been performed, individual colonies are dominated not only by one species, but by one strain within that species. An example would be the *Acropora palmata-Symbiodinium 'fitti'* association, where pairings of single host and symbiont genotypes produce holobionts that may each exhibit unique extended phenotypes (Figure 1-1; Baums et al. 2014, Parkinson et al. submitted). In fact, in studies where microsatellite markers have been used to characterize both partners, the host:symbiont genotype ratio is one:one in >70% of colonies (Goulet and Coffroth 2003a; b; Santos et al. 2003b; Kirk et al. 2005; Pettay and LaJeunesse 2007; 2009; 2013; Thornhill et al. 2009; Andras et al. 2011; Pettay et al. 2011; Pinzon et al. 2011; Thornhill et al. 2013a; Baums et al. 2014; Prada et al. 2014b). This outcome falls in line with the predictions of basic population theory, as closely-related organisms generally compete for similar resources, leading to competitive exclusion among similar species (Gause 1934; Hardin 1960). However,

there are certainly other associations where strains from multiple *Symbiodinium* species codominate in one host colony (*e.g.* Rowan et al. 1997; van Oppen et al. 2001), such that the holobiont can be viewed as a more complex community. The presence of low-abundance or 'background' symbionts representing <0.1% of the symbiont population may also shape some holobiont dynamics (see Box 1, Figure 1-5). This range of partnership complexity provides exciting potential for deconstructing the processes shaping the evolution of mutualisms across reef habitats.

Intraspecific functional diversity in corals: classic studies

Traditionally, common garden and reciprocal transplanting experiments have been used to test for functional differences of genotypes in plants (*e.g.* Hufford and Mazer 2003) and corals (Potts 1984; Edmunds 1994; Bruno and Edmunds 1998; D'Croz and Mate 2004; Smith et al. 2007). Typically, colonies from environmentally distinct sites (*e.g.* shallow vs. deep or inshore vs. offshore) are reciprocally transplanted to test how they perform relative to native corals. In parallel, colonies from both sites may be transplanted to a third location to test how they perform relative to each other in a common environment. As one might expect, studies on reef-building corals have found species that are characterized by generalist genotypes (Smith et al. 2007), species that show local adaptation (D'Croz and Mate 2004; Kenkel et al. 2013), and species that harbor both generalist and specialist genotypes (Potts 1984). Such studies address the performance of the specific combination of coral and *Symbiodinium* genotypes in the experimental units. However, the relative contribution of each partner to holobiont performance has been difficult to measure.

Prior to the mid-1990s, confirmation of the distinctness or clonality of coral colonies was difficult because of the lack of genetic data and the fact that coral clones are generally impossible to distinguish visually (even histo-incompatibility proved unreliable; Heyward and Stoddart 1985). For example, in a classic common garden reciprocal transplant experiment, Potts (1984)

mounted clonal fragments of *Acropora* sp. sourced from each of five environments from a single reef onto common wire grids. Five replicate grids were distributed among the five locations. Source location (a proxy for host genet) drove non-random differences in growth rate and survivorship among individual colonies in shared environments. After eight years of observation, colonies with different origins did not converge on a common morphology to match the native colonies at their new locations, indicating low phenotypic plasticity in this coral (at least morphologically) and further supporting a genetic component of coral performance. However, the corals sampled for this study may have included two cryptic species that in some environments can only be distinguished with molecular techniques (Potts 1984; Ayre et al. 1991).

In another example, host genotype effects on thermotolerance were examined (Edmunds 1994). To minimize the chance of incorrectly assigning genets, patches of *Orbicella (=Montastraea) annularis* complex that were physically clustered in groups attached by contiguous skeleton but unconnected by coral tissue were considered as clones of the same genotype, because such a formation suggests a common origin. The author showed that bleaching colonies were aggregated rather than randomly distributed on the reef, and that these aggregations corresponded to genotype identities. While the spatial distribution of bleaching colonies might alternatively be explained by the distribution of colonies with distinct *Symbiodinium* associations and therefore thermotolerances, it is unlikely that the experimental colonies harbored different symbiont species. This is because the corals were located at a common depth over a small spatial scale, reducing the number of light microhabitats that lead to unique symbiont associations within the host species complex (Rowan et al. 1997). In a second experiment, subfragments from large colonies of *Porites porites* located more than 15m apart (thus suggesting they belonged to different genets) were experimentally exposed to elevated temperatures for three days and their symbiont densities were measured. Despite having similar

densities at the start of the experiment, the putatively distinct genotypes showed different rates of symbiont loss (or, in one case, gain) after thermal stress exposure (Edmunds 1994).

The coral literature is rife with similar examples where genotype-level effects seemed apparent, but actual genotypes were not resolved explicitly. Given that the spatial range over which host ramets of the same genet have been distributed (*e.g.* from <1m to >70m in *Acropora palmata*; Baums et al. 2006), it may not be appropriate to assume that by swimming a certain distance, the chance of collecting a clonal colony is greatly reduced. For fine-scale ecological questions, it will be necessary to incorporate molecular confirmation of intraspecific diversity. As genomics-empowered tools become less expensive and more accessible, a greater number of studies are taking advantage of fine-scale resolution.

Intraspecific functional diversity in corals: genomics-empowered studies

A series of recent work on the Mediterranean Red Coral (*Corallium rubrum*) demonstrates the utility of a genomics approach to studies of marine evolutionary ecology. This particular coral lacks *Symbiodinium*, reducing the complexity of the system. First, neutral microsatellite markers were used to differentiate populations of *C. rubrum* (Ledoux et al. 2010a; Ledoux et al. 2010b; Costantini et al. 2011). Populations were structured along a depth gradient that reflected distinct, stable thermal environments. This genetic structure corresponded with variability in *C. rubrum* thermal stress limits (Torrents et al. 2008). Since the multi-locus genotypes of each colony were established, individuals from each population could be targeted to assess physiology. Colonies were subfragmented and exposed to various heat stress regimes in common garden aquaria, while the expression of key heat shock proteins were monitored via qPCR (Haguenauer et al. 2013). After assessing variability in gene expression among individuals within different populations, the authors found evidence consistent with local adaptation driven by environmental variability, and argued for a trade-off between reduced responsiveness of metabolic genes and frontloading of thermotolerance genes. Critically, environmental

heterogeneity at shallow sites seemed to select for phenotypically plastic individuals, as reflected by high genetic variability in the shallow population versus low genetic variability in the populations at depth. This work emphasizes the potential importance of cryptic diversity in coral communities and the significance of marginal populations in providing evolutionary novelty (Bell and Gonzalez 2011; Boulay et al. 2014). It also exemplifies a useful strategy for investigating genotype-level effects driving thermal adaptation in symbiotic corals.

The reductive approach of assessing the performance of either the host or symbiont in isolation is more difficult for symbiotic scleractinian corals. One methodology is to experiment with coral larvae, which often lack *Symbiodinium* prior to settlement. Crosses of gametes collected from distinct adult genets produce large batches of offspring with known heritage. Controlled crosses between adjacent *Acropora palmata* individuals showed that full sibling larval batches were unequally affected by thermal stress, which influenced swimming speeds and developmental rates (Baums et al. 2013). The same larval batches exhibited diverse transcriptional responses to thermal stress depending on their heritage (Polato et al. 2013), revealing a higher-than-expected degree of molecular variation in this endangered coral species. Among *A. palmata* adults, some individuals were sexually incompatible (Baums et al. 2013). This was not due to general infertility as most individuals were capable of producing viable larvae when crossed with a compatible genotype. Clearly, intraspecific diversity has fitness consequences in corals. In another experiment, Polato et al. (2010) identified colonies of *Orbicella faveolata* at two distant locations that belonged to one panmictic population according to neutral markers. At each location, locally-derived aposymbiotic larval batches were exposed to a common thermal stress. The larvae exhibited both shared and location-specific transcriptional responses, strongly suggesting the existence of local adaptation despite ongoing gene flow among locations.

Because some *Symbiodinium* can be maintained in culture, their performance can be measured independent of a host. *Symbiodinium goreaui* is a host-generalist symbiont featuring a global distribution (LaJeunesse 2005). In one study, *S. goreaui* was identified in two *Acropora tenuis* reefs located several hundred kilometers apart with average temperatures differences of ~2°C (Howells et al. 2009). After establishing via microsatellite genotyping that these reefs are likely inhabited by distinct populations of *S. goreaui*, symbionts from each population were isolated and cultured (Howells et al. 2012). Cultures were then exposed to elevated temperatures, and photochemical performance was monitored. *S. goreaui* cultured from the warmer reef population showed a smaller decline in photochemical performance at elevated temperature relative to the population from the cooler reef, even after >30 asexual generations in culture. Similar *in vitro* experiments have shown within-species differences in physiology (see below). Thus, when separated, both corals and *Symbiodinium* show intraspecific variation in thermotolerance that appears to have a heritable genetic component—the raw material of natural selection.

Howells et al. (2012) further tested whether intraspecific variation influences holobiont performance when the host and symbiont are combined. They used the distinct *Symbiodinium goreaui* populations to inoculate aposymbiotic larvae of the coral *Acropora millepora* from a third location. After growing to a sufficient size, symbiotic coral juveniles were then exposed to ambient or elevated temperatures, and both symbiont and host physiology were assessed. The symbiont population from the warmer reef showed optimal photochemical performance at elevated temperature, and coral juveniles associating with these symbionts grew rapidly with no signs of bleaching and minimal mortality at high temperature. In contrast, the symbiont population from the cooler reef experienced chronic photodamage at high temperature, and the juveniles inoculated with this population grew slowly and suffered high bleaching and mortality at high temperature. Symbiont and host thermotolerance correlated, showing a strong influence

of symbiont physiology on holobiont performance even below the species level. In a similar vein, Kenkel et al. (2013) used microsatellites and identified performance differences among two populations of the coral *Porites astreoides*. In this case, both hosted the same *Symbiodinium* species as determined by characterization of the symbiont community using high-throughput sequencing of the ITS2 marker. Host structure appeared to be maintained by differences in variable inshore vs. stable offshore thermal regimes. In a common garden, offshore holobionts were less tolerant of experimental heat stress, showing elevated bleaching and reduced growth compared to inshore holobionts. Despite the homogeneity of the symbiont population, *Symbiodinium* in offshore hosts experienced lower photochemical efficiency during heat stress than those associating with inshore hosts. These results support the contention that the host plays an important role in holobiont thermotolerance (Baird et al. 2009b). Moreover, it is not just the host species, but intraspecific populations that may determine performance.

To assess host and symbiont adaptive potential, Csaszar et al. (2010) identified two coral populations of a single species (*Acropora millepora*). Each population associated with a different symbiont species. Heritability estimates for key thermal response traits within each host population showed the symbionts to be relatively more capable of adapting to climate change than the host. However, as the authors recognized, while hosts were genotyped to the level of individuals, symbionts were only resolved to the sub-cladal type (approximately species) level. For the purpose of their study, Csaszar et al. made the assumption that each unique colony hosted a consortium of symbiont genotypes that were equally dispersed across the colony surface. Since then, most high-resolution studies have shown instead that coral colonies tend to be homogenous vessels of the dominant symbiont genotype except for rarer cases like *Orbicella*. The authors suggest that their heritability estimates would most likely biased upwards in such a situation, overestimating the extent of symbiont genetic variance relative to environmental variance. Moreover, if all unique colonies within a given host population shared a single clonal symbiont

strain (an unlikely possibility, but one that cannot be ruled out with the current data), the bias would be even greater, as accurate heritability estimates must come from measuring trait variation between unique individuals. Though the relative comparisons between host and symbiont heritability must be interpreted with caution, this study sets an excellent precedent, as it is one of the few to both measure intraspecific trait variation in coral hosts and confirm the unique identity of the host genets involved.

Preliminary evidence in a genomics age

While the previously mentioned studies mostly examined intraspecific variation at the population level, genotype level effects have only rarely been explored (Baums et al. 2013; Polato et al. 2013). Now that both major components of the coral holobiont can be genotyped to individuals, the doors have opened for high resolution investigations of partner interactions. Here we highlight preliminary evidence that variation at the genotype level may be extensive in both corals and *Symbiodinium*, and that unique partner pairings drive unique responses to stress. This work tests the first of our major hypotheses; that interactions between partners contribute to functional diversity that may subsequently be acted upon by selection. We argue that to truly understand how corals may respond to the myriad selective pressures of a changing climate it will be necessary to assess the contribution of intraspecific diversity to holobiont performance.

Coral growth in restoration nurseries: With global reef degradation reaching alarming levels, marine managers have developed methods to rear coral fragments *in situ* for restoration purposes. A typical 'coral gardening' approach involves several steps: donor colonies are identified and fragmented; the pieces are attached to artificial substrate; the fragments are grown together in a common nursery plot; ultimately, these aquacultured colonies are outplanted to depauperate reefs (Rinkevich 1995; 2005). The goal is to increase coral biomass, diversity, and reproductive capacity, as well as to restore the reef ecosystem and associated fauna (Precht 2006). During the growth phase, the underwater nurseries serve as common gardens where

environmental conditions are roughly equivalent for all colonies, and observed differences can be attributed mostly to genetic effects (Baums 2008). Maternal effects or acclimation to the donor colony's source environment can carry over to affect performance in the nursery, but these factors have been difficult to assess. Restoration nurseries have greatly expanded in the Caribbean, where the endangered *Acropora cervicornis* and *A. palmata* have been targeted for extensive management (Lirman et al. 2010; Johnson et al. 2011; Young et al. 2012). As part of the process, hundreds of colonies in the Florida Reef Tract have been genotyped at multi-locus microsatellite markers (*e.g.* Baums et al. 2010), and many have been monitored for growth and mortality for several years (Griffin et al. submitted; Lirman et al. submitted).

These nurseries provide a unique and under-utilized resource for investigations of genetic influence on coral performance. The few studies that have been conducted with nursery-reared colonies all point to intraspecific genotype effects on growth. For example, Bowden-Kerby (2008) reared genets of acroporid corals from both forereef and backreef environments in a common garden backreef nursery. In contrast to the study of Potts (1984), here source population (a proxy for host/symbiont genotype) was more important than environment in determining growth rate; source was determined to be a significant factor in 75% of tests compared to 44% for environment. Forrester et al. (2013) transplanted *A. palmata* fragments from two source locations to a common garden at a third. In the first year, there were no observed differences between groups, but when the experiment was repeated, growth rate varied by source. In a concurrent experiment, colonies were subdivided into fragments and reciprocally transplanted to 'home' and 'away' environments. Clonal fragments moved 'away' grew more slowly, revealing a slight home-field advantage and a combined influence of both environment and genotype.

Griffin et al. (2012) reared fragments of several *A. cervicornis* genotypes at a line nursery in Puerto Rico and confirmed the hypothesis that linear tissue extension rate varied among individuals. A re-analysis of this data set is presented here (Figure 1-2). In addition to

discriminating growth rates by host genotype, we also separated colonies into depth classes by their relatively shallow (9-10.5m) or deep (10.5-13m) positions in the line nursery, as depth was a significant factor in model analysis (Griffin et al. 2012). We removed measurements from individuals attached to the lines by cable ties, as this method was shown to negatively affect growth (Griffin et al. 2012). To use the terminology of that study, host genotypes are referred to by color names or capital letters. Repeat genotyping of host samples derived from the nursery (rather than the donor colony, as in the original study) revealed that genotypes 'A' and 'B' were actually identical, as were 'Blue' and 'Brown,' so their measurements were pooled. Additional genotyping of the dominant symbiont associated with each colony revealed that three of the four hosts shared a clonal *Symbiodinium 'fitti'* (ITS2 type A3) strain; host 'A/B' associated with a unique *S. 'fitti'* strain. The 'Green' host genotype grew faster than all others, regardless of depth. Identical individuals generally grew faster at greater depth. Interestingly, the 'Blue/Brown' genotype deviated significantly from the 'A/B' and 'Yellow' genotypes when reared in deep but not shallow depths. This indicates an interaction between host genotype and environment. Symbiont genotype did not appear to affect growth, since the most deviant host genotypes shared a clonal symbiont, while two of the hosts that did not differ in growth rate at either depth associated with distinct symbionts. To test this particular hypothesis rigorously, it will be necessary to track the growth rates of ramets of the same host genet each associating with distinct symbiont genotypes; such cases are difficult (though not impossible) to find in nature (Baums et al. 2014).

Symbiont growth rates in culture: It has long been possible to culture *Symbiodinium* independent of the host in artificial media (McLaughlin and Zahl 1959). By now a great many studies have been performed *in vitro*, revealing key physiological differences among *Symbiodinium* in terms of cold tolerance (Thornhill et al. 2008a; McBride et al. 2009), heat tolerance (Robison and Warner 2006; Suggett et al. 2008), light tolerance (Iglesias-Prieto and

Trench 1994; 1997a; Hennige et al. 2009), and acidification tolerance (Brading et al. 2011). Typical phenotypic traits that have been monitored under different environmental conditions include culture growth rates and photochemical efficiencies (*e.g.* Robison and Warner 2006; Thornhill et al. 2008). Given the state of *Symbiodinium* taxonomy prior to the 1990's, most early work assumed the physiology of a few cultures was representative of the entire genus. Over the years, more studies have incorporated clades, types, and species designations, broadening our understanding of the extensive physiological diversity within *Symbiodinium*, but none have resolved individuals within species.

Using a hierarchical molecular approach, two species of clade B *Symbiodinium* were recently delineated with a combination of nuclear, mitochondrial, and chloroplast markers (LaJeunesse et al. 2012). *S. minutum* associates with the globally-distributed anemone *Aiptasia* sp. in tropical waters, while *S. psygmophilum,* despite being present in the tropics, is cold-tolerant and typically engages in symbiosis with the scleractinian corals *Astrangia poculata, Cladocora caespitosa,* and *Oculina patogonica* in high latitudes of the Atlantic Ocean. In a preliminary experiment designed to test the hypothesis that phenotypic differences could be detected among genotypes within and between *Symbiodinium* species, we reared several monoclonal cultures of *S. minutum* and *S. psygmophilum* genotypes under identical temperature and light regimes and monitored growth rates (in terms of asexual propagation of cells). We used the micro-culture methods of Rogers and Davis (2006) as a guide, and reared all cultures in ASP-8A media (Ahles 1967). First, genotype uniqueness was confirmed with microsatellite repeat length variation (*i.e.* different alleles) at nuclear marker *Sym15* (Pettay and LaJeunesse 2007) and sequence variation at chloroplast $psbA^{ncr}$ (Moore et al. 2003; LaJeunesse and Thornhill 2011) for each culture of each species. Next, individual cells from synchronized cultures (n=3 genotypes per species) were transferred to 96-well plates via cell sorter such that each culture was represented in sixteen replicate wells with ~5 cells each at the start. Plates were incubated at 25°C and a 12:12

light/dark photoperiod at 60 microeinsteins. As cells divided asexually, plates were observed under a microscope at 400X magnification and total cell counts were recorded at noon every two days for two weeks. The growth rates were exponential, so data were log transformed and fit to a linear regression. The slope of the line was recorded as the growth metric per replicate well. The entire experiment was repeated twice.

The *S. psygmophilum* culture PurpPFlex failed to grow (as occasionally happens with recent transfers of older cultures, such as in this case), so ultimately we collected data from three *S. minutum* genotypes (Mf1.05b, rt-002, and rt-351) and two *S. psygmophilum* genotypes (Mf10.14b.02 and rt-141). Initial growth was highly variable until at least ten cells were present in each well, and cell counts became difficult after concentrations reached >200 cells/well, so we only included in our analysis wells with time series data between this count range. After failing to detect differences between experiments (t-test, $t_{(101)} = 1.25$, $p = 0.216$), data from each run were combined and analyzed together.

We noted a difference in average growth rate between species, reported here as ln(cells/day) ± 95% Confidence Interval. For *Symbiodinium minutum*, the growth rate was 0.34 ± 0.01, while for *S. psygmophilum* it was 0.31 ± 0.02 (ANOVA, $F_{(1,120)} = 4.97$, $p = 0.028$) . When separated by genotype, it became clear this effect was driven entirely by the *S. psygmophilum* culture rt-141, which had much lower growth rates than all other cultures regardless of species (ANOVA, $F_{(4,117)} = 7.39$, $p < 0.001$; Figure 1-3). The diversity in growth rates among *S. psygmophilum* may reflect the genetic diversity within this species, which exceeds that of *S. minutum* (LaJeunesse et al. 2012). The key result is that phenotypic variation among genotypes within *Symbiodinium* species can potentially exceed that found between members of different species. This situation is not uncommon in nature (Bangert et al. 2006), but to date, the concept of intraspecific variation within *Symbiodinium* species has largely been ignored. A vast preponderance of reef ecology studies only measure symbiont phenotypes at the low-resolution

'clade' or intermediate-resolution 'type' level. Using crude averages from these higher order taxonomic rankings may miss important dynamics taking place among or within species. Further experimentation with more *Symbiodinium* genotypes (both *in vitro* and *in hospite*) will be necessary to confirm these findings. The fact that such patterns can be found even among a small number of strains implies that, much like in corals, intraspecific variation in symbiont physiology may be extensive.

Host genotype effects on clonal symbiont performance: In their analysis of host and symbiont population interactions, Howells et al. (2012) showed that intraspecific variation among *Symbiodinium* influenced the growth of host juveniles in a laboratory setting. But does intraspecific variation among hosts influence symbiont performance? To address this question, we recently took advantage of the *Acropora palmata-Symbiodinium 'fitti'* association, wherein individual host colonies usually associate with only one clonal symbiont strain (Baums et al. 2014). Distinct coral genets that shared a clonal *S. 'fitti'* strain were identified growing close to each other within a natural common garden. Highly sensitive qPCR assays established that no other *Symbiodinium* could be detected within the colonies. Fragments were removed, exposed to cold shock *ex situ* (10°C for three days), and monitored for photochemical efficiency changes and acute host transcriptional responses. We found that the photochemical response of the symbiont strain varied depending on which host genotype it associated with (Parkinson et al. submitted). Because all measured *Symbiodinium* were clonal and environmental variation was reduced by the proximity of the colonies, the most parsimonious explanation was that physiological variation among host genotypes drove photochemical differences among the clonal symbiont strains. Experiments designed to test for intraspecific variation should make sure that individual histories are not a confounding factor; the natural common garden proved advantageous for that purpose here.

In a subset of the holobionts exposed to cold, symbiont photochemical efficiency was phenotypically buffered (Waddington 1942; Bradshaw 1965; Reusch 2014), meaning the reaction norm changed relatively little with environmental perturbation. In other host backgrounds, the symbiont strain's response was less buffered. Host expression of iron sequestering and oxygen stress signaling genes correlated with these differences in symbiont performance, suggesting that variation in iron microhabitat and/or redox sensitivity among hosts may mediate clonal symbiont performance during stress. Anecdotally (because sample size was small), the colonies that participated in the annual spawning event had the most buffered symbiont responses. Those colonies with less buffered symbiont responses did not spawn. This result suggests a possible fitness consequence of genotype interactions among holobionts, highlighting the potential evolutionary importance of intraspecific diversity among coral mutualists.

Metabolomic analysis of symbiotic and nonsymbiotic polyps: The *Astrangia poculata-Symbiodinium psygmophilum* association has been proposed as a model system for investigating coral-algal symbiosis. This scleractinian hard coral is more amenable to aquaculture than exclusively tropical species and exists across a broad latitudinal and temperature range. Uniquely, *A. poculata* colonies often feature both symbiotic and nonsymbiotic polyps within the same colony under nonstressful conditions. This attribute allows for experimental investigation into the molecular underpinnings that mediate successful symbiotic interactions among hosts and symbionts while controlling for partner genotypes. We generated metabolomic profiles for symbiotic and aposymbiotic polyps dissected from each of three *A. poculata* colonies to provide another example of the insights that can be gained when intraspecific diversity is accounted for in the experimental designs. We also analyzed a *Symbiodinium psygmophilum* monoclonal culture (isolated from a tentacle of *A. poculata*). Methods generally followed Gordon et al. (2013) with minor modifications. Target tissues were snap frozen in liquid nitrogen within 1 min of sampling, then metabolites were extracted in isopropanol:acetonitrile:water (3:3:2) solution. The

samples were separated on a Shimadzu 20R UFLC high-performance liquid chromatography system using a C_{18} column. Mass spectra and tandem mass spectra were obtained in both positive and negative ion mode on an AB SCIEX 5600 Triple TOF. The resulting LC-MS profiles were Pareto transformed to reduce bias from metabolites with large fold changes while preserving the rank and dimensionality of the data (van den Berg et al. 2006).

Principle component analysis (PCA) clustered polyps by symbiont state more strongly than host genotype (Figure 1-4a). PCA loadings revealed ~4000 compounds (including isotopic and monoisotopic peaks) that were mainly present in only one of the symbiotic states, driving group clustering. For example, a platelet activating factor (PAF) was observed at much higher levels in nonsymbiotic polyps (Figure 1-4b). This metabolite has multiple functions in humans, and may play a role in intracellular signaling (Venable et al. 1993). The single *Symbiodinium* sample fell far from either of the holobiont clusters in the PCA. Certain compounds were observed only in the *Symbiodinium* sample, such as 13E-Docosenamide, the function of which is unclear in *Symbiodinium* (it has been found in the cerebrospinal fluid of mammals; Cravatt et al. 1995). Unfortunately, a majority of metabolites could not be easily annotated, and further work will be required to characterize them. Controlled contrasts should reveal key players in the metabolic interactions that allow the symbiosis to persist. Being able to compare fragments of the same host genotype in two symbiotic states reduces the problem of working with non-model coral species that contain a large amount of genetic variation. That variation would otherwise obscure patterns. This is but one example of how new technologies, when applied to combined and isolated components of the holobiont, will facilitate new insights into marine endosymbiotic mutualisms.

Coevolutionary context and climate change

Mutualisms in general (Kiers et al. 2010) and coral-algal associations in particular (Hoegh-Guldberg et al. 2007) are threatened by a changing climate and anthropogenic

disturbance. Aside from the extreme case of mutual extinction (Dunn et al. 2009), other negative evolutionary outcomes of changing environmental conditions may include shifts from mutualism to antagonism, switches to inferior partners, and mutualism abandonment (Kiers et al. 2010). Unequal responses to climate shifts between partners can contribute to mutualism breakdown (Warren and Bradford 2014). Such breakdown is apparent in coral systems, where the 'coral bleaching' phenomenon (when hosts and symbionts dissociate due to stress) takes place at temperatures below the upper thermal limits of most free-living microalgae (Berry and Bjorkman 1980). There is a unique aspect to engaging in symbiosis that makes the intact association more sensitive to temperature changes; this is likely due to the consequences of an oxygen-sensitive animal taking on a photosynthetic symbiont that generates reactive oxygen species under elevated light and temperature conditions (Lesser 2006; Baird et al. 2009b). While many efforts have been made to assess the adaptive potential of coral holobionts facing rising sea surface temperatures, almost none have considered intraspecific trait variation (but see Csaszar et al. 2010). Such investigation will be needed to more accurately predict the role of coevolution in the coral holobiont response to climate change.

Many corals transmit their symbionts vertically by provisioning eggs with *Symbiodinium* cells (Hirose et al. 2008), but most corals spawn symbiont-free gametes or larvae (Baird et al. 2009a), and therefore must acquire their algal complement from the environment. In a closed vertical system it is easier to accept that tight coevolution takes place; it is less clear how changes in the symbiont genome could be considered heritable when partner genomes are uncoupled every host generation. And yet, there is remarkable stability between horizontally-transmitted host and symbiont species. The Caribbean broadcasters in the *Orbicella* genus appear flexible at the clade level (associating with members of Clades A, B, C, and D), but are actually quite specific, hosting only a few species within each clade (Thornhill et al. 2014). The two lineages of the Caribbean gorgonian *Eunicea flexuosa* each associate exclusively with a corresponding Clade

B symbiont (Prada et al. 2014b), while the Caribbean scleractinian *Acropora palmata* typically associates with *Symbiodinium 'fitti'* (Baums et al. 2014). These examples, along with a number of other studies and data sets, clearly demonstrate that coevolution takes place in coral-algal systems, with unique host and symbiont combinations (holobionts) being the units of selection (Iglesias-Prieto and Trench 1997b; LaJeunesse et al. 2004; LaJeunesse et al. 2005; Reshef et al. 2006; Correa and Baker 2011; Finney et al. 2010; LaJeunesse et al. 2010; Thornhill et al. 2013; 2014; Lesser et al. 2013; Prada et al. 2014b).

To paraphrase Thornhill et al. (2014), coevolution despite vertical *Symbiodinium* transmission can be explained by the processes of ecological selection via host-specialization in geographic isolation. Divergent selection acting on intraspecific variation favors adaptations that increase *Symbiodinium* fitness in a given host intracellular habitat, removing suboptimal generalist genotypes. Subtle physiological differences correlated with reproductive incompatibilities among hosts may also affect which symbiont genotypes are favored. Some evidence for such differences is presented below. Reinforced by assortative mating, ongoing divergence within symbiont populations would closely mirror that of the host, as each host generation selects for its corresponding partner lineage. The *Eunicea* association provides a good example where both host and symbiont lineages are relatively recently diverged and the *Symbiodinium* are host-specialized (Prada et al. 2014b). The processes that lead to such ecological speciation occur at the scale of interactions between partners at the level of individuals within species, which is why the holobiont can be viewed as a unit of selection.

An aspect of population biology that may shed light on coevolutionary capacity is patterns of population genetic structure and gene flow. Based on the current evidence, population genetic structure does not match between coral host and algal symbiont (Andras et al. 2011; Andras et al. 2013; Baums et al. 2014). Adaptation to thermal and ocean acidification stress is likely ongoing but those adaptations that require reciprocal changes in the mutualistic partners

(*e.g.* pathways involved in exchange of nutrients) will be spread inefficiently if dispersal scale is not matched between partners. For example, in *Acropora palmata* the host is divided into two large populations encompassing the eastern and western Caribbean (Baums et al. 2005b). At the same time, the dominant symbiont (*Symbiodinium 'fitti'*), consists of seven populations, each found over smaller geographic regions (Baums et al. 2014). Thus a beneficial adaptation arising in *S. 'fitti'* may only efficiently rise to high frequency in parts of the host range. However, even weak selection can be sufficient to spread advantageous alleles throughout structured populations, in part because fixation times for such alleles are greatly reduced relative to their neutral counterparts (Slatkin 1976; Rieseberg et al. 2004). Patterns of gene flow can vary substantially among coral hosts from small to large geographic scales (reviewed by Baums 2008). We expect the same to be true for *Symbiodinium* species. Hence, additional studies are needed that resolve the population genetic structure of both partners simultaneously.

Little theoretical work has been done to understand how population genetic structure should be matched between hosts and symbionts. Work on parasites suggests that population structure should be smaller scale in the parasite compared to the host population (as found by Dybdahl and Lively 1996), though there are examples of the opposite case (Martinez et al. 1999) and balanced structure (Mulvey et al. 1991). However, the traditional Red Queen model of rapid antagonistic coevolution does not seem appropriate for mutualisms, where fitness consequences of interactions are measured in gains rather than losses. An alternative model for mutualisms based on game theory, the Red King hypothesis (Bergstrom and Lachmann 2003), predicts that unbalanced evolutionary rates among partner species can be stable. Currently, this model is not spatially explicit—it cannot account for local adaptation to environmental gradients such as light, for example—but nevertheless makes interesting predictions. According to Red King, the host is assumed to be 'enslaving' the faster-evolving symbiont (Hilbe et al. 2013) by repeatedly 'demanding' over evolutionary time scales that more opportunistic symbiont genotypes evolve

back toward being more generous. The Red King hypothesis may need to be modified to account for the one-to-many interactions between a coral colony and individual *Symbiodinium* cells (Gokhale and Traulsen 2012). Finally, such models will require empirical data accounting for both inter- and intraspecific diversity and population structure in both partners. Results might provide important insight when predicting the effects of climate change on marine mutualisms.

Future directions

Consideration of intraspecific diversity in experimental designs will likely improve the predictive value of models of climate adaptation in corals. For example, when climate projections do not incorporate adaptive processes such as genetic adaptation, they predict 20-80% more mass bleaching events in a given period than when such processes are included (Logan et al. 2014). Adaptation-free models over-predict the current frequency of bleaching, which indicates that adaptive processes are likely ongoing. Indeed, rapid adaptation and acclimation to thermal stress have been demonstrated among corals exposed to highly variable temperatures (Palumbi et al. 2014). Intraspecific diversity may represent a component of adaptive capacity to increased temperature in corals (Baums 2008; Baums et al. 2013), although rare beneficial alleles can spread rapidly even when diversity is low. We would predict a link between intraspecific diversity and bleaching resistance, much like the classic link between diversity and infectious disease resistance (O'Brien and Evermann 1988). If an empirical link can be made, this information can be incorporated into models projecting the survival of corals.

There are several areas where the development of new techniques will provide further insight in to the nature of marine mutualisms. The difficulty of aquaculturing corals has always presented a challenge to molecular studies in this system. Rearing of an F2 generation for traditional genetic experiments has previously been intractable. Only recently has successful culturing of corals from gametes to sexual maturity taken place (Iwao et al. 2010; Baria et al. 2012). These colonies spawned after three or four years of growth, indicating that the rearing of

F2 generations to sexual competence for backcrosses will require at least six years for these species. Further complications stem from the symbiotic promiscuity of larvae, which may take more than three years to reflect the algal complement of stable adult colonies (Abrego et al. 2009b). Despite these issues, new technologies are providing different avenues for molecular characterization of corals. For example, Lundgren et al. (2013) recently used next generation sequencing to characterize a suite of single nucleotide polymorphisms (SNPs) that correlate with environmental variables in populations of scleractinian corals on the Great Barrier Reef. Five SNPs for *Acropora millepora* and three SNPs for *Pocillopora damicornis* exhibited likely signatures of selection. These markers may serve as quantitative trait loci for stress tolerance, a critical tool for managers attempting to identify particularly resilient genotypes for restoration purposes.

In parallel with the development of microsatellite markers to distinguish coral and algal individuals, efforts have been made to elucidate the taxonomic diversity of coral-associated microbes, cryptic invertebrates, and more transient associates such as reef fish. An integrative approach that simultaneously assesses diversity across all these community levels would provide a comprehensive understanding of how coral genotypic diversity affects and is affected by reef community diversity. This can be accomplished by combining surveys of natural coral stands, manipulation of *in situ* common gardens, and *ex situ* experiments. Even at small spatial scales, natural variation in genotypic evenness and richness is common within and across species, ranging from minimal clonal replication to reefs dominated by just one genet (Hunter 1993; Ayre and Hughes 2000; Miller and Ayre 2004; Baums et al. 2006; Boulay et al. 2014). By tracking the functional and taxonomic diversity of associated micro- and macro-scale assemblages over time in plots of varying host and symbiont genotypic diversity or composition, it will be possible to quantify the link between diversity and community dynamics. We would predict that host and *Symbiodinium* genotypic diversity positively correlate with microbial and epifaunal community

diversity. The incorporation of environmental stressors in such designs will help to assess the direct effects of those stressors as well as the indirect effects of diversity and composition on both ecosystem function and resilience, potentially informing conservation and restoration strategies (Srivastava and Vellend 2005). Again, we would predict a positive association between holobiont genotypic diversity and resilience. These types of studies would address our second major hypothesis; that reef community dynamics are influenced by intraspecific diversity among corals.

An interesting application of fine-scale techniques will be to examine the coral colony landscape in terms of the distributions of different symbiont genotypes throughout host tissues. Do *Symbiodinium* stratify not only based on light regime (*e.g.* top, bottom, and sides of colonies), but also within specific host tissues (*e.g.* tentacles)? Can multiple symbiont species or genotypes within a species occupy a single symbiosome within a single host cell? Laser-capture microdissection (Espina et al. 2006) has already been used to isolate targeted bacterial endosymbionts of *Siboglinum fiordicum*, a tube worm (Thornhill et al. 2008b). The same technology could be applied to isolate *Symbiodinium* among non-calcifying hosts *in hospite*, and be coupled with transcriptomic or metabolomic profiling. Because somatic mutations in the undifferentiated host germ line can propagate as corals age (reviewed by van Oppen et al. 2011), and early larval fusion can generate chimeras (Frank et al. 1997; Barki et al. 2002; Puill-Stephan et al. 2009), it will also be interesting to map host genotypic mosaicism within a colony and to see if this influences symbiont associations in any way.

Further research into the physiology and ecology of background *Symbiodinium* is required to determine the role of this diversity in coral holobionts. Manipulating background strains will be difficult. A first step would be rearing healthy, completely symbiont-free corals, much like sterile mice reared without gut bacteria. With current aquaculture techniques, this is impossible for scleractinian hard corals. Progress has been made in the model anemone *Aiptasia*

sp. (Weis et al. 2008). Though they lack the biomineralization processes of hard corals, *Aiptasia* represent a promising first step for several reasons. It is easy to produce clonal replicates, novel associations with heterologous symbionts are possible, and the same individuals can be inoculated, bleached, and re-inoculated experimentally in an aquarium setting. Moreover, genomic resources are available for the host and the homologous symbiont, *S. minutum* (Sunagawa et al. 2009; Bayer et al. 2012; Lehnert et al. 2012; Shoguchi et al. 2013). This system may be well-suited for establishing whether background *Symbiodinium* are functionally relevant during normal and stressful conditions. Additional transcriptomic, metabolomic, and proteomic characterizations of different *Symbiodinium* are ongoing. By contrasting molecular phenotypes at both coarse resolution (*e.g.* between clades; Ladner et al. 2012; Barshis et al. 2014) and fine-scale resolution (*e.g.* between species within clades and between individual within species), we will begin to decipher the mechanisms by which evolution gave rise to the current diversity of *Symbiodinium.*

Conclusion

Intraspecific variation is a major component of terrestrial mutualisms, affecting ecological interactions between proximate symbiotic species as well as higher order community dynamics. Our understanding of such forces in marine endosymbiotic associations is lacking. We have reviewed some of the current literature and presented additional preliminary evidence suggesting intraspecific variation is extensive in coral hosts and algal symbionts, and that such variation interacts to affect the function of the combined holobiont. The holobiont is both a key ecological feature (being the physical structure that shapes reef ecosystems) and a unit of natural selection. Future research should incorporate fine-scale molecular genotyping of both partners to address key questions about marine symbiosis ecology and evolution, and to characterize the role of holobiont extended phenotypes in an era of changing climate.

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Figures

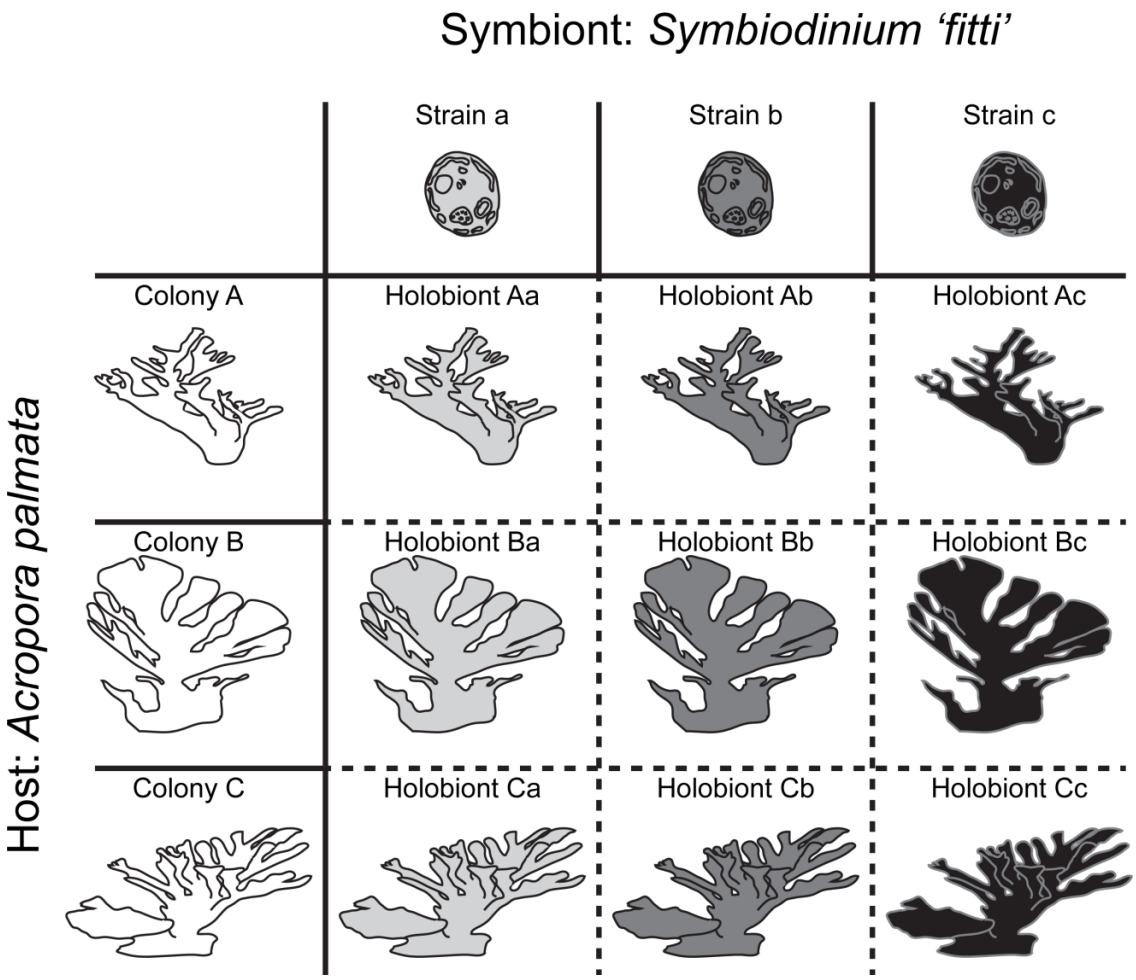

Figure 1-1 Diagram showing how coral-algal holobionts represent unique pairings of partner genotypes in the *Acropora palmata-Symbiodinium 'fitti'* association. Host genotype (colony) is indicated by shape; symbiont genotype (strain) is indicated by color. In this association, the host:symbiont genotype ratio is one:one in most colonies. Typically, >99% of each colony's symbiont population is composed of clonal cells representing a single genotype (that is, one strain).

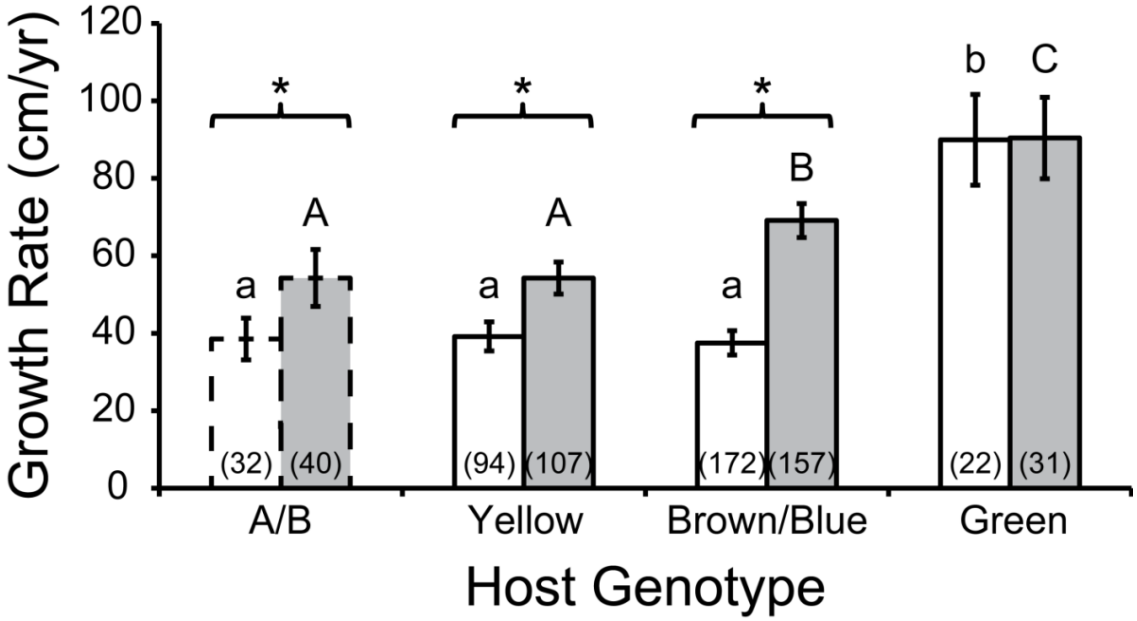

Figure 1-2 *Acropora cervicornis* colony growth as a function of host genotype. The Green host genotype had higher growth rates (cm/year) then genotypes A/B, Yellow, and Brown/Blue in shallow water (white bars; similar growth rates indicated by common lower case letters, Tukey's HSD; $p < 0.05$) and in deep water (gray bars, similar growth rates indicated by common upper case letters, Tukey's HSD; $p < 0.05$). Growth rates were usually higher in deep compared to shallow colonies of a given genotype (asterisks: t-test; $p < 0.05$). Numbers in parentheses indicate sample size (number of colonies). Error bars depict 95% confidence intervals. Host A/B is bordered by dashed lines to emphasize that for this particular holobiont, the corresponding *Symbiodinium 'fitti'* strain was distinct from the strain that was common to the other three holobionts. Data reanalyzed from Griffin et al. (2012).

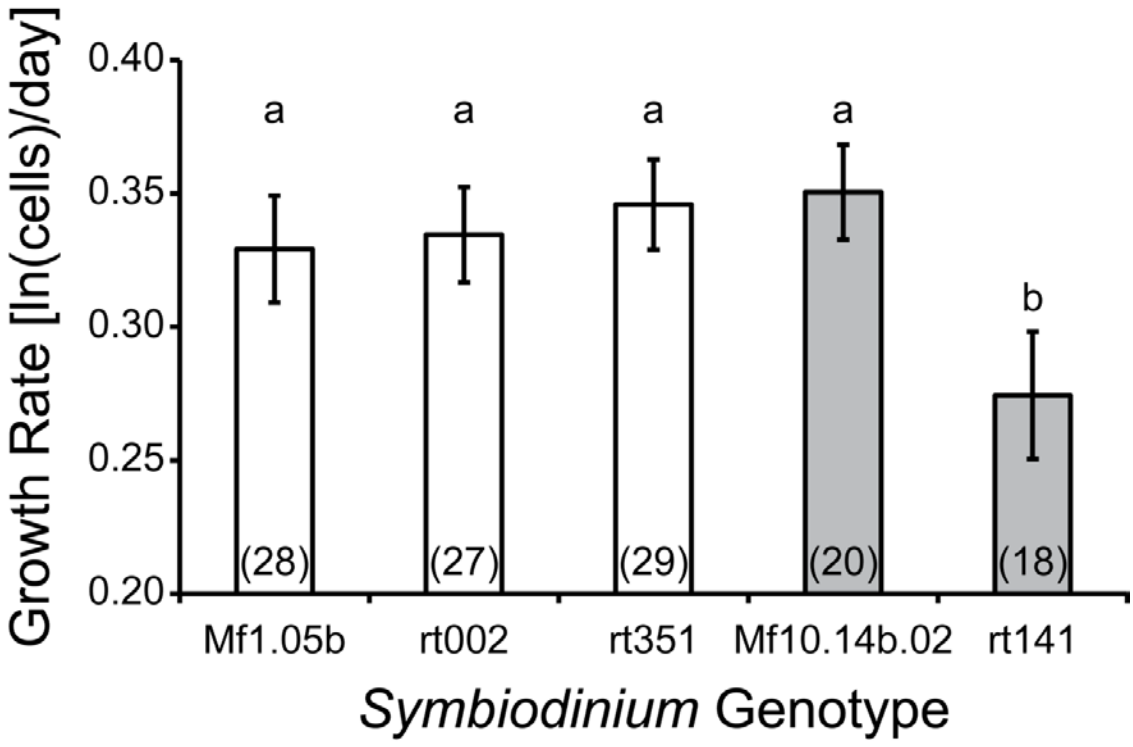

Figure 1-3 *Symbiodinium* culture growth as a function of genotype. *S. minutum* genotypes (white bars) showed little variation in growth rates compared to *S. psygmophilum* genotypes (gray bars). Letters indicate statistically different growth rate groupings (Tukey's HSD; $p < 0.05$). Numbers in parentheses indicate sample size (number of wells). Error bars depict 95% confidence intervals. S. Denecke et al. (unpublished data).

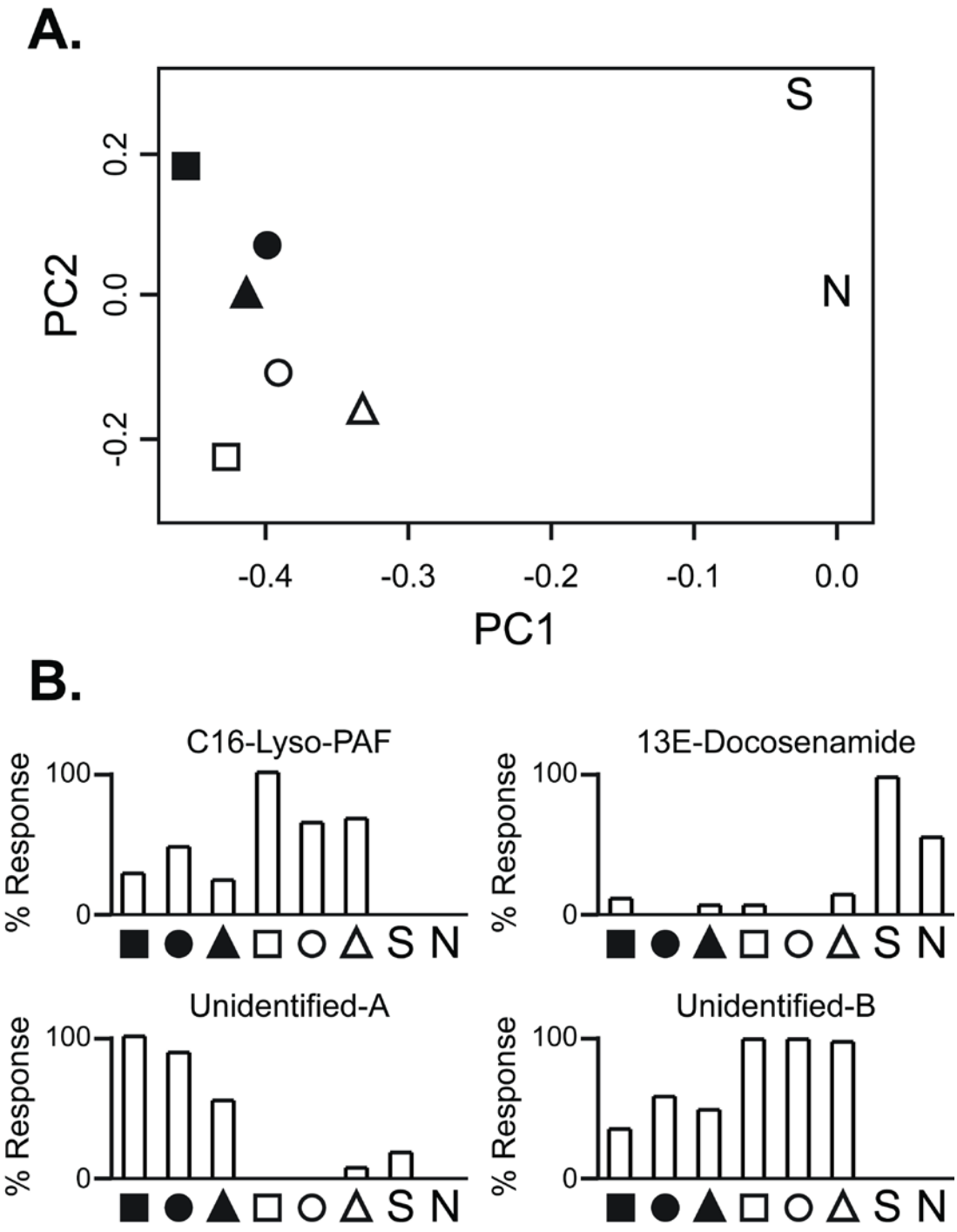

Figure 1-4 Preliminary analysis of the *Astrangia poculata-Symbiodinium psygmophilum* metabolome. **(a)** Principle component analysis of metabolite profiles. Shown are principle components 1 and 2 (x- and y-axis, respectively) of Pareto-transformed metabolite data. Shapes

indicate host genotype (n=3). Black fills correspond to symbiont-rich polyps. White fills correspond to nearly symbiont-free polyps. 'S' indicates a sample of a *Symbiodinium psygmophilum* monoculture. 'N' indicates a negative control (purified water). *A. poculata* samples cluster by the symbiotic state of the polyps rather than by host genotype. **(b)** Representative profiles for specific metabolites. C16-Lyso-PAF was abundant in nonsymbiotic polyps but low in symbiotic polyps and absent in *Symbiodinium* culture. 13E-Docosenamide was mainly present in *Symbiodinium* culture but not in coral tissue. The two unidentified compounds are characteristic of metabolites with greater detection in symbiotic (Unidentified-A) or nonsymbiotic (Unidentified-B) polyps. N. Polato et al. (unpublished data).

Figure 1-5 Box 1. Low abundance *Symbiodinium*

Given that DNA evidence is the primary means by which *Symbiodinium* are both detected and identified, our ability to quantify symbiont diversity is restricted by the molecular techniques used. Not all techniques and markers have equal resolving power (Sampayo et al. 2009). One of the most common markers, the internal transcribed spacer 2 (ITS2) of the ribosomal array, is multicopy and undergoes concerted evolution, maintaining functional and nonfunctional rare variants in the species population (Dover 1982). Much debate has focused on the information lost when using denaturing gradient gel electrophoresis to screen out rare intragenomic variants (Apprill and Gates 2007; Thornhill et al. 2007). This methodology conservatively underestimates total symbiont diversity within a coral colony while revealing the dominant or codominant taxa (i.e. the most numerically abundant and presumably ecologically-relevant species). In the process, minor strains that comprise <5% of the total symbiont population within host tissues go unrecognized. With the development of several sensitive qPCR assays (Ulstrup and Van Oppen 2003; Ulstrup et al. 2007; Correa et al. 2009; Mieog et al. 2009) and the advent of next generation sequencing (Kenkel et al. 2013; Green et al. 2014), it has been possible to survey the diversity of 'background' populations of *Symbiodinium* below the detections limits of DGGE and traditional PCR.

In a recent survey of 26 coral taxa previously thought to be 'specific' (restricted to associations with one *Symbiodinium* clade), background symbionts from multiple clades could be detected with qPCR assays in nearly all host species (Silverstein et al. 2012). When a nonsymbiotic coral species was screened as a control, the assays returned false positives from putatively contaminant symbionts trapped in the mucus or gut cavity 9% of the time. This rate of natural contamination is quite high, but nevertheless, background strains are more common than previously thought. It is understood that most corals that acquire their symbionts from the environment each generation are promiscuous during early ontogeny, associating with multiple

symbiont taxa that are not dominant in adults (Coffroth et al. 2001; Santos et al. 2003a; Little et al. 2004; Coffroth et al. 2006; Abrego et al. 2009a; Byler et al. 2013; Cumbo et al. 2013; Poland et al. 2013; Yamashita et al. 2013). Since the capacity for non-specific associations is present in juveniles, it is not necessarily surprising that multiple clades were detected in low abundance in adult corals (Santos et al. 2004; Baird et al. 2007; Baker and Romanski 2007). It is currently unclear whether the presence of a background symbiont implies that it is functionally relevant to the holobiont. Though corals may have always been open to infiltration by background symbionts, host-symbiont specificities have evolved multiple times regardless.

Detection of low-abundance *Symbiodinium* cells in corals suggest that hosts may be open environments where small numbers of heterologous symbionts are entering and exiting the system on a regular basis. If commensal, these symbionts may move passively through the system without engaging in symbiosis. If parasitic, they may trigger a host rejection response or may be competitively displaced by the dominant symbiont, such that only a small number are present in a coral at a given time. Finally, if mutualistic, they may be fully engaged in the fitness of the holobiont despite their rarity. For example, rare symbionts may be important if they contribute a different but essential metabolic resource than the dominant symbiont strain (analogous to rare members of the bacterial biosphere; reviewed by Pedros-Alio 2012), or if they can increase sufficiently in number to replace a compromised dominant symbiont should environmental conditions change (Buddemeier and Fautin 1993; Baker et al. 2004; Berkelmans and van Oppen 2006).

Studies are needed to distinguish between these competing scenarios. So far, the few experiments that have successfully tracked background symbionts during natural environmental extremes suggest that they are not viable sources of persistent acclimation to stress, at least in terms of replacing the dominant symbiont. After a cold-water bleaching event in the Gulf of Mexico, most *Pocillopora damicornis* colonies with mixed symbiont communities did not

'shuffle' (*c.f.* Baker 2003) to the more thermally tolerant species (McGinley et al. 2012), instead remaining stable despite environmental variability. In corals sampled before, during, and after a 2005 bleaching event in Barbados, background populations of the thermally tolerant *Symbiodinium trenchii* increased in prevalence prior to bleaching, but declined to pre-stress levels over the next two years of non-stressful conditions (LaJeunesse et al. 2009). However, functional relevance may not be tied directly to cell numbers (a rare strain may always be rare and yet essential). Such a hypothesis has yet to be tested in corals, though bacterial analogues are known. For example, a single rare bacterium representing 0.006% of the total cell count in peat accounted for a much larger proportion of the biome's sulfate reduction relative to its abundance (Pester et al. 2010). This is an active research area, and despite our current data deficiency, future studies may provide more convincing evidence of the functional relevance of background *Symbiodinium*.

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Chapter 2

LIMITED BENEFIT OF LARVAL SYMBIONT MANIPULATION FOR *EX SITU* CORAL RESTORATION

Abstract

Restoration of threatened Caribbean Acroporid coral populations has traditionally relied on *in situ* cultivation of asexual fragments from adult colonies. Rearing of genetically diverse larvae derived from sexual reproduction remains challenging in part due to size-dependent mortality during early development. Manipulation of the larval symbiont community during *ex situ* aquarium rearing may be one avenue for increasing larval growth rates and reducing mortality. To determine whether the growth of recently-settled coral larvae was influenced by their symbiont composition, full-sibling *Acropora palmata* juveniles were reared at six public aquaria. To test whether growth might be faster for symbioses that matched adult associations, half of the aposymbiotic settlers were pre-exposed to homologous symbionts in isolation. Then all settlers were transferred to display tanks containing sources of both homologous and heterologous symbionts. After 1 month, symbiont cladal diversity was high among host juveniles, including representatives from all four clades known to commonly associate with corals. Pre-exposure had no influence on symbiont composition. By 30 months, only 2 clades were detected, though the reduction in diversity is confounded with mortality. These results are consistent with previous studies showing early promiscuity in the symbiosis with winnowing over time. While polyp growth was strongly influenced by the aquarium setting, symbiont composition had no effect, indicating that the artificial introduction of a particular symbiont type

is not the most effective way to reduce the early post-settlement mortality bottleneck during *ex situ* restoration efforts.

Introduction

A combination of factors has led to the significant decline of the Caribbean Elkhorn coral, *Acropora palmata* (Bruckner 2003), resulting in its listing as a threatened species under the US Endangered Species Act (Hogarth 2006). At present, sexual recruitment is nearly absent, consistent with a lack of wide-scale recovery of *A. palmata* populations (Williams et al. 2008). The fast-growing Acroporid species have been the focus of extensive restoration efforts, mainly via coral gardening methods (Rinkevich 1995,2005). In a typical restoration project, a few local donors are transplanted to *in situ* nurseries, where colonies are fragmented, mounted, grown, and finally outplanted back to the reef. These asexual propagation techniques preserve a portion of extant genetic diversity, but cannot contribute to further diversity until the fragments grow to reproductive size (at least 4 years; Lirman 2000). Breeding attempts are underway to provide sources of sexually-produced offspring through *ex situ* (Petersen et al. 2006; Bosch and Morse 2012) and *in situ* (Omori 2005; Nakamura et al. 2011; Cooper et al. 2014) methodologies.

Sexually-produced offspring would help maintain the genetic diversity of these threatened species, minimizing risks such as founder effects and inbreeding depression (Baums 2008). However, larval rearing in particular remains challenging due to the extremely high (often ~99%) combined pre- and post-settlement mortality rates typical in nature (Babcock and Mundy 1996; Wilson and Harrison 2005; Vermeij and Sandin 2008) and in restoration projects (Raymundo and Maypa 2004; Richmond 2005; Miller and Szmant 2006; Guest et al. 2010; Cooper et al. 2014; this study). Settler mortality is both size- and age-dependent (Babcock and Mundy 1996; Raymundo and Maypa 2004; Vermeij and Sandin 2008), with those corals that

reach a larger size at a younger age having a much greater chance of surviving. Thus, one goal of sexually-based coral restoration efforts is to maximize growth rates during early development, hopefully reducing the impact of the size-dependent post-settlement mortality bottleneck.

Acropora palmata generates aposymbiotic larvae via broadcast spawning of gametes (Szmant 1986). Upon settlement, the coral enters into an obligate symbiosis with unicellular dinoflagellates of the genus *Symbiodinium*, which have been divided into multiple evolutionary lineages based on molecular markers (for reviews, see Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). *A. palmata*'s acquisition of symbionts occurs horizontally from the environment rather than vertically through the maternal line (Wallace 1985), making novel associations possible each generation. Despite this, most *A. palmata* adults in shallow habitats associate with one symbiont species: *Symbiodinium* ITS2 type A3 (LaJeunesse 2002; Thornhill et al. 2006). The Carribbean A3 type is distinct from the Pacific A3 type (LaJeunesse et al. unpublished data) and is referred to as *Symbiodinium 'fitti' nomen nudum* (Pinzon et al. 2011) but currently lacks a formal species description.

Promiscuity in symbiosis during early development, where the symbiont complement found in juveniles is different than that found in stable adults, is evident in many symbiotic cnidarians, including Caribbean octocorals (Coffroth et al. 2001; Santos et al. 2003; Coffroth et al. 2006; Poland et al. 2013) and Pacific Acroporid corals (Little et al. 2004; Abrego et al. 2008; del C. Gomez-Cabrera et al. 2008; Abrego et al. 2009a,b; Bay et al. 2011; Cumbo et al. 2013). The onset of specificity during maturation can reduce initial symbiont diversity over time. This 'winnowing effect'(Nyholm and Mcfall-Ngai 2004) can occur in as little as 3 months (Coffroth et al. 2001) or be delayed 3.5 years or more (Abrego et al. 2009a). Variable windows of flexibility and subsequent winnowing are probably common in scleractinian corals (Rodriguez-Lanetty et al. 2006), as most adults associate predominantly with few kinds of symbionts over their geographic ranges (LaJeunesse 2005; Goulet 2006; but see Silverstein et al. 2012 for a discussion of

background *Symbiodinium*). It is unknown when the mature association between *Symbiodinium* A3 and *A. palmata* stabilizes.

The 'clade' is a low-resolution designation, as nearly all *Symbiodinium* clades are composed of multiple types or species (LaJeunesse 2001; LaJeunesse et al. 2012). Nevertheless, a majority of studies of coral symbiosis ecology have contrasted physiology between rather than within clades. Functional consequences for corals harboring symbionts from different clades have been measured via symbiont density rates per host colony and percentage of symbiotic juveniles (Weis et al. 2001; Baird et al. 2009; Bay et al. 2011), patterns of symbiont localization (Rodriguez-Lanetty et al. 2006), physiological responses of both the coral and *Symbiodinium* under heat and light stress (Abrego et al. 2008; Fisher et al. 2012), gene expression differences (DeSalvo et al. 2010), and juvenile growth rates (Little et al. 2004). Knowing which symbionts maximize the health of developing *A. palmata* settlers may improve captive breeding attempts by enhancing early growth rates, and thereby reducing the settler mortality bottleneck. One might intuitively expect larvae that begin life associating with the same (homologous) symbionts as their parents will outperform larvae that associate with different (heterologous) symbionts belonging to other species or clades. However, the ideal symbiont might be different for juvenile versus adult colonies (*e.g.* Little et al. 2004), and for artificial versus wild settings.

The goal of this experiment was to determine if manipulating the symbiont complement of *A. palmata* settlers reared in captive environments might reduce early mortality during *ex situ* restoration. Toward that end, aposymbiotic larvae were exposed to homologous *Symbiodinium* A3 as well as heterologous symbionts found associating with other coral species (members of clades B, C, and D, as well as other types within clade A). Settler growth and symbiont diversity were monitored over time. Given the specificity of adult *A. palmata* toward *Symbiodninium* A3 it was hypothesized that homologous symbionts would result in the best performance as reflected by faster juvenile growth rates.

Materials and Methods

Coral and Symbiodinium *collection*

During a mass spawning event in Tres Palmas Marine Reserve (Rincón, Puerto Rico; N 18° 20.800', W 67° 15.811'; August 20th-21st, 2008), *Acropora palmata* gametes were collected from two previously genotyped adult colonies and crossed following Baums et al. (2005). This single 2-parent cross produced tens of thousands of full sibling larvae. The aposymbiotic larvae were maintained on-site in 0.2 µm filtered seawater. At 4 days post-fertilization and prior to settlement, batches of ~8,000 larvae were shipped overnight to 6 public aquaria that participated in the annual SECORE workshop (SExual COral REproduction; secore.org). The aquaria included the National Aquarium, Washington and Baltimore; Chicago's Shedd Aquarium; Columbus Zoo and Aquarium; Dallas Zoo and Children's Aquarium at Fair Park; Omaha's Henry Doorly Zoo; and the Smithsonian's National Zoological Park. Hereafter, aquaria are referred to as Baltimore, Chicago, Columbus, Dallas, Omaha, and Smithsonian, respectively.

Each aquarium was provided with tissue homogenate containing homologous *Symbiodinium* type A3 isolated from 1 of the parental colonies. To isolate *Symbiodinium*, tissue was blasted from small coral fragments using a WaterPik filled with 1 µm-filtered seawater. The slurry was lightly centrifuged (4,000 rpm for 10 min), decanted, homogenized, and resuspended in filtered seawater. The process was repeated until symbiont cells appeared clean under a microscope. Final homogenates consisted of 15mL volumes at 10^6 cells/mL. The caps were left ajar for air exchange, and only secured during transport. *Symbiodinium* cells can survive up to 6 days under these conditions with little decrement in their viability or capacity to establish symbioses with juvenile corals (Carter and Hagedorn, unpublished data).

Larval rearing

After arriving at each aquarium, ~8,000 swimming larvae (4-5 days old) were divided into two separate 40 L tanks (~1 larva/mL) lined with unconditioned ceramic settlement tiles (22 x 22 mm; Petersen et al. 2005). Though acroporids are generally considered to be incapable of acquiring symbionts pre-settlement, stable symbioses have been established in the swimming planulae of at least one species (Van Oppen 2001). Thus, the entire 15 mL of *Symbiodinium* homogenate was added to one of the tanks in an attempt to inoculate half of the larvae with the parental strain. This was termed the 'pre-exposure' treatment; the other larvae were 'unexposed.' Final symbiont concentrations were ~375 cells/mL, which is lower than usual for this type of experiment (*e.g.* 1,000 cells/mL; Voolstra et al. 2009), but still higher than concentrations known to successfully establish symbioses in octocorals (e.g. 100 cells/mL; Coffroth et al. 2001). Corals were exposed only once, and water changes began after 24 h.

Larval rearing conditions varied: temperature ranged between 26 (at Dallas) and 27.8°C (at Chicago); daily water changes ranged between two times (at Chicago and Omaha) and three times (at all others); and light conditions ranged between indirect natural sunlight (at Dallas) and 40 watt fluorescent tubes (at Chicago). Given time and resource limitations, more detailed records of environmental conditions could not be maintained at all aquaria. After 1-2 weeks, all tiles with surviving settlers were transferred to display tanks containing Indo-Pacific corals. These served as sources of heterologous symbionts (clades B, C, D, and A types other than A3), as corals can expel thousands of viable symbionts into the water column every day (Hoegh-Guldberg et al. 1987; Titlyanov et al. 1996). Chicago, Columbus, and Omaha also contained adult fragments of Caribbean *A. palmata* in their display tanks (likely hosting homologous A3), while Baltimore, Dallas, and Smithsonian did not (though other sources of Pacific A3, such as tridacnid clams, were present). The symbionts hosted by the source Indo-Pacific corals were not

typed, with the assumption that they still associated with foreign strains and had not switched once translocated to the aquarium (Smith et al. 2009).

Growth measurements and symbiont genotyping

Haphazardly selected tiles (n=5-11 depending on the aquarium, with 1-9 settlers per tile) were preserved in 95% ethanol at 1 month (Baltimore, n = 43 settlers; Chicago, n = 32; Columbus, n = 10; Dallas, n = 23; Omaha, n = 12), 4 months (Omaha, n = 11; Smithsonian, n = 30), and 30 months (Chicago, n = 8; Omaha, n = 14). Logistical issues prevented sampling of Smithsonian at 1 month and Chicago at 4 months. Sampling at 4 and 30 months was limited by extreme mortality. Settlers were individually photographed at 1 and 4 months and measured along the longest diameter using a Zeiss AxioCam MRm digital camera and the software Axiovision v. 4.8.1.0 (Carl Zeiss Microimaging Inc., Thornwood, NY). By 30 months, the corals had grown so large that clippings of the colony were sampled; these were not measured for size.

For each individual settler recovered during the experiment (n = 183), symbiont DNA was extracted using the DNEasy animal tissue kit (Qiagen, Valencia, CA). The internal transcribed spacer one (ITS1) rDNA region was analyzed using a combination of denaturing gradient gel electrophoresis (DGGE) screening and direct sequencing of dominant bands. First, the entire ITS region was targeted using the symbiont-specific primers 'ZITSUP' and 'ZITSDN' with a touchdown polymerase chain reaction (PCR) following Santos et al. (2001). This product was used as template for a second PCR using the primers 'ITS1 clamp' and 'ITSintrev2' (LaJeunesse et al. 2008) with a touchdown PCR following LaJeunesse et al. (2003). Repeat PCR reactions introduce errors (Tindall and Kunkel 1988), but were necessary given the low density of symbionts in each settler. Products were run on denaturing gradient gels (50-90% internal gradient) following Sampayo (2009) to generate distinct ITS1-DGGE fingerprints. Prominent bands were excised, reamplified, and directly sequenced (LaJeunesse 2002). Not all bands in all fingerprints were sequenced—most were identified by comparison to previously sequenced

fingerprints from other settlers in the same aquarium. Traditional DGGE-PCR detects numerically dominant or codominant *Symbiodinium*; it does not detect background strains representing <5-10% of the population (Thornhill et al. 2007). This is a conservative estimate of the cutoff given that the double PCR methodology may have been more sensitive to low-abundance strains. Regardless, only clearly dominant bands were chosen for sequencing. Chromatograms were checked and sequences aligned using CodonCode Aligner software (CodonCode, Dedham, MD).

Statistical analyses

Maximum parsimony phylogenetic analyses on aligned data sets were performed using Paup* 4.0b10 (Swofford 2002) combined with the PaupUp graphical interface (Calendini and Martin 2005). Under maximum parsimony, sequence gaps were designated as a fifth character state. A two-way Type III ANOVA ($p = 0.05$) was used to examine the effects of factors '*Symbiodinium* Clade' (levels: A, A/C, A/D) and 'Aquarium' (levels: Baltimore, Chicago, Dallas, Omaha) on mean settler diameter at 1 month. Unweighted marginal means were used to account for unequal sample sizes. Size data were normalized by reciprocal transformation; results are reported in original units. IBM SPSS Statistics v.22 (IBM, Inc., Armonk, NY) was used for all statistical analyses.

Results

Of the 183 total settlers analyzed, 151 were successfully typed for *Symbiodinium* composition via DGGE fingerprinting. Most samples that failed to amplify were taken at the 1 month time point, when symbiont DNA quantities were at their lowest. Amplification failures appeared randomly distributed across aquaria. Phylogenetic analysis of representative ITS1 dominant band sequences (n=78) for settlers at 1 or 4 months revealed 14 total sequence variants

across all aquaria (clade A: 3; B: 1; C: 9; D: 1). Nearly all clade A sequences (31 of 33) shared 100% identity with type A3 (GenBank Accession AF333507). The other two variants differed by 1-2 base pairs; these may represent sequencing errors, contamination from intragenomic variants (Thornhill et al. 2007), or truly distinct A types. Caribbean A3 (*Symbiodinium 'fitti'*) and Pacific A3 are likely different species based on hierarchical molecular markers (LaJeunesse et al. unpublished data), but are not distinguishable at ITS1. All clade B sequences matched type B1 (AF333511); this type includes further diversity not captured at this marker (Finney et al. 2010). The members of clade D were also monotypic (AF334660); at the ITS1 marker, types D1 and D1a are indistinguishable. Members of clade C accounted for 9 sequence variants. Thus, for the *A. palmata* settlers used in this study, *Symbiodinium* clades A, B, and D were represented by one or few species each, whereas clade C diversity was greater.

Symbiont communities were genetically diverse at clade-level resolution after 1 month (n = 84 settlers) and 4 months (n = 29 settlers) [Figure 2-1]. Clades C and D were present in the settlers of 6 of 6 aquaria; clade A was present in 5 of 6 aquaria; clade B was present in 2 of 6 aquaria (Figure 2-1). Where present, clade A was found alone or codominant with another clade in the majority of settlers at a given aquarium (at 1 month: Baltimore, 100%; Chicago, 89%; Dallas, 86%; Omaha, 100%; at 4 months: Omaha, 91%; Smithsonian, 100%). After 30 months (n=22 settlers), symbiont community diversity declined, as did the total number of corals due to high post-settlement mortality. Only clades A and D were represented in the few remaining Chicago settlers (n = 5) and Omaha settlers (n = 13) at the end of the experiment.

Our attempt to 'pre-expose' half of the settlers to the parental A3 strain did not appear to influence symbiont composition (Figure 2-2). However, it is not clear that the larvae actually acquired the symbionts at that stage. Ultimately, membership to a particular exposure treatment was known for 88 settlers with typed symbionts. For all hosts where only clade A was detected, roughly 50% were 'pre-exposed' and 50% were 'unexposed,' indicating that pre-exposure did not

improve the likelihood of acquiring clade A, nor did a lack of exposure prevent the acquisition of clade A. The ratios were similar for other clade combinations. We attempted to genotype the A3 strains using ten microsatellite markers (Pinzon et al. 2011), but were hindered by the small amount of DNA available in 1-4 month old settlers. Nevertheless, we identified the parental A3 strain in 2/11 corals successfully typed at 30 months post-settlement from Omaha (data not shown).

A two-way ANOVA on the filtered data set (n=53 settlers, see Discussion) showed a strong aquarium influence on settler diameter after 1 month ($F_{3,44} = 10.620$, $p < 0.001$), but no symbiont clade influence ($F_{2,44} = 0.277$, $p = 0.759$) [Figures 2-3, 2-4]; there was no interaction effect ($F_{3,44} = 0.197$, $p = 0.898$). Omaha settlers grew twice as large on average as juveniles in other aquaria (Tukey's HSD, $p < 0.05$; Figure 2-3). Though they were excluded from analysis due to low sample size (n = 2 each), settlers associating with codominant clades A/C/D or clade D alone were roughly equivalent in size to the averages for clades A, A/C, and A/D (Figure 2-4).

Discussion

This experiment faced several setbacks, especially mortality, resulting in a small data set with low power. Ultimately, it was unclear if our attempt to manipulate coral-symbiont associations worked. However, a sufficient number of settlers with diverse symbionts survived at each aquarium to measure simultaneously the contribution of symbiont association and aquarium setting to coral growth rate during early ontogeny. Despite small sample sizes, the trends show that local environment is more influential to host growth than symbiont composition at 1 month. This key result should guide aquarists weighing methodologies to enhance larval survival during *ex situ* rearing.

As has been reported previously for Pacific acroporids, this study found that Caribbean *Acropora palmata* larvae are initially capable of taking up a variety of *Symbiodinium* from diverse clades, despite the specificity of adult *A. palmata* colonies for type A3. Most settlers harbored clade A in most aquaria (132 of 151, or 87%). However, no clade A types were found in settlers at Columbus, where larger fragments of Caribbean *A. palmata* were present in the display tank. Thus, A3 was presumably available, but either not readily taken up or stochastically undetected. Members of clade B were found in 1 settler at Dallas and 2 settlers at Columbus. A likely source of clade B (and its member species *S. minutum*, in particular) is the aquarium pest *Aiptasia* (Thornhill et al. 2013). All aquaria reported the presence of these anemones in the display tanks, and *S. minutum* is known to establish opportunistic, albeit temporary, symbioses (LaJeunesse et al. 2010). Members of clades C and D were detected in multiple settlers from every aquarium; all aquaria contained other corals known to associate with clades C and D that could have served as symbiont sources.

Based on these observations, settlers at all aquaria were exposed to members of 4 major *Symbiodinium* clades after being transferred to display tanks. They were capable of initially acquiring both homologous and heterologous symbionts, either monotypically or in combination. By the end of the experiment, only representatives from 2 clades (A and D) remained stable symbionts in the 2 aquaria where settlers survived long enough for resampling (Figure 2-1). This is not a surprising progression: though most adults associate with clade A, some colonies in deeper or more turbid waters associate with clade D, but clade B and C associations are rare (Thornhill et al. 2006; Baums et al. 2010). It is also possible that some diversity went undetected due to the low number of settlers available at the last time point. However, the sample size at Omaha at 30 months was actually larger than at 1 month, so the power to detect other clades in this aquarium was technically greater at the final time point. Despite this, clade C was no longer observed. Since individuals had to be sacrificed entirely at 1 and 4 months to type symbionts,

settlers could not be sampled repeatedly over time. This made it impossible to determine whether the community shift was the result of an ontogenetic change in *Symbiodinium* associations or differential mortality between corals hosting members of different clades. This is a common confounding issue in temporal studies of larval symbiont composition (but see Poland et al. 2013).

'Pre-exposure' of settlers to the homologous parental strain appeared to have no effect on symbiont composition (Figure 2-2). This contrasts with other studies, where similar pre-exposure limited (but did not prevent) further acquisitions (Poland et al. 2013; Mellas et al. 2014). One possible explanation is that our exposure attempt failed, and all A3 were acquired after transfer to the display tanks containing other corals. This scenario seems unlikely; at least 2 settlers at Omaha acquired the parental strain. However, other adult *A. palmata* colonies from Puerto Rico were present in this aquarium's display tank; we cannot rule out the possibility that one of these adults shared a clonal strain with the parental colony and acted as a secondary source. Ideally, we would have confirmed successful inoculation after 'pre-exposure' and before transfer, but several aquaria did not have compound microscopes readily available at the time of the experiment. The difficulties presented by low symbiont DNA concentrations in young settlers prevented testing our original hypothesis that a parental strain might confer growth advantages relative to a non-parental A3 or a heterologous clade. However, we could still test for growth rate variation among different clade combinations.

Many settlers were damaged in transport back to Penn State, preventing accurate diameter measurements but allowing for symbiont typing. For two-way ANOVA, the data set was restricted to settlers for which both clade and size were recorded. Analyzing the restricted data set at 1 month (n=53), aquarium setting appeared to be far more predictive of settler size than symbiont clade composition. Aquarium conditions that may have affected the growth of juvenile settlers include but are not limited to water flow (Schutter et al. 2010), lighting (Kinzie et al.

1984), bicarbonate concentrations (Herfort et al. 2008), crustose coralline algal substrate (Ritson-Williams et al. 2010), microbial communities (Vermeij et al. 2009), and food quality and availability (Petersen et al. 2008). Settlers at Omaha grew twice as large on average compared to those at other aquaria, suggesting that differences in environmental conditions resulted in drastically different growth rates (Figure 2-3). The rapid growth of Omaha settlers is not uniquely attributable to the presence of A3 derived from adult Caribbean *A. palmata* in the display tank because similar fragments and symbionts were also present at Chicago, where growth was much slower. At Omaha, ~60% of *A. palmata* settlers had budded secondary polyps after 1 month. For the coral *Siderastrea radians*, this greatly increases the probability of settler survival (Vermeij and Sandin 2008). At all other aquaria, the proportion of secondary budding was ~25% or lower.

Symbiont composition did not affect growth rate, at least for the clade combinations A, A/C, and A/D (Figure 2-4). Unfortunately, Columbus settlers were too damaged for size measurement, excluding the only settlers where clades B or C were detected in isolation rather than in combination with another clade. There were only two representatives each of clade combination A/C/D and clade D alone; these were removed from consideration given the low sample size. As a result, all analyzed groups included clade A, and it is difficult to say with statistical rigor that the lack of a strong clade effect is not attributable to this common denominator (but note that the two settlers containing D only have similar average diameters to all other symbiont combinations; Figure 2-4). Regardless, the significant aquarium effect despite the presence of clade A in most settlers at most aquaria suggests that controlled inoculation with a specific algal strain will be less effective at reducing size-dependent mortality than maintaining optimal environmental conditions. This is the main implication for restoration workers. Additionally, because *A. palmata* larvae are symbiotically promiscuous, and because many *ex situ* restoration programs are located in facilities where corals from around the world are on display,

care should be taken to prevent contamination from sources of *Symbiodinium* that would not be encountered naturally. Restoration settlers may inadvertently act as vessels for the introduction of foreign *Symbiodinium* species when outplanted to the reef.

Though no symbiont clade combination in this study appeared to help or harm early host growth relative to any other combination, associating with particular types of *Symbiodinium* likely confers advantages to *A. palmata* in the wild on longer time scales than addressed here, as inferred from the specificity of adult *A. palmata* for A3. The time frame for the maturation of the symbiosis is not known but present results indicate that it takes longer than 30 months in aquarium-reared *A. palmata*. The molecular mechanisms underlying the disparity between juvenile and adult symbiotic associations merit further study. Future restoration work should focus on determining which environmental factors are most critical in reducing early settler mortality.

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Figures

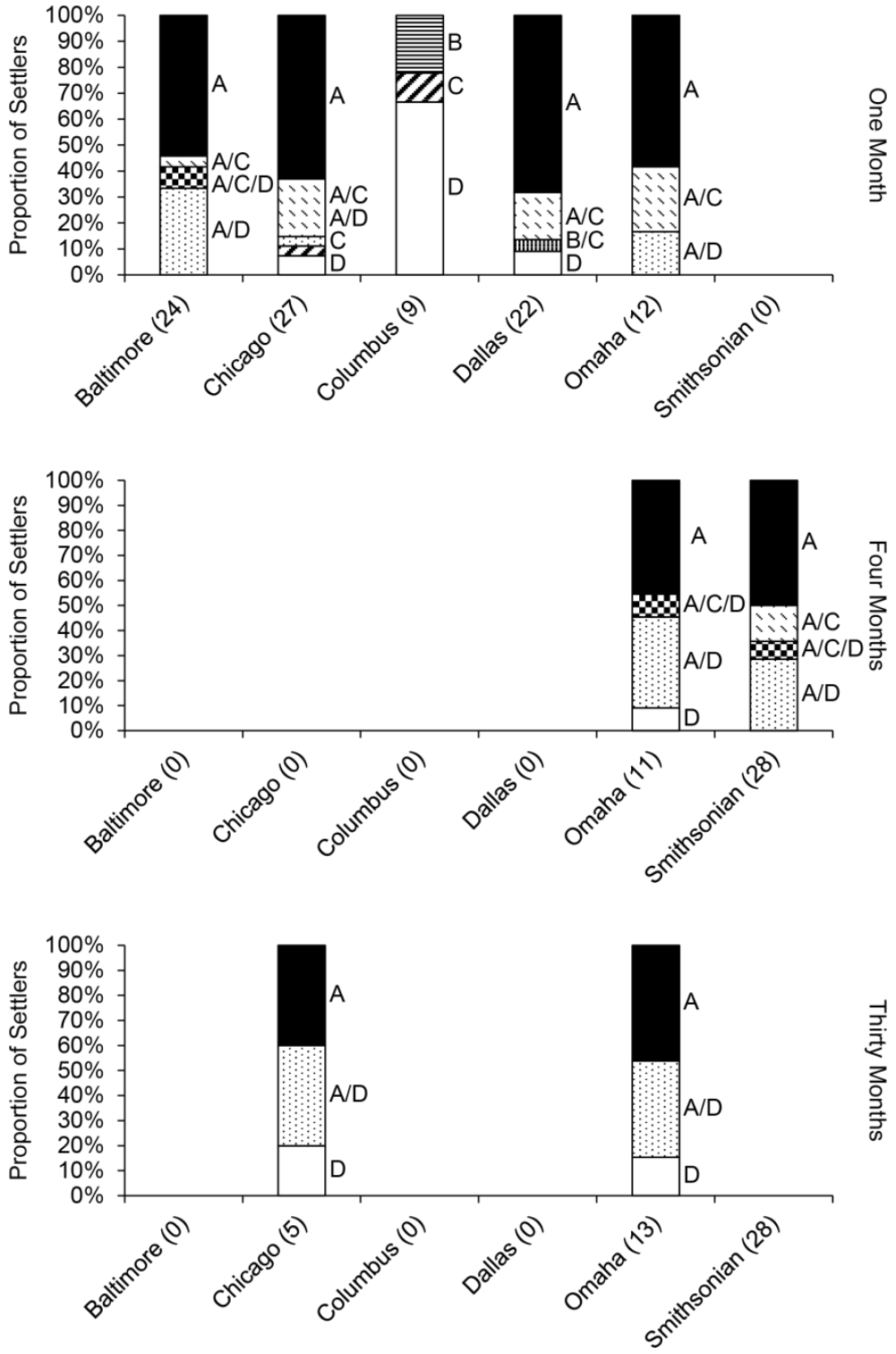
Proportion of Settlers
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
One Month
A
A/C
A/C/D
A/D
A
A/C
A/D
C
D
B
C
D
A
A/C
B/C
D
A
A/C
A/D
Baltimore (24)
Chicago (27)
Columbus (9)
Dallas (22)
Omaha (12)
Smithsonian (0)
Four Months
A
A/C/D
A/D
D
A
A/C
A/C/D
A/D
Baltimore (0)
Chicago (0)
Columbus (0)
Dallas (0)
Omaha (11)
Smithsonian (28)
Thirty Months
A
A/D
D
A
A/D
D
Baltimore (0)
Chicago (5)
Columbus (0)
Dallas (0)
Omaha (13)
Smithsonian (28)

Figure 2-1 Symbiont clade diversity in *A. palmata* settlers at public aquaria. The proportion of settlers harboring each clade or mixture of clades (A-D) are reported at 1, 4, and 30 months. The total number of samples from each aquarium is given in parentheses. Chicago's Shedd Aquarium was not sampled at 4 months, though settlers persisted. Elsewhere, settlers expired after 1 month, except at Smithsonian, which was only sampled at 4 months.

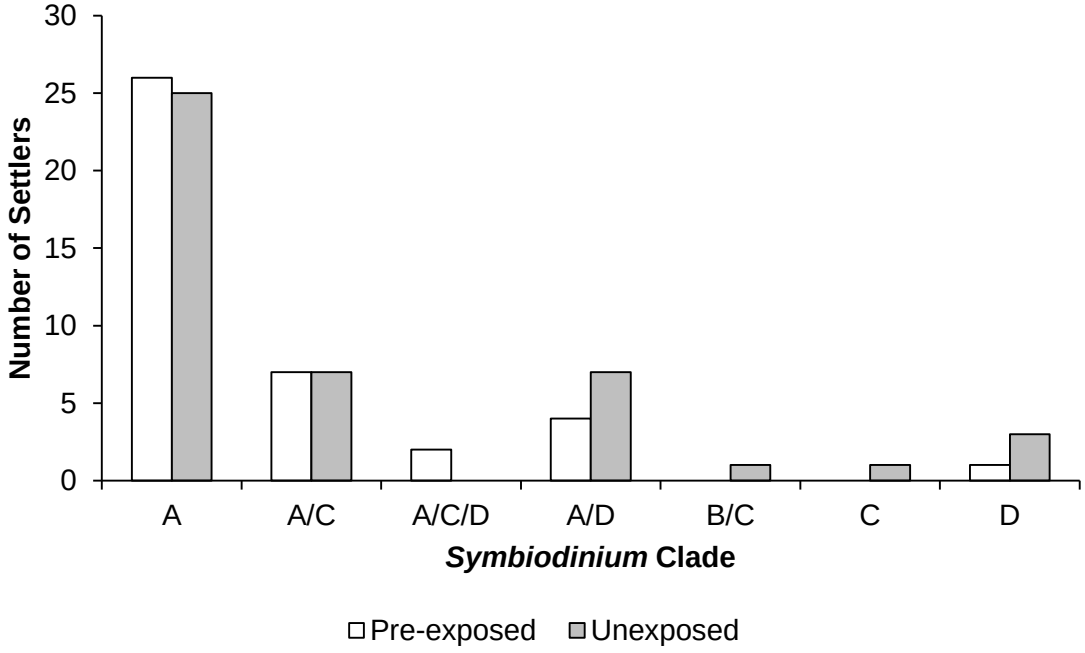

Figure 2-2 Distribution of all settlers belonging to each detected clade combination based on exposure status. "Pre-exposed" settlers were subjected to an attempt at inoculation with the parental *Symbiodinium* A3 strain prior to transfer into aquarium display tanks with other symbiont sources. "Unexposed" settlers were not (see text for details).

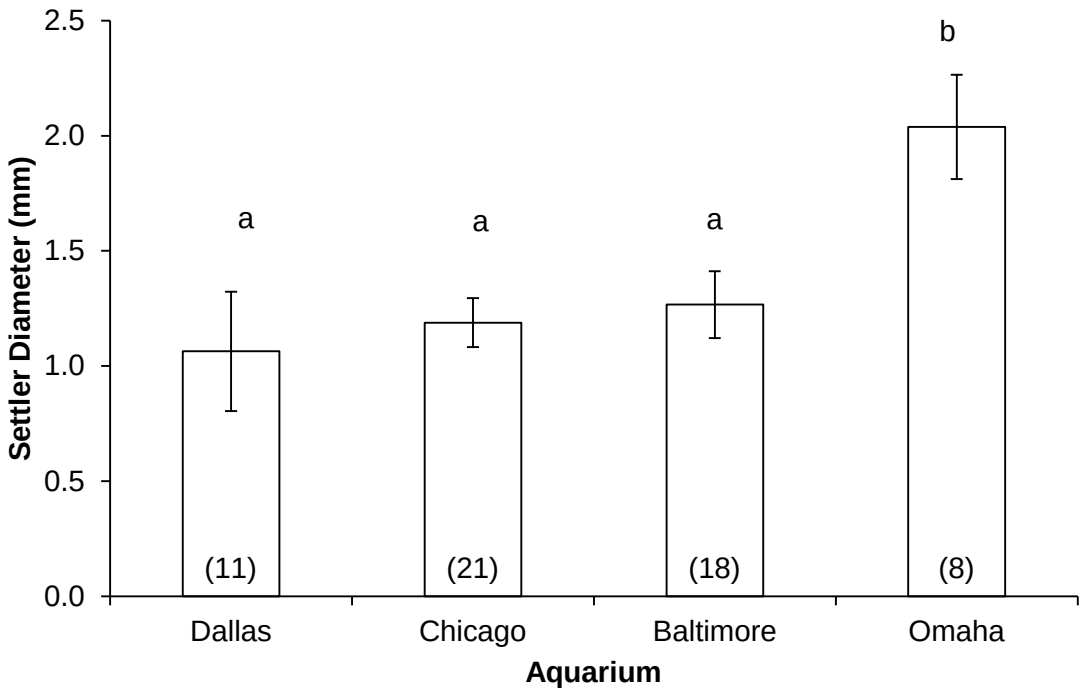

Figure 2-3 Comparison of mean settler length (± 95% CI) across aquaria after one month. Distinct letters represent statistically different means (Tukey's HSD, $p < 0.05$). Statistical analysis is based on log-transformed data. The total number of samples from each aquarium is given in parentheses. Columbus and Smithsonian settlers are excluded since they were not measured at this time point.

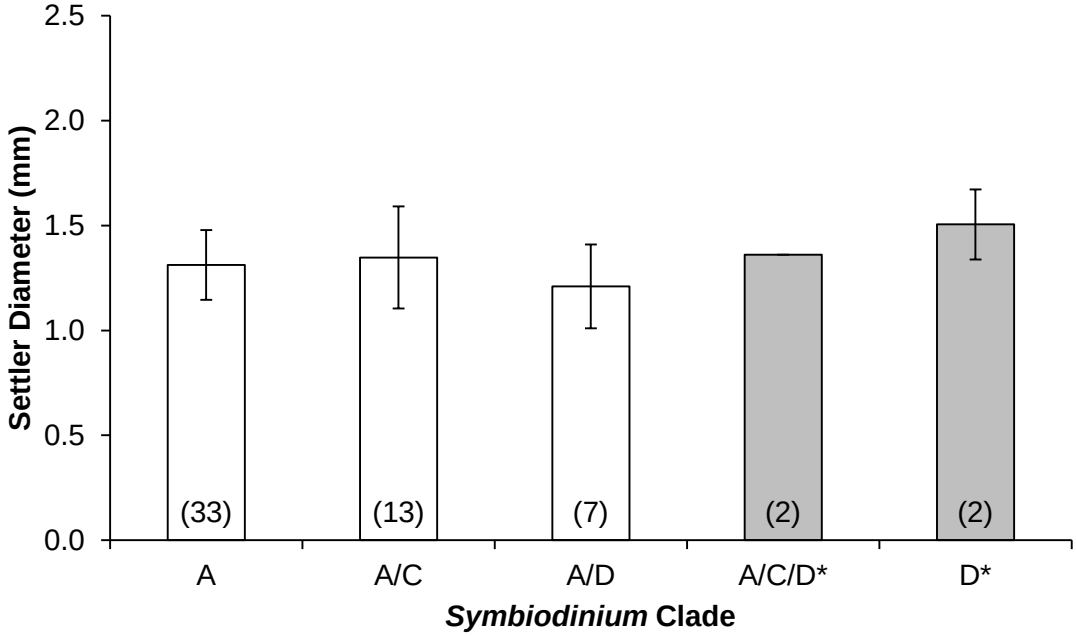

Figure 2-4 Comparison of mean settler length (± 95% CI) across *Symbiodinium* clades after one month. There were no significant differences at $p < 0.05$ based on reciprocal-transformed data. Total number of samples associating with each clade or combination is given in parentheses. Due to low replication (n=2 each) of larvae associating with the combination A/B/C or D alone, these measurements were not included in statistical analysis. Accurate size measurements for settlers associating with B alone (n=2), C alone (n=2), and B/C in combination (n=1) were not attainable due to damage during transport.

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Chapter 3

THE MOLECULAR, ECOLOGICAL, AND TAXONOMIC DIVERSITY OF *SYMBIODINIUM* (DINOPHYCEAE) CLADE B IN THE NORTHWESTERN ATLANTIC OCEAN, INCLUDING DESCRIPTIONS OF *S. AENIGMATUM* SP. NOV., *S. ANTILLOGORGIA* SP. NOV., *S. BRIAREUM* SP. NOV., *S. MADRACIS* SP. NOV., AND *S. PSEUDOMINUTUM* SP. NOV.

Abstract

To expedite the formal recognition of genetic diversity within the dinoflagellate genus *Symbiodinium*, we recently adopted a hierarchical molecular approach to delineate two species within Clade B: *S. minutum* and *S. psygmophilum.* These genetics-based designations were supported by ecological, physiological, and some morphological data. Here, we use the same framework to describe five additional Clade B species: *S. aenigmatum* sp. nov., *S. antillogorgia* sp. nov., *S. briareum* sp. nov., *S. madracis* sp. nov., and *S. pseudominutum* sp. nov from the northwestern tropical Atlantic Ocean. In each case, cohesive molecular data delineate these species lineages as evolutionarily distinct. 'Host-stable' species are ecologically dominant but are not receptive to the culturing process, whereas 'enigmatic' species culture easily but lack ecological context. Our data highlight both the spectrum of *Symbiodinium* lifestyles within a single Clade and the broad applicability of hierarchical molecular classification. This work sets the stage for further study into the evolution of distinct dinoflagellate symbiosis strategies among closely-related lineages.

Introduction

The genus *Symbiodinium* encompasses a diverse array of unicellular dinoflagellates, many of which participate in mutualisms with cnidarian host taxa including scleractinian corals (for reviews, see Trench 1993; Baker 2003; Coffroth and Santos 2005). Based mostly on ribosomal DNA markers, the group is currently divided into nine major clades (A-I; Rowan and Powers 1991; Coffroth and Santos 2005; Pochon and Gates 2010) and a multitude of subcladal types (LaJeunesse 2001; 2002). In some cases, formal species have been described (Freudenthal 1962; Trench and Blank 1987; Blank and Huss 1989; Trench and Thinh 1995; Hansen and Daugbjerg 2009; LaJeunesse et al. 2012; Jeong et al. 2014; LaJeunesse et al. 2014). Because different types possess different physiologies and stress tolerances (LaJeunesse 2001; Robison and Warner 2006; Frade et al. 2008; Thornhill et al. 2008; Hennige et al. 2009; Brading et al. 2011), *Symbiodinium* diversity has been implicated in the variable response of coral holobionts to stressors associated with climate change (Baker et al. 2004; Berkelmans and van Oppen 2006; Sampayo et al. 2008; LaJeunesse et al. 2009; LaJeunesse et al. 2010a). Despite their importance, taxonomic description of *Symbiodinium* species has lagged far behind recognition of molecular diversity within the system, leading to confusion in the field.

Given the morphological similarity of evolutionarily divergent lineages in the group, recent assessments of *Symbiodinium* diversity have relied almost entirely on molecular markers (Sampayo et al. 2009), particularly the internal transcribed spacer (ITS) of ribosomal DNA. However, because the gene is multi-copy, distinguishing intra- and intergenomic variants presents a challenge (Thornhill et al. 2007), particularly when concerted evolution maintains ancestral sequences in the population. There is a lack of consensus among researchers regarding the interpretation of ITS molecular diversity as it relates to taxonomy (Apprill and Gates 2007; Thornhill et al. 2007; Correa and Baker 2009; Stat et al. 2011). This has led to calls for

standardizing the formal description of *Symbiodinium* species by using genetic evidence from multiple genomic regions and complementary phenetic data (LaJeunesse et al. 2012; Stat et al. 2012). Microsatellite markers have recently been used to show that sexual recombination occurs within but not between sympatric *Symbiodinium* populations with distinct ecological preferences (LaJeunesse et al. 2010b; LaJeunesse et al. 2014; Thornhill et al. 2014), reflecting reproductive isolation and serving as a powerful basis for species delineation (LaJeunesse et al. 2014). However, developing such markers can be resource-intensive, and their utility is usually restricted to a few closely-related lineages.

In the absence of population genetic markers, the combined analysis of more conservative phylogenetic markers spanning nuclear, mitochondrial, and plastid genomes can effectively distinguish evolutionarily divergent lineages (Sampayo et al. 2009). We recently used this hierarchical molecular approach to describe two species of Clade B *Symbiodinium*: *S. minutum* and *S. psygmophilum* (LaJeunesse et al. 2012). These taxa lent themselves to formal description for several reasons. First, they were known to function as the numerically dominant symbionts in certain hosts, so their ecology and biogeography had been well studied. Second, they were readily maintained in culture, so their physiology had been tested independently of the host. Third, their cell sizes were very distinct; this is one of the only obvious morphological signals viewed under a light microscope that two *Symbiodinium* likely belong to different species. Fourth, and least subjectively, the two species exhibited clear differentiation and reciprocal monophyly across several genomic loci, indicating a lack of sexual recombination consistent with the Biological Species Concept. However, it is rare for all four lines of concordant evidence to be available for a given *Symbiodinium* lineage. Here, we argue that strong genetic signatures along with just one additional criterion should be sufficient to draw *Symbiodinium* species boundaries.

Traditionally, only species that could be cultured were formally described. However, culturing is a highly selective process (e.g. von Wintzingerode et al. 1997), particularly for

symbiotic dinoflagellates (Rowan et al. 1996; Rowan 1998; Carlos et al. 1999; Santos et al. 2001). Efforts to culture the numerically dominant *Symbiodinium* have often failed (Santos et al. 2001; LaJeunesse 2002; Goulet and Coffroth 2003; Krueger and Gates 2012). Efforts to maintain evolutionarily derived, 'host-stable' lineages in long-term artificial culture have failed (LaJeunesse et al. 2005; Krueger and Gates 2012), yet these are some of the most abundant and ecologically important *Symbiodinium*. At the same time, those *Symbiodinium* that *are* favored by culturing—often low abundance background contaminants distinct from the dominant mutualist *in hospite* (Santos et al. 2001; LaJeunesse 2002)—are important to describe. These 'enigmatic' lineages are potentially free-living or commensal, occupying different niches than classically endosymbiotic species (Jeong et al. 2014). Without any predictable associations with particular cnidarian hosts, their ecology remains difficult to study.

Continuing with the hierarchical molecular classification scheme outlined by LaJeunesse et al. (2012), we use a combination of phylogenetic markers to describe several new species within *Symbiodinium* Clade B: two host-stable species associated with the cnidarian genera *Briareum* (an octocoral) and *Madracis* (a stony coral) that are apparently unculturable with current techniques and available culture media; two enigmatic, potentially free-living species that thrive in culture, and one additional species that bridges the gap, showing a degree of host-specialization and culturability (*S. antillogorgia*). We place these new species in a larger phylogenetic context by comparing them with Clade B diversity surveyed throughout the northwestern tropical Atlantic Ocean, and highlight the extent of ecological variation found within just one *Symbiodinium* clade.

Materials and Methods

Specimen collection

Tissues from the host cnidaria *Antillogorgia bipinnata, A. elisabethae, Briareum asbestinum,* and *Madracis* sp. were collected from the Florida Keys (USA), San Salvador (Bahamas), Barbados, Belize, Curaçao, and Puerto Rico to target symbionts with host-stable associations. Some of the samples had been used in an earlier study (Finney et al. 2010) and were previously screened for the presence of Clade B *Symbiodinium* using denaturing gradient gel electrophoresis (DGGE) of the ITS region as in Sampayo et al. (2009). Whole tissues were preserved in high-salt, 20% DMSO buffer (Seutin et al. 1991) and stored at -20°C.

Cultures and cell size analyses

Monoclonal cultures of Clade B *Symbiodinium* isolated from Western Atlantic cnidarian hosts were acquired from the Robert K. Trench collection and the Buffalo Undersea Reef Research Culture Center collection. Cultures were maintained in liquid media (ASP8-A; Ahles, 1967) and kept in incubators delivering 80–120 µmol quanta • m^{-2} • s^{-1} photosynthetically active radiation (PAR) on a 14:10 (light: dark) photoperiod under Philips fluorescent tubes (Koninklijke Philips Electrons, Amsterdam, the Netherlands).

Cultures were photographed during log phase growth under bright-field illumination at a magnification of 400–1000× using an Olympus BX61 compound microscope (Olympus Corp., Tokyo, Japan) with a Jenoptik ProgRes CF Scan digital camera (Jenoptik, Jena, Germany). Uncultured tissue homogenates were photographed under identical conditions. Cell length and width for at least 40 individuals per culture or tissue sample were calculated with the program ImageJ (Abramoff et al. 2004), and used to calculate ellipsoid volume as $(4/3)\pi \times abc$, where a, b, and c are equal to half the length, width, and height , respectively (cell height was assumed to be identical to cell width). Cell volumes were normalized via natural logarithm transformation. Size

differences between putative species were assessed with ANOVA. Each species was represented by 4 strains (either unique cultures or unique host colonies). Previously published measurements from *S. minutum* and *S. psygmophilum* (LaJeunesse et al. 2012) were included for comparison.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses

Whole tissue DNA extractions were performed as described by LaJeunesse (2001); culture DNA extractions were performed as described by Coffroth et al. (1992). In addition to the nuclear, mitochondrial, and plastid DNA sequences used for species delineation in LaJeunesse et al. (2012), we also sequenced the nuclear eukaryotic 60S large ribosomal subunit (*LSU*); the primers and thermal cycler conditions for all markers are listed in Table 3-S1.

To amplify DNA, reactions were performed in 25 µL volumes containing 2.5 µL of 2.5 mM dNTPs, 2.5 µL of 25 mM MgCl2, 2.5 µL standard Taq Buffer (New England Biolabs, Ipswich, MA, USA), 0.13 µL of 5 U • μL^{-1} Taq DNA Polymerase (New England Biolabs), 1 µL of each forward and reverse primer at 10 µM, and 1 µL of 5–50 ng DNA template. Products were cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA, USA) at the Pennsylvania State University Genomics Core Facility. Chromatograms were checked and sequences aligned using CodonCode Aligner software (CodonCode, Dedham, MD, USA) and MEGA v. 6 (Tamura et al. 2013).

Previously published sequence information for cultures of *S. minutum* and *S. psygmophilum* (LaJeunesse et al. 2012) were included in the primary alignment for reference. A complete list of sample identities, host origins, and collection locations for this primary data set are provided in Table 3-S2. Sequences aligments for each gene can be found in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g52r). To place the new species in a larger phylogenetic context, Sym15 flanker sequences from 160 additional samples from a

previous study (Finney et al. 2010) were added to the data set for a subsequent alignment incorporating much of the Clade B diversity found in the northwestern tropical Atlantic Ocean.

Phylogenetic analyses were performed on aligned data sets in PAUP* 4.0b10 (Swofford, 2000) under maximum parsimony with indels included as a 5th character state. Bootstrap support was calculated with 1000 replicates. Bayesian posterior probabilities were calculated with the software Mr. Bayes (Huelsenbeck and Ronquist 2001), using the optimal nucleotide substitution model for each gene (or a partitioned model for the concatenated sequence) based on corrected Akaike Information Criterion as calculated with ModelTest 3.7 (Posada and Crandall 1998).

To resolve phylogenetic diversity within lineages associated with *Briareum*, we applied the Automatic Barcode Gap Discovery (ABGD) algorithm (Puillandre et al. 2012) to all species at *ITS1/5.8S/ITS2*, Sym15 flanker, and *cp23S* separately, using the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) and default parameters. This method sorts samples into putative species based on the automatically detected and recursively tested gap between inter- and intraspecific genetic distance, making it a somewhat objective method for establishing species hypotheses.

Results

Taxonomic assignment of species

Complementary genetic evidence from multiple genetic markers with different evolutionary rates spanning mitochondrial (*cob*: 915bp), nuclear (partial *ITS1/5.8S/ITS2*: 253bp; and *LSU*: 597bp), and plastid genomes (*cp23S*: 600bp), along with the single copy microsatellite flanker (Sym15: 223bp) supported the designation of five new *Symbiodinium* species in Clade B. We concatenated all sequences (2588bp from 5 markers), producing a phylogeny with monophyletic lineages and high bootstrap and Bayesian support (Figure 3-1). Well-supported

monophyly indicated a lack of genetic recombination between lineages, validating that the new groups adhered to the precepts of the Biological Species Concept. The Ecological Species Concept was also supported by the specific host associations among host-stable *Symbiodinium* and the rarity with which enigmatic *Symbiodinium* known only from culture were detected in the wild.

The newly described *S. briareum* showed strong division among sublineages (indicated by Greek letters in Figure 3-1), but there was insufficient evidence to delimit each branch as cohesive species at this stage; the group is instead presented as a species complex awaiting further resolution with population genetic data. For most new species, comparative physiological data were unavailable, but a recent study revealed that the formerly described *S. minutum* and newly described *S. pseudominutum* exhibit distinct photosynthetic carbon assimilation responses under thermal stress (Oakley et al. 2014). Though some new species had obvious cell size differences, others were nearly identical despite membership in opposite radiations within Clade B (Figure 3-2). Based on these genetic and, where available, ecological, physiological, and morphological data, we assigned formal binomials to *S. aenigmatum, S. antillogorgia, S. briareum, S. madracis,* and *S. pseudominutum*.

***Symbiodinium aenigmatum*, sp. nov.**

Diagnosis. Coccoid cells range in average size from 6.7 to 8.4 μm at maximum diameter during log phase growth (Figure 3-2). The combined nucleotide sequences of *cp23S*, Sym15 microsatellite flanker, partial nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob* define this species. Complete sequence alignments can be found in the Penn State ScholarSphere database (URL).

Type locality. Cultured from tissue of the scleractinian mustard hill coral, *Porites astreoides*, Florida Keys, USA, though it is not one of the dominant symbionts in that host.

Appears closely related to ITS2 type B23 found in hydrozoan fire corals of the genus *Millepora*, but differs by two insertions and five substitutions at this marker.

Etymology. The Latin 'aenigma' (mystery) refers to the obscure ecology of this species, which thrives in culture but only been detected in the wild once, likely as a background or contaminant symbiont.

Other notes. The authentic strain was originally isolated in 2004 by Mary Alice Coffroth; it is also known as culture mac04-180 from the Buffalo Undersea Reef Research Culture Center collection.

***Symbiodinium antillogorgia*, sp. nov.**

Diagnosis. Coccoid cells range in average size from 7.1 to 8.1 µm at maximum diameter during log phase growth (Figure 3-2). The combined nucleotide sequences of *cp23S*, Sym15 microsatellite flanker, partial nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob* define this species. Complete sequence alignments can be found in the Penn State ScholarSphere database (URL).

Type locality. Cultured from the gorgonian soft coral *Antillogorgia elisabethae*, Florida Keys, USA.

Etymology. 'Antillogorgia' refers to the genus name of the host, *Antillogorgia*, for which this symbiont appears to have a specialized association.

Other notes. The authentic strain was originally isolated in 2007 by Mary Alice Coffroth; it is also known as culture mac08-0689 from the Buffalo Undersea Reef Research Culture Center collection. The ITS2 type of this species is B1 (LaJeunesse 2001), which matches *S. minutum* and other ecologically distinct B1 lineages that possess identical *cob* and partial *ITS1/5.8S/ITS2* sequences but are distinguished at more rapidly evolving markers. This species is also detected in host *A. bipinnata*, but not outside the genus *Antillogorgia*.

***Symbiodinium briareum*, sp. nov.**

Diagnosis. Coccoid cells have an average size of 10.7 µm at maximum diameter *in hospite* (Figure 3-2). The combined nucleotide sequences of *cp23S*, Sym15 microsatellite flanker, partial nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob* define this species. Complete sequence alignments can be found in the Penn State ScholarSphere database (URL).

Type locality. Collected from the octocoral *Briareum asbestinum*, Florida Keys, USA.

Etymology. 'Briareum' refers to the genus name of the host *Briareum asbestinum*, for which this symbiont appears to have a specialized association.

Other notes. This species has yet to be cultured successfully for more than a few months. So far, it has only been detected in *Briareum asbestinum*, though this host associates with at least three other closely-related symbiont types. Together, these lineages make up the *S. briareum* complex, which may be resolved further with finer-scale markers in the future.

***Symbiodinium madracis*, sp. nov.**

Diagnosis. Coccoid cells range in average size from 6.1 to 7.2 µm at maximum diameter *in hospite* (Figure 3-2). The combined nucleotide sequences of *cp23S*, Sym15 microsatellite flanker, partial nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob* define this species. Complete sequence alignments can be found in the Penn State ScholarSphere database (URL).

Type locality. Collected from the scleractinian hard coral *Madracis decactis*, Curaçao.

Etymology. 'Madracis' refers to the genus name of the host *Madracis*, for which this symbiont appears to have a specialized association.

Other notes. This species has yet to be cultured successfully for more than a few months. The symbiont has not been detected outside the genus *Madracis*. Though species designations within *Madracis* are disputed (Diekmann et al. 2001), this symbiont has been recovered from

morphotypes of all currently named species (Frade et al. 2008). It can be competitively displaced in certain *Madracis* hosts by ITS2 types B13 or B15 depending on light or depth conditions (Frade et al. 2008).

***Symbiodinium pseudominutum*, sp. nov.**

Diagnosis. Coccoid cells range in average size from 7.8 to 8.5 µm at maximum diameter during log phase growth (Figure 3-2). The combined nucleotide sequences of *cp23S*, Sym15 microsatellite flanker, partial nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob* define this species. Complete sequence alignments can be found in the Penn State ScholarSphere database (URL).

Type locality. Cultured from the tissue of the ivory bush coral, *Oculina diffusa*, Florida Keys, USA, though it is not one of the dominant symbionts in that host.

Etymology. The Latin 'pseudo' (false) refers to the morphological similarity but molecular and ecological disparity between this species and the closely-related *S. minutum*.

Other notes. The authentic strain was originally isolated in the mid-1970s by David A. Schoenberg; it is also known as culture rt146 from the Robert K. Trench collection. The ITS2 type of this species is B1 (LaJeunesse 2001), which matches *S. minutum* and other ecologically distinct B1 lineages that possess identical *cob* and partial *ITS1/5.8S/ITS2* sequences but are distinguished at more rapidly evolving markers. This species has also been cultured independently from the tissues of diverse hosts including *Cassiopeia xamachana*, *Aiptasia sp.*, and *Antillogorgia sp.* from the Caribbean.

Subdivision within Symbiodinium briareum

At this time we are describing *Symbiodinium briareum* as a species complex. Based on the concatenated phylogeny, the group partitioned into 5 lineages, referred to as α, β, γ, δ, and ε (Figure 3-1). Although the majority of these branches were well-supported due to divergence at *cp23S*, there was a degree of overlap among incongruent branches at *ITS1/5.8S/ITS2* and

microsatellite Sym15 flanker. Though some branches grouped by geography, others did not. We therefore applied the ABGD algorithm to these three markers separately, to see if the objective distance-based method partitioned the samples into separate species groups consistently. For comparative purposes, we included sequence data from all species in the analysis.

ABGD performed moderately well at separating the other species populations described in both LaJeunesse et al. (2012) and this study (Figure 3-3). For example, *S. aenigmatum* was separated as a unique species at all three markers. *S. psygmophilum, S. antillogorgia,* and *S. madracis* were resolved at two of three markers (though twice they were over-resolved, splitting a known species into two subgroups). *S. minutum* and *S. pseudominutum*, the two most closely related species, were only resolved unambiguously at one marker each—this break is also supported at the hypervariable noncoding region of the *psbA* chloroplast gene (data not shown). Among the *S. briareum* complex, only the α-lineage was resolved at more than one marker. At *ITS1/5.8S/ITS2*, the complex split into two groups: the α-lineage vs. all others. At Sym15 flanker, the complex again split into two groups: in this case, the β -lineage vs. all others.

Diversity in nature and in culture

Clade B is comprised of two major groups based on ancestral ITS sequences (LaJeunesse 2005): the B1 and the B19 radiations. The split between these major radiations was apparent in the new species at all markers (Figure 3-S1). *S. antillogorgia, S. madracis,* and *S. pseudominutum* belong to the B1 radiation; *S. aenigmatum* and *S. briareum* belong to the B19 radiation. Currently, 3 B1 radiation species are culturable (*S. antillogorgia, S. pseudominutum,* and *S. minutum*), while 2 B19 radiation species are cultureable (*S. aenigmatum* and *S. psygmophilum*). After surveying Clade B *Symbiodinium* from the tissues of >20 host cnidarian genera from 10 locations throughout the Caribbean, we recovered at least 13 distinct phylogenetic lineages with >90% bootstrap and/or Bayesian support at the Sym15 microsatellite flanker (Figure 3-4). Many of these lineages corresponded to breaks between ITS2 types. An important

exception was ITS2 type B1, which subdivided into at least 5 lineages at the higher-resolution Sym15 flanker (see also Finney et al. 2010).

Discussion

The recognition of ecological diversity within *Symbiodinium* is not a new concept (Schoenberg and Trench 1980a; b; c), nor is the call for taxonomic revision within the genus (Blank and Trench 1986). However, prior to the advent of molecular genetic approaches, species delineation among these morphologically nondescript dinoflagellates had been a challenge. With the threat of climate change driving additional interest in coral-*Symbiodinium* associations, there is a pressing need for investigators to share a common, robust nomenclature. Genetics-based species descriptions are appropriate for such a purpose (LaJeunesse et al. 2012; Jeong et al. 2014, LaJeunesse et al. 2014). Here, we formally classified five new, ecologically distinct species of Clade B *Symbiodinium* using a hierarchical molecular approach (Figure 3-1). Expanding on the findings of Finney et al. (2010), we placed these species in a phylogenetic context within the northwestern tropical Atlantic Ocean (Figure 3-4). We confirmed that the diversity of *Symbiodinium* in culture is a small fraction of natural diversity (Stern et al. 2010). Below, we describe the genetic, morphological, and ecological evidence supporting the description of these five species.

Genetic evidence

All five new species were well resolved in the concatenated gene tree (Figure 3-1). However, not all species were reciprocally monophyletic at all genes (Figure 3-S1). The concatenated tree represents an ‘average’ of evolutionary history among lineages and genomes, and in simulations recaptures the true species tree better than consensus trees formed by analyzing each gene separately (Gadagkar et al. 2005). Differences among gene trees can occur

for many reasons, including incomplete lineage sorting, introgressive hybridization, unrecognized multi-copy genes, inadequate sampling, and sample misidentification (Funk and Omland 2003). A lack of resolution can also result from comparing genes with different rates of evolution, such as those used in this study (LaJeunesse et al. 2012). For example, the mitochondrial *cob* gene is highly conserved, with only 11/915 variable sites among all species described here, and thus, when used alone, provides very little resolution (though it usefully supports the split between the B1 and B19 radiations). At the more rapidly evolving genes, there very few discrepancies. The only major differences involved the placement of culture rt141 outside of the main *S. psygmophilum* branch at the Sym15 flanker, the degree of diversity among what we are calling the *S. briareum* species complex at the Sym15 flanker vs. *cp23S*, and the fact that *S. minutum* and *S. pseudominutum* cultures share identical sequence at *cp23S* despite being clearly distinct at the Sym15 flanker.

The utility of microsatellite flankers in species delineation

Non-coding, independently sorting microsatellite flanker sequences sometimes offer better species-level resolution in *Symbiodinium* than slower-evolving mitochondrial, ribosomal, or chloroplast phylogenetic markers. For example, three Clade D species readily distinguished by microsatellite flankers and population genetic data nevertheless shared identical *cp23S* sequences (LaJeunesse et al. 2014). Within Clade B, *cp23S* seems to distinguish well between some species and not others, while the Sym15 microsatellite flanker provides the greatest resolution. As previously acknowledged, the ideal markers may vary from clade to clade, which is why the use of multiple markers are necessary for *Symbiodinium* species delineation (LaJeunesse et al. 2012).

The utility of ITS in species delineation

Intragenomic variation at multicopy rDNA can give the impression of more diversity than actually exists (Thornhill et al. 2007; Sampayo et al. 2009; LaJeunesse et al. 2014; Thornhill et al. 2014). For example, multiple ITS2 sequences correspond to the Clade D species *S.*

eurythalpos (LaJeunesse et al. 2014). Conversely, it is also evident that a lack of intergenomic variation at ITS2 can mask diversity (Finney et al. 2010). At least six lineages in the B1 radiation all share the same numerically dominant ancestral ITS2 sequence in their genomes (Figure 3-2). This condition appears to have been maintained by concerted evolution for millions of years in these separately evolving lineages (Dover 1982; Thornhill et al. 2014). Nevertheless, distinct ecological associations and sequence divergence at other markers clearly resolve these entities as genetically isolated (i.e. species). Care must be taken when using ITS to approximate species diversity, as it can bias estimates in both a positive and negative direction. Though less useful for resolving the B1 radiation, ITS sequences are generally unique among species in the B19 radiation.

The S. briareum *species complex*

The new species *S. briareum* is monophyletic and well-supported (Figure 3-1), but its sublineages may in fact represent unique species. The Automated Barcode Gap Discovery algorithm partitioned this diversity inconsistently (Figure 3-3). ABGD also had issues separating other, less ambiguous Clade B species at certain markers. Because the α-lineage was supported as a unique entity in two out of three markers, we chose to use it to define the holotype for *S. briareum*, with the caveat that higher resolution population genetic data may be required to establish whether the remaining lineages represent intraspecific variation, introgressed hybrids of distinct species, or reproductively isolated species still undergoing lineage sorting (Funk and Omland 2003).

Morphological evidence

We base our description of *Symbiodinium* primarily on hierarchical molecular delineation; other lines of evidence were less conclusive. Cell sizes among these species were distinct in only a minority of cases (Figure 3-2). *S. aenigmatum*'s size range overlapped with *S. antillogorgia*, *S. madracis*, *S. minutum*, and *S. pseudominutum*, even though *S. aenigmatum*

belongs to a separate radiation. A lack of size differences is clearly not evidence that two *Symbiodinium* belong to the same species. For example, *S. pilosum* and *S. goreauii* cell diameters both range from 9 to 13 μm, though they belong to highly divergent Clades A and C, respectively (Trench and Blank 1987). Moreover, Kofoidian plate counts—the previous gold standard for morphological diagnoses among armored dinoflagellates—are time-consuming and sometimes unreliable, as variation in plate numbers exist even within purportedly isoclonal lines (Jeong et al. 2014). Given the morphological uniformity of coccoid *Symbiodinium in hospite*, we find that morphological characterization is an unnecessary step that should not be a requirement for species delineation in this group.

Historical issues with culturing

Over the past several decades of culturing *Symbiodinium* from host Cnidaria, researchers have recovered the numerically-dominant endosymbionts, background symbionts (those that dominate at some hosts but persist in low abundance in others), and other truly cryptic species. This fact was difficult to recognize prior to the molecular characterization of diversity in the genus, which is why some species named for their hosts (*e.g. S. linuchea*) may not actually be representative of the host's true symbiont (Santos et al. 2001; LaJeunesse 2002). Extra care must be taken when reading literature that refers to cultures by their strain names, which often logically reflect the host from which they were isolated, whether they represent the ecologically-relevant symbiont or not. For example, the well-studied culture Mf1.05b was named after of its 'host' *Montastrea (=Orbicella) faveolata* (and is often referred to as its symbiont), but the culture is actually a strain of *S. minutum*, which associates stably with *Aiptasia* sp. (Thornhill et al. 2013) but has yet to be detected as a functionally important, dominant symbiont in *O. faveolata*.

Host-stable species

We use the term 'host-stable' to refer to mutualistic *Symbiodinium* that associate persistently with their hosts as the numerically dominant symbiont. Though likely facultative,

they give the impression of being obligate endosymbionts, rarely being cultured from or detected in the water column. Instead, they predictably associate with certain hosts, and are always recovered *in hospite*. Host-stable *Symbiodinium* are restricted to a small number of host species. Two new Clade B *Symbiodinium, S. briareum* and *S. madracis*, fall into this host-stable category, and are therefore named after their hosts: the octocoral genus *Briareum* and scleractinian genus *Madracis*, respectively. *S. antillogorgia* is also host-stable (see below).

Despite multiple culturing efforts, including the use of macerated host tissue as a 'host factor,' host-stable symbionts rarely survive for more than a few months without dying out or being replaced by contaminants (LaJeunesse et al. 2005; Krueger and Gates 2012). These symbionts appear to suffer diminished vitality when removed from the host habitat, while free-living or generalist species have a competitive advantage *in vitro*. Host specialization likely develops over millions of years of coevolution. The inability to culture these *Symbiodinium* from host tissues is likely a technical limitation rather than a biological one—we have simply failed to recreate the appropriate conditions. Importantly, the newly described *S. antillogorgia* is a host-stable species that nevertheless thrives in culture. It is named for the octocoral genus *Antillogorgia*, and has been found to be dominant in *A. elisabethae* and *A. bipinnata*. The Clade B species S. *minutum* and *S. psygmophilum* are also both culturable and ecologically dominant (LaJeunesse et al. 2012), though *S. psygmophilum* appears to associate with a broader range of hosts, whereas *S. antillogorgia* and *S. minutum* have yet to be identified as dominant in hosts outside the genus *Antillogorgia* and *Aiptasia*, respectively.

Enigmatic species

The 'contaminants' that supplant host-stable symbionts during the culturing process may be well-known (*e.g. S. pilosum*) or new to science (such as those characterized here). They might be completely free-living, commensal, or perhaps endosymbiotic but restricted in growth potential. The presence of a competitively dominant symbiont may prevent them from reaching

larger population sizes *in hospite*, and thus they have remained undetected using traditional techniques. We refer to these *Symbiodinium* as enigmatic. The new Clade B species *S. pseudominutum* exemplifies this ecology. Despite its close evolutionary history with *S. minutum*, which is commonly detected in nature, *S. pseudominutum* has only been isolated three times (from *Aiptasia*, *Antillogorgia*, and *Cassiopea*). It grows well in culture, features a lower half-saturation constant of both photosynthesis for dissolved inorganic carbon and relative electron transfer rate under thermal stress (Oakley et al. 2014), and exhibits a distinct gene expression profile when compared to *S. minutum* (Parkinson et al. unpublished data).

The other new enigmatic species, *S. aenigmatum*, is genetically distinct from other members of its Clade B radiation. It has only been cultured from the tissues of one host population: *Porites astreoides* from the Florida Keys. The coral is never dominated by *S. aenigmatum*, and yet 11 independent cultures from multiple individuals recovered this symbiont (Coffroth personal observation), indicating that it is incredibly successful *in vitro* despite its background or contaminant status in relation to *P. astreoides*. Interestingly, *S. aenigmatum*'s ITS2 sequence (B23) has also been detected in six samples of the fire coral genus *Millepora* from collections in Barbados (Finney et al. 2010). However, this ITS2 sequence B23 is distinct from *S. aenigmatum* by two insertions and five substitutions, likely indicating that they are separate species. Nevertheless, the two lineages share identical Sym15 flanker sequence, and thus appear to belong to the same group when only Sym15 is considered (Figure 3-4). Further typing of *Millepora* symbionts in the Eastern Caribbean should clarify whether *S. aenigmatum* is ever a dominant symbiont in hydrozoans, but the current evidence supports the conclusion that the species is enigmatic.

Conclusion

The proposed ecological roles of these species are provisional and may alter in light of future environmental change. Though we have yet to detect certain enigmatic species associating

with cnidarian hosts, this does not mean that they won't be identified in the future with more sampling effort or deep-sequencing techniques (Green et al. 2014). Nevertheless, given what we currently know about *Symbiodinium* biogeography, physiology, and ecology, it is clear that many ecologically distinct species exist within Clade B. The degree to which particular *Symbiodinium* may shift roles when hosts are stressed or abiotic conditions change is a topic for further investigation.

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Figures

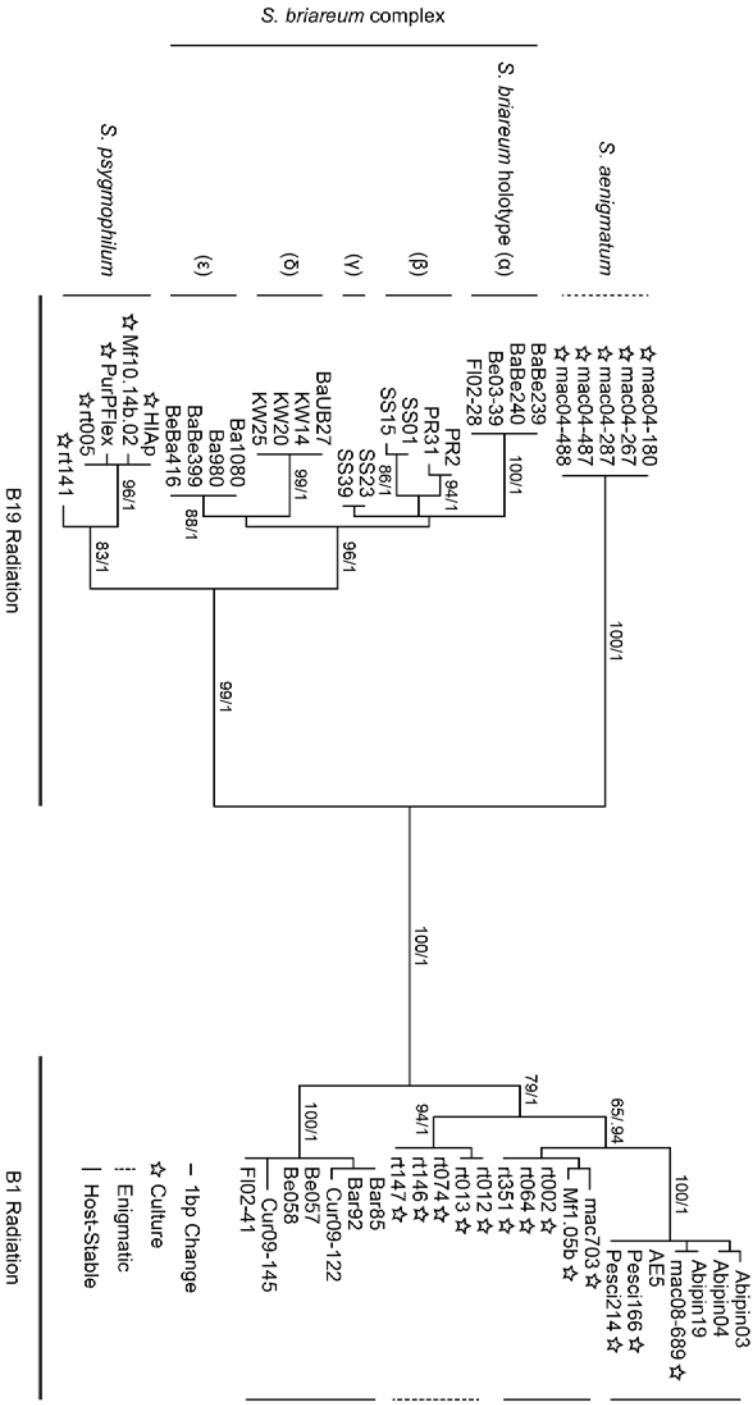
S. briareum complex
S. briareum holotype (α)
S. aenigmatum
(β)
(γ)
(δ)
(ε)
S. psygmophilum
☆ mac04-180
☆ mac04-267
☆ mac04-287
☆ mac04-487
☆ mac04-488
BaBe239
BaBe240
Be03-39
FI02-28
PR2
PR31
SS01
SS15
SS23
SS39
BaUB27
KW14
KW20
KW25
Ba1080
Ba980
BaBe399
BeBa416
☆ Mf10.14b.02
☆ HIAp
☆ PurPFlex
☆ rt005
☆ rt141
100/1
86/1
94/1
99/1
88/1
96/1
96/1
83/1
99/1
100/1
B19 Radiation
79/1
65/ 94
100/1
94/1
100/1
Abipin03
Abipin04
Abipin19
mac08-689 ☆
AE5
Pesci166 ☆
Pesci214 ☆
mac703 ☆
Mf1.05b ☆
rt002 ☆
rt064 ☆
rt351 ☆
rt012 ☆
rt013 ☆
rt074 ☆
rt146 ☆
rt147 ☆
Bar85
Bar92
Cur09-122
Be057
Be058
Cur09-145
FI02-41
S. antillogorgia
S. minutum
S. pseudominutum
S. madracis
B1 Radiation
– 1bp Change
☆ Culture
Enigmatic
Host-Stable

Figure 3-1 Maximum parsimony phylogeny of the 5 new and 2 previously described Clade B *Symbiodinium* based on the concatenated sequences of *cp23S*, Sym15 microsatellite flanker, nuclear ribosomal *ITS1/5.8S/ITS2* and *LSU*, and mitochondrial *cob*. Branch support is indicated by bootstrap values followed by Bayesian posterior probabilities. Bootstrap values <65% were omitted. The tree is split along the major axis separating the B1 and B19 radiations.

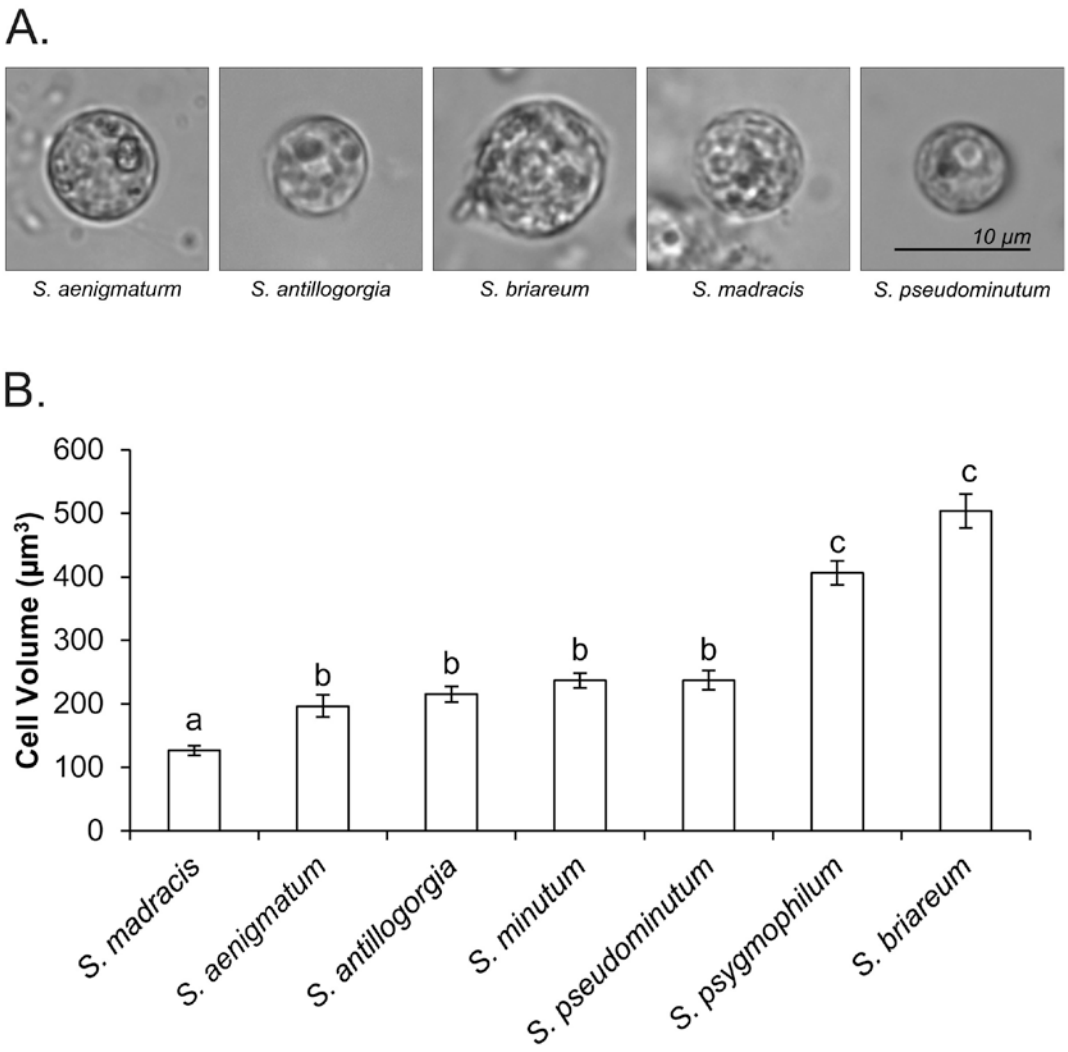

Figure 3-2 (a) Light micrographs for each of the 5 new Clade B *Symbiodinium* species. All images are equivalently scaled. Note that *S. briareum* is represented by a cell from relatively old preserved host tissue, and has therefore lost its pigmentation. **(b)** Average cell volume for each of the 5 new and 2 previously described Clade B *Symbiodinium* species. Error bars represent 95% confidence intervals. Letters indicate membership in statistically significant groupings (K-W test; post hoc $p_{adj} < 0.05$). For each species, n = 4 cultures or host colonies, each with n > 40 measurements. Note that for *S. briareum*, measurements were taken from pseudoreplicates of the same host colony due to a lack of preserved host material.

Sample	Species	*ITS2*	Sym15	*cp23S*
mac04-180	*S. aenigmatum*			
mac04-267	*S. aenigmatum*			
mac04-287	*S. aenigmatum*			
mac04-487	*S. aenigmatum*			
mac04-488	*S. aenigmatum*			
BaBe239	*S. briareum (α)*			
BaBe240	*S. briareum (α)*			
Be03-39	*S. briareum (α)*			
Fl02-28	*S. briareum (α)*			
BaUB27	*S. briareum (δ)*			
KW14	*S. briareum (δ)*			
KW20	*S. briareum (δ)*			
KW25	*S. briareum (δ)*			
Ba1080	*S. briareum (ε)*			
Ba980	*S. briareum (ε)*			
BaBe399	*S. briareum (ε)*			
BeBa416	*S. briareum (ε)*			
SS23	*S. briareum (γ)*			
SS39	*S. briareum (γ)*			
PR02	*S. briareum (β)*			
PR31	*S. briareum (β)*			
SS01	*S. briareum (β)*			
SS15	*S. briareum (β)*			
HIAp	*S. psygmophilum*			
MF10.14b.02	*S. psygmophilum*			
PurPFlex	*S. psygmophilum*			
rt005	*S. psygmophilum*			
rt141	*S. psygmophilum*			
Abipin03	*S. antillogorgia*			
Abipin04	*S. antillogorgia*			
Abipin19	*S. antillogorgia*			
mac08-689	*S. antillogorgia*			
AE5	*S. antillogorgia*			
Pesci166	*S. antillogorgia*			
Pesci214	*S. antillogorgia*			
mac703	*S. minutum*			
Mf1.05b	*S. minutum*			
rt002	*S. minutum*			
rt064	*S. minutum*			
rt351	*S. minutum*			
rt012	*S. pseudominutum*			
rt013	*S. pseudominutum*			
rt074	*S. pseudominutum*			
rt146	*S. pseudominutum*			
rt147	*S. pseudominutum*			
Bar85	*S. madracis*			
Bar92	*S. madracis*			
Be57	*S. madracis*			
Be58	*S. madracis*			
Fl02-41	*S. madracis*			
Cur09-122	*S. madracis*			
Cur09-145	*S. madracis*			

Figure 3-3 Sequence-based clustering by the Automated Barcode Gap Discovery algorithm. Read from top to bottom for a given gene, shading is used to indicate transitions between cluster memberships for each sample.

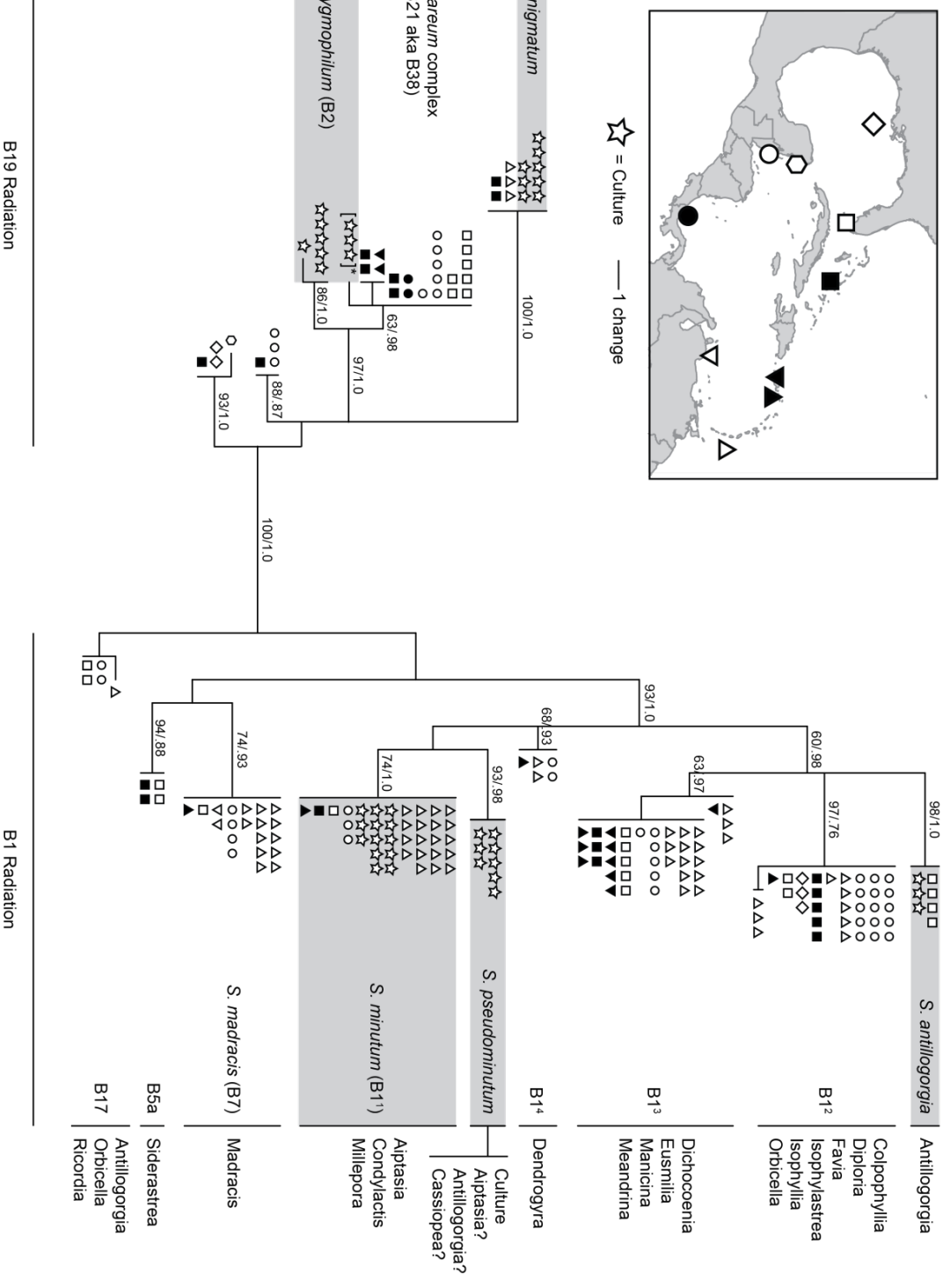
= Culture
1 change
S. aenigmatum
S. briareum complex (B21 aka B38)
S. psygmophilum (B2)
S. antillogorgia
S. pseudominutum
S. minutum (B11)
S. madracis (B7)
Culture
Porites?
Millepora
B23
Briareum
Astrangia
Diploria
Madracis
Oculina
Plexaurella
B19
Colpophyllia
B6
Colpophyllia
Diploria
Favia
Isophylastrea
Isophyllia
Orbicella
B12
Dichocoenia
Eusmilia
Manicina
Meandrina
B13
Dendrogyra
B14
Culture
Aiptasia?
Antillogorgia?
Cassiopea?
Aiptasia
Condylactis
Millepora
Madracis
B5a
Siderastrea
Antillogorgia
Orbicella
Ricordia
B17
98/1.0
97/1.76
60/.98
63/.97
93/1.0
68/.93
93/.98
74/1.0
74/.93
94/.88
100/1.0
88/.87
93/1.0
97/1.0
63/.98
86/1.0
100/1.0
B19 Radiation
B1 Radiation

Figure 3-4 Western Atlantic *Symbiodinium* Clade B maximum parsimony phylogeny based on the flanking region of microsatellite Sym15. Branch support is indicated by bootstrap values and Bayesian posterior probabilities, respectively; where omitted, neither value exceeded 85%. The tree is split along the major axis separating the B1 and B19 radiations. For each lineage the host origin, species name, and ITS2 type are provided (if known). Symbols represent locations where samples were collected on the inset map. Cultured material is indicated by a star. Shading indicates which lineages are currently in available in culture.

Table 3-S1 Gene regions targeted for analyses, gene types, primer pairs used for PCR, primer sequences, approximate sizes of amplified DNA fragments, and annealing temperatures used to delineate species in Clade B of the genus *Symbiodinium*. For analysis of ITS regions using denaturing gradient gel electrophoresis, a GC-rich area (clamp) is attached to the primer (underlined).

Table 3-S1 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g52r).

Table 3-S2 Additional details for samples used in species delineation, including sample ID, species membership, material type (either culture or tissue), host species (note that cultures isolated from a given "host" may not be representative of the dominant symbiont in that species), and collection location.

Table 3-S2 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g52r).

Figure 3-S1 Maximum parsimony trees for each gene: (a) *cob*, (b) *LSU*, (c) partial *ITS1/5.8S/ITS2*, (d) Sym15 flanker, and (e) *cp23S*. Thick lines indicate branches with >60% bootstrap support (n=1000 replicates). A phylogeny based on the concatenation of all sequences is presented in Figure 3-1.

Figure 3-S1 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g52r).

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Chapter 4

INTRASPECIFIC DIVERSITY AMONG HOST-SYMBIONT PAIRINGS YIELDS FUNCTIONAL VARIATION IN CORAL-ALGAL SYMBIOSES

Abstract

Coral reefs are declining globally. Temperature anomalies disrupt coral-algal symbioses at the molecular level, causing bleaching and mortality events. In terrestrial mutualisms, diversity in pairings of host and symbiont individuals (genotypes) results in ecologically and evolutionarily relevant performance differences as a result of stress. The extent to which such intraspecific diversity provides functional variation in coral-algal systems is unknown. Here, we assess functional diversity among unique pairings of coral and algal individuals. We targeted six genetically distinct *Acropora palmata* coral colonies that all associated with a single, clonal *Symbiodinium 'fitti'* strain in a natural common garden. No other species of algae or other strains of *S. 'fitti'* could be detected in host tissues. When colony branches were experimentally exposed to cold stress, host genotype influenced the photochemical efficiency of the symbiont strain, buffering the stress response to varying degrees. Gene expression differences among host genotypes with buffered vs. non-buffered symbiont responses included biochemical pathways that mediate iron availability and oxygen stress signaling—critical components of molecular interactions with photosynthetic symbionts. Spawning patterns among hosts reflected symbiont performance differences under stress. These data suggest that interactions between particular coral-algal genotype pairings may be an important source of physiological variation in coral symbioses, contributing raw material available to natural selection.

Introduction

Many coral animals host endosymbiotic, single-celled dinoflagellate algae from the genus *Symbiodinium*. The association is fundamental to reef ecosystems; the photosynthetic capacity of the algae allows scleractinian corals to accrete calcium carbonate skeletons in nutrient-poor waters (Muscatine and Porter 1977). Sustained thermal stress can cause the association to break down, resulting in symbiont loss known as 'coral bleaching' (Glynn 1993, Fitt et al. 2001). The fitness consequences of bleaching range from reduced host reproductive output to colony death (Szmant and Gassman et al. 1990; Ward et al. 2000, Jones 2008). Bleaching can be triggered by either hot or cold temperatures (Jokiel and Coles 1990, Saxby et al. 2003). These selective pressures may lead to local acclimation or adaptation of both the host and symbiont (Polato et al. 2010, Howells et al. 2012, Howells et al. 2013). Sea surface temperature anomalies are predicted to increase in magnitude and frequency in the future, along with their impacts on coral communities (Donner et al. 2005, Hoegh-Guldberg et al. 2007). The physical interaction between symbiont and host cells is intimate (Wakefield and Kempf 2001), and physiological changes in one of the partners have direct consequences for the other (Davy et al. 2012). If a component of these interactions is genetically-determined, there is potential for reciprocal fine-scale genetic effects on host and symbiont responses to stressors associated with climate change.

Interactive genetic effects have been observed in diverse systems including insect-bacteria (Feldhaar 2011) and legume-*Rhizobia* (Parker 1995, Heath 2010, Heath et al. 2012), among others. Such effects act synergistically to expand the range of functional diversity subject to selective pressures (Rodriguez et al. 2009). Selection among reef-building coral mutualisms is intensified by climate change, but whether and at what rate corals may acclimate or adapt are debated (Baird et al. 2007, Brown and Cossins 2011). Variable bleaching susceptibilities among adjacent colonies with identical host and symbiont compositions at the species level suggest that

intraspecific variation in stress responses exist (Glynn et al. 2001, Goulet et al. 2008, LaJeunesse et al. 2010). Despite renewed emphasis on the fundamental concept that natural selection acts on variation among individuals within species populations (Bolnick et al. 2003, Bolnick et al. 2011), our understanding of the adaptive significance of fine-scale genetic effects in coral holobionts is still in its infancy.

To date, most studies have focused on how relationships between coral species and *Symbiodinium* taxa (i.e. species, types, or clades) affect stress performance (e.g. Loya et al. 2001, Berkelmans and van Oppen 2006, Frade et al. 2008, Sampayo et al. 2008, Mieog et al. 2009). Within a particular coral species, colonies paired with 'stress-resistant' rather than 'stress-sensitive' symbiont taxa might be more tolerant to increased temperatures (Abrego et al. 2008), show altered transcription (Voolstra et al. 2009, DeSalvo et al. 2010b), and possess distinct heritabilities for holobiont traits (Csaszar et al. 2010), though perhaps at the cost of slower growth rates (Little et al. 2004). Interactions below the species level have received less attention (Baums 2008), though they may produce similar effects. For example, holobiont thermotolerance varies between two genetically-differentiated populations of a single host species despite sharing one symbiont species across sites (Kenkel et al. 2013a, Kenkel et al. 2013b). Conversely, juveniles of a single host species population reared in a common garden show distinct thermotolerances when associating with either of two populations of a single symbiont species (Howells et al. 2012).

Recent evidence suggests coral performance may depend on the identity of individual genotypes within species. Functional differences among *Symbiodinium* strains have yet to be measured robustly, but experimental crosses between genotyped corals show incompatibilities among parents in terms of fertilization rates and larval survival (Baums et al. 2013). Moreover, batches of symbiont-free, half-sibling larvae exhibit different physiological and molecular responses to temperature stress (Baums et al. 2013, Polato et al. 2013). Similar host genotype

effects have not been documented conclusively in adult corals with stable symbioses because symbiont community has not been controlled at the same resolution.

Here, we investigate functional diversity among coral holobionts at the finest scale possible, resolving both partners to the level of individuals with neutral molecular markers. We take advantage of the *Acropora palmata—Symbiodinium 'fitti'* system, where the host-to-symbiont genotype ratio is generally one-to-one (Baums et al. 2014). We use physiological experiments involving symbiont photochemistry and gene expression during temperature stress to test the hypothesis that host genotype affects the performance of a single clonal symbiont strain *in hospite*. We identify biochemical pathways affected by host gene expression at different temperatures that may mediate variation in symbiont photochemistry and damage by photosynthetically generated oxygen radicals. Finally, we find that host reproduction is correlated with stress response phenotypes unique to particular host-symbiont genotype pairings, perhaps influencing the evolutionary trajectory of coral holobionts in stressful environments.

Materials and M**ethods**

Study system

The Caribbean Elkhorn coral, *Acropora palmata*, associates with one dominant phylogenetic lineage of *Symbiodinium*: ITS2 type A3 (Thornhill et al. 2006). Based on hierarchical molecular markers (LaJeunesse et al., unpubl. data), the Caribbean A3 lineage represents a cohesive species (LaJeunesse et al. 2012), and is provisionally termed *Symbiodinium 'fitti' nomen nudum* (Pinzon et al. 2011). Formal description of the species is underway (LaJeunesse et al. unpubl. data). *A. palmata* spawns symbiont free eggs, therefore *S. 'fitti'* cells must be taken up from the environment by the larva or after settlement, potentially along with other *Symbiodinium* species (Voolstra et al. 2009). Though background strains from other clades

have been detected in adult colonies (Silverstein et al. 2012), only strains belonging to *S. 'fitti'* are present at appreciable levels (≥95% of the symbionts in host tissue) in most colonies throughout the Caribbean (Thornhill et al. 2006, Baums et al. 2010, Baums et al. 2014). The haploid symbiont occasionally reproduces via sexual recombination, but *in hospite* mostly propagates via cell division (Santos et al. 2004, Baums et al. 2014), such that each coral can be viewed as a culture vessel for a single symbiont strain.

Host and symbiont genotyping

In Spring 2011, colonies of *A. palmata* (n = 20) were sampled from La Bocana Chica Reef in the Puerto Morelos Reef National Park, Mexico (N 20°52.461', W 86°51.073'). For each colony, the host was genotyped at 5 neutral microsatellite loci according to Baums et al. (2005) to identify multilocus genotypes. The *S. 'fitti'* strain in each colony was genotyped at 10 microsatellite loci (Pinzon et al. 2011). Probability of identity was on the order of 10^{-7} for the host and 10^{-5} for the symbiont (Baums et al. 2014). Host and symbiont samples sharing identical alleles at all loci were deemed to be clonemates of the same host genotype and symbiont strain, respectively. Multilocus genotypes were collapsed using GenAlEx vers. 6.4 (Peakall and Smouse 2006).

To study host genetic effects on holobiont phenotype, we controlled for both symbiont strain and the natural environment. There were at least 15 host genotypes and 9 symbiont strains present on the reef. A total of 6 *A. palmata* genotypes (referred to as *A*, *B*, *D*, *X*, *Y*, and *Z*) harbored the same strain of *S. 'fitti,'* therefore these 6 colonies were targeted for temperature experiments. The colonies were found within 30 m of each other, and each colony was located within 2 m of at least one other colony, minimizing environmental heterogeneity. Depth ranged from 2.4-3.9 m. All colonies exceeded minimum reproductive size (Lirman 2000).

Because corals sometimes host more than one clade of *Symbiodinium*, samples of the 6 target colonies were screened with traditional DGGE-PCR of the ITS2 region, which detects all

sub-cladal types that represent ≥5% of the total symbiont community (LaJeunesse 2002, Thornhill et al. 2006). The region was amplified using the primers ITSintFor2 and ITS2CLAMP following the touchdown protocol of LaJeunesse and Trench (2000). Bands were visualized on a denaturing gradient gel as in Sampayo et al. (2009), excised, reamplified with the same primers excluding the GC-rich clamp, and directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA, USA) at the Pennsylvania State University Genomics Core Facility. Chromatograms were checked using CodonCode Aligner software (CodonCode, Dedham, MD, USA).

Additionally, clade-specific rt-PCR assays (Correa et al. 2009) were used to test for background symbionts below the DGGE detection limit as in McGinley et al. (2012). Thresholds for detection were determined by serial ten-fold dilution of DNA from monocultured *Symbiodinium* (1-0.0001 ng/µL); clade A: rt272 (Pacific A3); clade B: mac703 (*S. minutum*); clade C: rt152 (*S. goreaui*); clade D: A001 (*S. trenchi*). Each assay included a positive culture control and a no-template control. Each reaction was run in triplicate. Melting curves were used as in Correa et al. (2009) to confirm that "no detection" products were likely primer dimer (T_m < 80°C) and "detection" products were likely target template (T_m > 80°C). Dilution series analysis was conservatively restricted to C_T values with standard deviations within 5% of the mean across all three replicates. Within *Symbiodinium* type A3, distinct strains can be detected using the 10 microsatellite markers provided the minor strain represents ≥5% of the A3 population (Baums et al. 2014).

Temperature stress experiment

In summer 2011, single fragments (~30 cm^2) from the 6 target corals were collected with hammer and chisel from the top of each colony at the growing tip and transported to a 45 L polycarbonate bin containing filtered seawater maintained at 29°C to match that day's reef conditions. The outdoor bin was covered with a shade cloth that reduced irradiance by ~50%.

The fragments were then subdivided into separate pieces of ~10 cm^2 each. After 2 d of acclimation, replicate pieces of each genotype were transferred to each of 3 separate shaded 45 L polycarbonate bins containing filtered seawater maintained at temperatures of 20 (cold), 27 (ambient), or 34°C (hot) with aquarium chillers (Current-USA, CA, USA). Water was circulated with an aquarium pump and changed daily with additional preheated filtered seawater. The fragments were maintained in the treatments for three days; HOBO data loggers (Onset Co., MA, USA) in each bin indicated temperatures stayed within ±0.4°C of the target for each treatment.

The ambient treatment temperature (27°C) was set two degrees below reef conditions (29°C) to reduce the risk of unintended bleaching in the aquaria. This temperature is well within the previous year's range (25.4-29.7°C) and is close the historical summer average (27.7°C; NOAA Coral Reef Watch 2011). The cold treatment was set 5°C below the historical minimum (to 20.1°C from 25.1°C), while the hot treatment was set 4°C above the historical maximum (to 34.1°C from 30.1°C). The treatments were extreme compared to what these colonies would naturally experience on the reef. Temperatures were not ramped, such that the corals were exposed to instantaneous temperature shock. Given time restrictions at the field site and concerns that intraspecific differences might be subtle, the exposure was designed not to mimic natural conditions, but to accentuate acute cold shock differences among individuals.

Symbiont photochemical efficiency

The *in situ* photopchemical performance of the *S. 'fitti'* strain was estimated using a Diving PAM fluorometer (Walz, Germany) with the following settings: Measuring Intensity 12, Saturation Intensity 8, Saturation Width 0.6 s, Damping 2, and Gain 3. Visual symbiont cell loss began in all fragments placed in the hot treatment after 6 h of exposure. After 9 h, these fragments were completely bleached and sloughing tissue, rendering photochemical efficiency measurements impossible. Neither the cold nor ambient treatment fragments visibly bleached during the three days of exposure.

For the cold and ambient treatments, maximum excitation pressure over photosystem II (Q_m) was calculated daily for each colony as $Q_m = 1 - [(\Delta F/F'_m$ at noon)/(F_v/F_m at dusk)] (Iglesias-Prieto et al. 2004). Q_m ranges from 0, where photochemistry is light-limited, to 1, indicating photoinhibition. For a set of fragments of the same host genotype origin, the Q_m of ambient treatment was subtracted from the Q_m of the cold treatment to calculate ΔQ_m values, or the relative ability of the clonal *S. 'fitti'* strain to tolerate cold stress in different host genotypic backgrounds. Larger ΔQ_m values indicate greater impacts of temperature stress on the symbiont.

Other symbiont phenotypes

Additional *S. 'fitti'* phenotypes included average symbiont density and average symbiont cell volume. Replicate hemocytometer cell counts (n = 5) were taken from a single $1cm^2$ tissue plug per colony. Ellipsoid cell volumes were estimated as $4\pi(abc)\bullet 3^{-1}$, where *a* is half the cell's longest diameter and *b* and *c* are taken as half the perpendicular diameter (n = 40 cells per colony).

Spawning observations

All *A. palmata* colonies at the study site in Puerto Morelos, including those that were not used in this experiment, were observed for annual spawning between 20:00 h and 22:30 h from the 15^{th} to the 19^{th} of August 2011 by SCUBA divers.

Microarray experiment

To investigate the molecular underpinnings of the host genotype effect on symbiont photochemistry, the host's acute response to temperature stress was assessed through gene expression using a microarray designed from the *A. palmata* transcriptome (Polato et al. 2011). To achieve biological replication, we included RNA extracts from the two host genotypes with the smallest symbiont ΔQ_m (hosts *Z* and *B*) and the two host genotypes with the largest symbiont ΔQ_m (hosts *D* and *Y*). Hot treatments samples were included on the microarray and coded the same way as the cold and ambient treatment samples with respect to symbiont physiology.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA, USA) from subsamples of each host genotype (n = 4) at each temperature (n = 3; hot stress included) taken 3.5 h after exposure to treatment. Concentration and quality of RNA extracts were quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to ensure that high molecular weight RNA was present. High-quality mRNA was hybridized to custom 2-channel microarrays (Nimblegen 6019040401) and analyzed as in Polato et al. (2013). Associated raw data and a more detailed description of hybridization conditions can be accessed in the NCBI Gene Expression Omnibus database (Edgar et al. 2002) through GEO Series accession number GSE50926 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50926).

Statistical analysis

To analyze photochemistry, ΔQ_m values of each fragment from all three days were included in a repeated measures and a one-way ANOVA in the R statistical environment (R Development Core Team 2008). The potential correlation between host codominant genotypic distance and ΔQ_m was explored using a Mantel test with 999 replicates in GENALEX 6 (Peakall and Smouse 2006). To test the hypothesis that spawning behavior and holobiont phenotype were related, we implemented Barnard's exact test (Barnard 1945), a more powerful alternative to Fisher's exact test for 2x2 contingency tables (Mehta and Hilton 1993), using the R package Exact (Calhoun 2013).

Expression data were analyzed in R as in Polato et al. (2013) using the Bioconductor package LIMMA (Smyth 2005). A log base 2 fold change cut-off of 1.5 (= fold change cut-off of 2.8) and a *Q*-value threshold of 0.05 were used to filter significant results. To visualize transcription profiles, expression values were transformed gene-wise into scaled coefficients (standard scores above or below the probe mean) and plotted as a heatmap with MultiExperiment Viewer v4.9 (Saeed et al. 2003). An unrooted sample tree was created through hierarchical

clustering of expression profiles using the hclust function and complete linkage agglomeration method in R. Principle component analysis was carried out using the prcomp function and a covariance matrix in R. Lists of differentially expressed genes were analyzed for functional enrichment with the online tool GOEAST v1.3 (Zheng and Wang 2008) based on the original annotation file associated with the microarray. Redundancies in Gene Ontology (GO) terms were removed with REVIGO (Supek et al. 2011). Gene lists were also analyzed through DAVID Bioinformatics Resources v6.7 (Huang et al. 2009b, Huang et al. 2009a) at the GO level of Biological Processes. For these analyses, default parameters were used for FDR correction and semantic similarity. A list of all differentially expressed genes and the R code used in the expression analysis can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g45b).

Results

Host diversity and symbiont uniformity

The six *Acropora palmata* corals analyzed in this experiment had unique multilocus genotypes as determined with five neutral microsatellite loci (Table 4-S1a). Each colony associated with the same, genetically uniform *Symbiodinium 'fitti'* strain, with an identical multilocus genotype at ten neutral microsatellite loci (Table 4-S1b). Only one band was present on the ITS2 DGGE profiles of each colony; as expected, its sequence corresponded to *Symbiodinium* type A3 (Genbank Accession: AF333507). No other subcladal types were present above the 5% detection limit of this technique. Furthermore, no clade B, C, or D symbionts were detected with highly sensitive rt-PCR assays (Figure 4-S1). Thus, each holobiont could be viewed as a unique pairing of a single host and single symbiont genotype, where all hosts were distinct and all symbiont cells identical.

Symbiont photochemistry

The photochemical performance of the *S. 'fitti'* strain, as measured by the maximum excitation pressure over photosystem II (Q_m), varied with host individual genotype under ambient and cold conditions (Figure 4-1). The three-day average cold treatment effect (ΔQ_m) ranged from 0.12 ± 0.08 SD (in host *B*) to 0.43 ± 0.03 SD (in host *D*). A repeated measures analysis revealed that the day of observation was not a significant factor (ANOVA, $F_{(2,10)} = 0.738$, $p = 0.502$); we therefore treated values from different days as technical replicates to obtain measures of error for each host background.

Holobionts with clonal symbionts differed in ΔQ_m (ANOVA, $F_{(5,12)} = 7.582$, $p = 0.002$). In some host genotypes, the *S. 'fitti'* strain's photochemical efficiency changed little with cold shock (small ΔQ_m) while in other host genotypes, its photochemical efficiency changed greatly with cold shock (large ΔQ_m). ΔQ_m did not correlate with host genetic distance (Mantel test, $p = 0.36$). Due to bleaching and mortality, hot treatment corals could not be measured with PAM fluorometry. When testing for other differences among holobionts, we observed deviations in symbiont density (ANOVA, $F_{5,24} = 76.34$, $p < 0.001$; Figure 4-S2a, b) but not cell ellipsoid volume (ANOVA, $F_{5,234} = 1.78$, $p = 0.119$; Figure 4-S2a, b). ΔQ_m did not correlate with either metric (density: $R^2 = 7.4\%$, $p = 0.603$; volume: $R^2 = 8.1\%$, $p = 0.536$; Figure 4-S2c, d).

Host gene expression

The combination of representative of two photochemical phenotypes (small ΔQ_m: hosts *Z* and *B;* large ΔQ_m: hosts *D* and *Y*) and three temperature treatments produced six expression profiles (Figure 4-2a). Hierarchical clustering and principle component analysis (PCA) on expression profiles supported similar groupings (Figure 4-2bc). The first PCA axis explained a majority of total variation (59.69%) and corresponded to a split between the hot treatment and all other temperature treatments. The distribution of differentially expressed probes (DEPs) reflected this split: there were 18,609 DEPs in the hot vs. ambient contrast, but only 184 DEPs in the cold

vs. ambient contrast (Figure 4-3). The second PCA axis explained 12.63% of total variation and corresponded to a split between small and large ΔQ_m holobionts. In holobionts with small ΔQ_m, the host was transcriptionally dynamic when responding to cold stress (184 DEPs); we refer to these hosts as Dynamic. In colonies with large ΔQ_m, the host was transcriptionally static (14 DEPs); we refer to these hosts as Static. No transcripts showed a photochemical phenotype by temperature treatment interaction. ΔQ_m and total number of DEPs were significantly correlated at $\alpha = 0.1$ ($R^2 = 87.1\%$, $p = 0.067$; Figure 4-4), but note the small sample size (n=4).

We distinguished genes that were differentially expressed within either small ΔQ_m or large ΔQ_m holobiont hosts by contrasting cold and ambient treatments among each group separately. Under cold shock, small ΔQ_m holobiont hosts significantly altered the expression at 184 probes comprising 54 unique genes with annotation information (Table 4-S2); we refer to these holobionts as 'Dynamic.' In contrast, large ΔQ_m holobiont hosts only differentially expressed 14 probes comprising 2 unique genes with annotation information (Table 4-S2); we refer to these holobionts as 'Static.' These 2 cold-responsive DEPs were were *NUDT9*, involved in a catabolic process, and *structural maintenance of chromosomes protein 5*, involved in cell division and DNA binding. When comparing cold stress response side-by-side (Figure 4-5), most of the significantly upregulated genes in Dynamic hosts were also upregulated in Static hosts, but the magnitude of the change was smaller, resulting in insignificant changes for Static hosts.

Genes upregulated in Dynamic hosts were involved in redox maintenance and signaling (e.g. *glutaredoxin, RAS protein, aldehyde dehydrogenase*), stress signaling (e.g. *nuclear factor kappa B, rho-related protein racA,* and *tumor necrosis factor receptor-associated factor 3*), calcium signaling (e.g. *calcium binding protein P22* and *fibropellin-3*), and transmembrane transport (e.g. *sugar phosphate exchanger 2, glycerol-3-phosphate transporter,* and *ABC transporter*). Nine transcription factors were adjusted, including zinc finger proteins. Seven protein products contained disulfide bonds, and were therefore potential targets of redox signaling

(see Discussion). According to analysis in GOEAST and REVIGO, the list of genes differentially expressed between cold and ambient temperatures were enriched for the GO functional categories signal transduction, response to stimulus, (ribo)nucleotide metabolic processes, and biological regulation. DAVID Bioinformatics Resources produced concordant results, with enriched clusters belonging to the categories transcription regulation, signal peptides, (ribo)nucleotide binding, alternative splicing, ion binding, and membrane proteins.

Next, we compared Dynamic and Static hosts at each temperature separately. A total of 130 DEPs were detected, reducing to 26 unique, annotated genes (Table 4-S3). Static hosts constitutively expressed more genes involved in oxidative stress response and redox signaling at all temperatures. For example, two *ferritins* were roughly 20-fold and 5-fold upregulated in static hosts relative to Dynamic hosts across all controls and treatments. This molecule binds iron ions, thereby reducing radical oxygen production via Fenton reactions (see Discussion). Accordingly, this gene list was functionally enriched for ion binding. Under cold and ambient conditions, Static hosts also expressed more *RHS family protein* and *glutaredoxin-like protein*, both belonging to the glutaredoxin (GRX) subfamily, important in redox balance and cell signaling.

In the hot treatment, all colonies bleached and died by the end of the first day. After only 3.5 h of exposure to high temperature, fragments were stressed and exhibited extensive differential expression relative to ambient (18,609 DEPs); many transcripts were shared between Dynamic and Static host genotypes (8,028 DEPs), with roughly equal proportions being upregulated or downregulated (Figure 4-3).

Host spawning behavior

The two Dynamic hosts spawned on 18 Aug. along with most of the rest of the *A. palmata* on the reef. We observed no spawning among the four Static hosts during this date or any other from 15-19 Aug. Such a pattern is unlikely the result of chance (Barnard's test, $p = 0.031$).

Discussion

We set out to determine whether intraspecific diversity among host-symbiont pairings yields functional variation in coral-algal symbioses. We found that a single *Symbiodinium 'fitti'* strain differed in photochemical performance during cold shock when engaged in symbiosis with six different *Acropora palmata* genotypes in a natural common garden (Figure 4-1). These differences correlate with detectable host transcription changes (Figures 4-2, 4-3, 4-4). Differences among colonies could not be attributed to background symbiont strains, symbiont cell density, symbiont size, host genetic distance, or environmental heterogeneity. Because the dominant symbiont cells were genetically uniform, and no other *Symbiodinium* were detected, host genotype emerges as the likely factor determining holobiont stress response phenotype in our experiment. However, even single cells from isoclonal lines of algae vary at the protein level (Garz et al. 2012), and other potential sources of variation such as endolithic fungi and algae, bacterial communities, and viruses were not evaluated. Nevertheless, in this first physiological study of scleractinian coral-algal symbioses using high-resolution markers to resolve both partners to the level of individual genotypes, we show that important physiological patterns can be missed when intraspecific diversity is overlooked.

Cold shock increases maximal excitation pressure

All photosynthetic organisms must contend with the issue of energy imbalance resulting from differences in the rates of rapid, temperature-independent absorption of solar energy and slower, temperature-dependent reactions of electron transport and metabolic growth (Huner et al. 1998). Cold temperatures slow the rates of carbon fixation reactions and electron transport, leading to an over-reduction of the plastoquinone pool. With normal rates of photon flux but slowed turnover of electron sinks, there is less capacity to oxidize the primary electron acceptor (that is, convert photosystem II from the 'closed' to 'open' state). This induces a decrease in

photosystem II yield ($\Delta F/F_m'$), driving an increase in maximal excitation pressure (Q_m) and an increase in photodamage. As expected, we observed Q_m elevation in all six holobiont fragments exposed to cold temperature. However, the magnitude of change in pressure (ΔQ_m) varied significantly among holobionts, despite the clonality of the *S. 'fitti'* strain shared by all hosts, indicating host modulation of symbiont stress response.

Distinct holobiont phenotypes

Because heat shocked corals were moribund, we focus here on cold shock. When the transcriptional profiles of colonies with small vs. large cold-induced changes in symbiont photochemistry (ΔQ_m) were compared under ambient conditions, we found that the hosts differentially expressed genes associated with iron availability and oxidative stress signaling pathways. After being subjected to cold, multiple host expression changes took place in colonies where symbiont photochemistry responded minimally (Dynamic holobionts), whereas in the remaining colonies host transcription changes were barely detectable and symbionts experienced greater fluctuation in photochemical efficiency (Static holobionts).

We interpret these data as indicative of a phenotypic buffering effect (Waddington 1942; Bradshaw 1965; Reusch 2014). The correlation between host transcription and symbiont photochemistry suggests that below the species level, differences in molecular signaling between partners lead to distinct physiological outcomes. Dynamic and Static hosts survived cold treatment for at least three days, but only Dynamic hosts appeared to participate in the annual synchronized spawning event. Together, these data suggest that in addition to photochemical and transcriptional stress responses, holobionts composed of different host-symbiont pairings may also have different fitness outcomes, providing raw material for natural selection. However, while a host-symbiont interaction that leads to reduced energy reserves may lower reproductive output, it is also possible that the observed lack of reproduction was solely a host genotype effect.

Ferritin and oxidative stress

We propose that the capacity of the host to manage its cellular environment, specifically iron availability and redox state, affects the resident symbiont (Figure 4-6). Based on the identity of genes differentially expressed in this and other studies (e.g. DeSalvo et al. 2010a), it appears that interactions and signals between partners may be mediated by reactive oxygen species (ROS). ROS production in the symbiont increases when the light reactions of photosynthesis are damaged, such as during hot and cold shock conditions (Allen and Ort 2001, Allakhverdiev et al. 2008). ROS can also be generated spontaneously in the host by way of Fenton reactions, which use free iron as a cofactor to convert H_2O_2 to extremely damaging $HO^{\bullet}$ (Lesser 2006).

Ferritin acts as an iron-sequestration molecule, reducing the free iron available for Fenton reactions (Torti and Torti 2002). Because H_2O_2 produced by *Symbiodinium* as a byproduct of damaged photosynthesis can leach out into the host cell (Lesser 2006; Saragosti et al. 2010), ferritin is consistently upregulated in thermally-stressed coral colonies (Csaszar et al. 2009; DeSalvo et al. 2010a). In this experiment, Static *A. palmata* constitutively expressed two ferritin genes up to 20-fold higher than Dynamic hosts both before and after cold shock (Table 4-S3). This suggests that steady-state ferritin production and therefore iron availability (which are linked; Cairo et al. 1995) can vary substantially among coral genotypes independent of stress, yielding distinct cellular environments for *S. 'fitti'* depending on the *A. palmata* colony with which the symbiont associates.

Symbiodinium are iron-limited *in hospite* (Entsch et al. 1983), and likely rely on the host as an iron source, so variation in ferritin expression may directly affect symbiont performance. The *S. 'fitti'* strain had greater photochemical efficiency (lower basal Q_m; Figure 4-1a) in Static hosts than in Dynamic hosts at ambient temperature (t-test, $p = 0.049$). Nutrient availability can dictate bleaching susceptibility (Wiedenmann et al. 2013), so variation in the expression of genes that mitigate nutrient limitation may be targets of selection. Indeed, at least one ferritin in *A.*

palmata is under intense positive selection (Schwarz et al. 2008). Holobiont performance appears to vary depending on how a given host balances iron availability for symbionts under ambient conditions against iron-enhanced oxygen toxicity under stress. Hosts with high levels of free iron available to their nutrient-limited symbionts may benefit from improved *Symbiodinium* performance provided conditions remain stress-free. During temperature anomalies, however, excess iron may become a liability as it promotes ROS production and tissue damage. Such a trade-off may support polymorphism within the mutualism. Future work incorporating the direct measurement of intracellular iron pools (Kakhlon and Cabantchik 2002) may yield further insights into the maintenance of intraspecific variation in host ferritin expression and the cellular mechanisms involved.

Glutaredoxin and redox signaling

Our data show that two redox maintenance genes encoding glutaredoxin-family proteins (GRXs) were constitutively upregulated in Static corals (Table 4-S2). As mediators of electron transfer in the glutathione cycle, GRXs are central to many redox regulatory processes (Noctor and Foyer 1998, Holmgren and Fernandes 2004), some of which may be particularly relevant for corals associating with photosynthetic symbionts. For example, GRXs reduce the oxidized form of ascorbic acid and other important antioxidants (Wells et al. 1990). GRX showed elevated rates of evolution in a comparison of *A. palmata* and *A. millepora* transcriptomes (Voolstra et al. 2011).

Glutathione redox reactions couple electron exchange to the creation of disulfide bonds in target proteins. This process, called S-glutathionylation, is reversible, and therefore serves as a biochemical signaling mechanism (Ghezzi and Bonetto 2003, Buchanan and Balmer 2005). Energy imbalances are known to be sensed in plants by alterations in photosystem II excitation pressure (Huner et al. 1998). This triggers a chloroplastic redox signal that communicates stress to the nucleus. Our data suggest that a similar signaling pathway may convey messages between

corals and *Symbiodinium*. Eight genes differentially expressed in Dynamic hosts responding to cold shock encoded protein with disulfide bonds; it is possible that these genes and/or their proteins were targets of redox signaling (Table 4-S3). If corals perceive symbiont-induced changes in redox balance through this type of signaling, the high level of GRXs in Static hosts may reflect the relatively greater photochemical efficiency (and likely ROS production) of their symbionts at ambient temperature. H_2O_2, so important in the ferritin oxidative stress response pathway, is also implicated as a messenger in redox signaling (Winterbourn and Hampton 2008, Messens and Collet 2013). Normal cell function depends on tight control of redox state, and an excess of oxidative molecules can threaten the equilibrium of cellular compartments. H_2O_2 can upset this balance by directly participating in thiol-sulfide redox exchanges (Noctor and Foyer 1998, Buchanan and Balmer 2005, Winterbourn and Hampton 2008).

Stress response polymorphisms

The maintenance of phenotypic polymorphisms may be favored in the presence of spatial and temporal environmental variability (Levins 1968). We observed at least two holobiont phenotypes that acted differently under normal or stressed conditions. The Static phenotype was associated with high but constant expression of ferritin and GRX before and after cold shock. While cold-induced transcriptional changes occurred, their magnitude was small, and symbiont performance worsened up to 9.9-fold (mean: 2.6-fold). At ambient temperature, Static hosts appeared to support greater symbiont photochemical efficiency than Dynamic hosts, perhaps because of high iron availability. Improved photochemistry benefits the holobiont by potentially increasing energy reserves, thus the Static phenotype may be favored when temperature conditions are stable.

In contrast, Dynamic hosts were more responsive to temperature changes; they upregulated GRX and other signaling genes under cold stress to a significant degree, and appeared to buffer their symbionts from changing environmental conditions. Though Dynamic

holobionts may shelter their symbionts from stress by maintaining smaller iron pools, they may suffer the cost of lower photosynthate production at ambient conditions because of an iron-limiting host environment.

According to this hypothesis, the Dynamic phenotype is low risk/low reward and suited to fluctuating environments, while the Static phenotype is high risk/high reward and suited to constant environments. Clearly both phenotypes are functional, but their relative success is context-dependent. These phenotypes likely represent points on a spectrum; were more genotypes included in the experiment, more extreme and intermediate phenotypes might be recovered. The degree to which distinct genotype pairings differ physiologically may vary from association to association. The *Acropora palmata—Symbiodinium 'fitti'* symbiosis is incredibly stable (Thornhill et al. 2006, Baums et al. 2014); one might expect less variation in host gene expression in mutualisms where genotype turnover is more common, such as with *Pocillopora damicornis—Symbiodinium 'glynni'* association in the Gulf of California (Pettay et al. 2011).

Climate change and adaptation

Hot and cold temperature anomalies can lead to wide-spread coral mortality (eg. Guzman and Cortes 1992, Hoegh-Guldberg and Fine 2004, Kemp et al. 2011; Lirman et al. 2011), guiding the outcomes of natural selection (Guest et al. 2012). Based on our observations here, we propose that host genotypes may differ in how they respond to extreme temperature changes by the way they recognize and act on the status of their symbionts through redox signaling, providing a target for selection. Given that physiological variation clearly exists within species of hosts and symbionts, it is important to recognize that the holobiont is a unit of selection in coral-algal symbioses (Iglesias-Prieto and Trench 1997; LaJeunesse et al. 2010; Thornhill et al. 2014; Prada et al. 2014). Though it remains unclear whether adaptation in corals can take place on ecological time scales, the variation resulting from interactions between particular host and symbiont genotypes may play an important role in the evolutionary response to climate change

(Barshis et al. 2010, Barshis et al. 2013). Selection may be particularly strong for alleles affecting the molecular pathways linked to symbiosis maintenance and performance during temperature stress, such as those outlined here.

Conclusion

This study provides new evidence of fine-scale functional diversity in corals. We have shown that symbiont photochemistry and host gene expression vary among distinct *Acropora palmata-Symbiodinium 'fitti'* genotype pairings. Evidence comes from the alteration of symbiont performance within a clonal cell line (Figure 4-1), contrasting host transcriptional responses to temperature stress (Figures 4-2, 4-3, 4-4, 4-5), and distinct spawning patterns in a single year. The potential for genotypic interactions to influence population-level dynamics emphasizes the need to preserve existing genotypic richness of both partners when managing reefs or designing coral restoration nurseries (Baums 2008). Future studies should test whether these metrics vary by symbiont genotype and over longer temporal scales. Though it is rare to find colonies sharing the same host genotype but associating with distinct symbiont genotypes, such cases will be necessary to test the converse of this experiment, to provide a more concrete link between genotype interactions and fitness effects, and to draw conclusions about micro-coevolution. Host-symbiont interactions at this level of genetic resolution have rarely been studied in coral holobionts in the past, but given their potential ecological and evolutionary significance, they merit further investigation.

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Figures

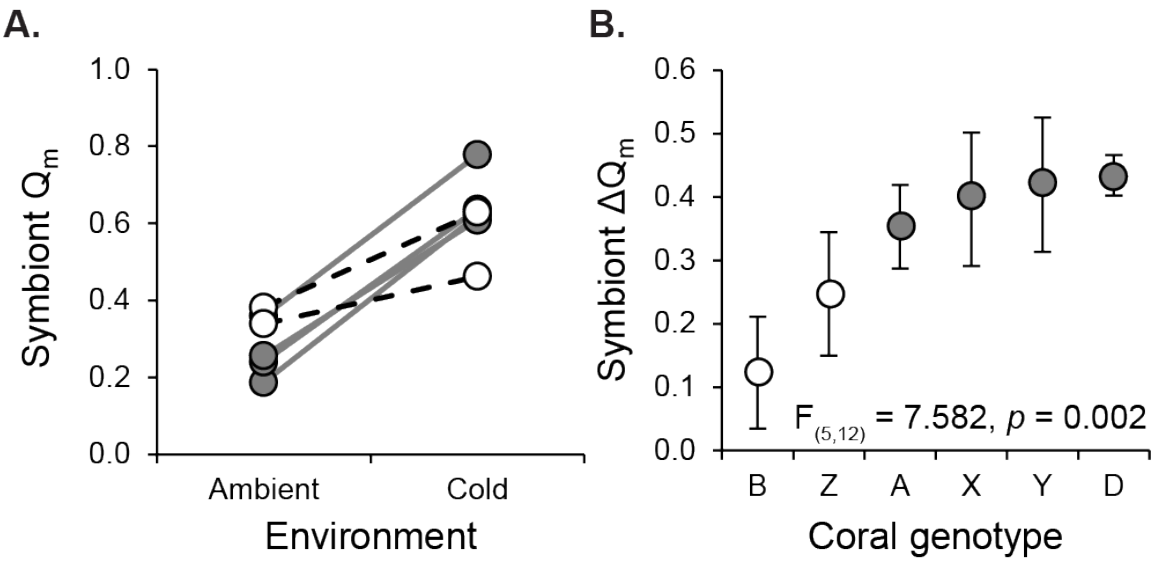

Figure 4-1 Physiology of a clonal *Symbiodinium 'fitti'* strain found in six distinct *Acropora palmata* genotypes. **(a)** Reaction norm of pressure over photosystem II (Q_m) for ambient and cold exposure. **(b)** The difference in pressure over photosystem II between cold and ambient exposure (ΔQ_m). In host genotypes B and Z (members of Dynamic holobionts; see text), the *S. 'fitti'* strain performed similarly regardless of temperature (small ΔQ_m; white fills/dashed lines) while in host genotypes A,X, Y and D (members of Static holobionts; see text), the symbiont strain's photochemical efficiency was greatly influenced by the cold shock (large ΔQ_m; gray fills/solid lines). Error bars represent 95% confidence intervals for three replicate measurements (one per day of exposure).

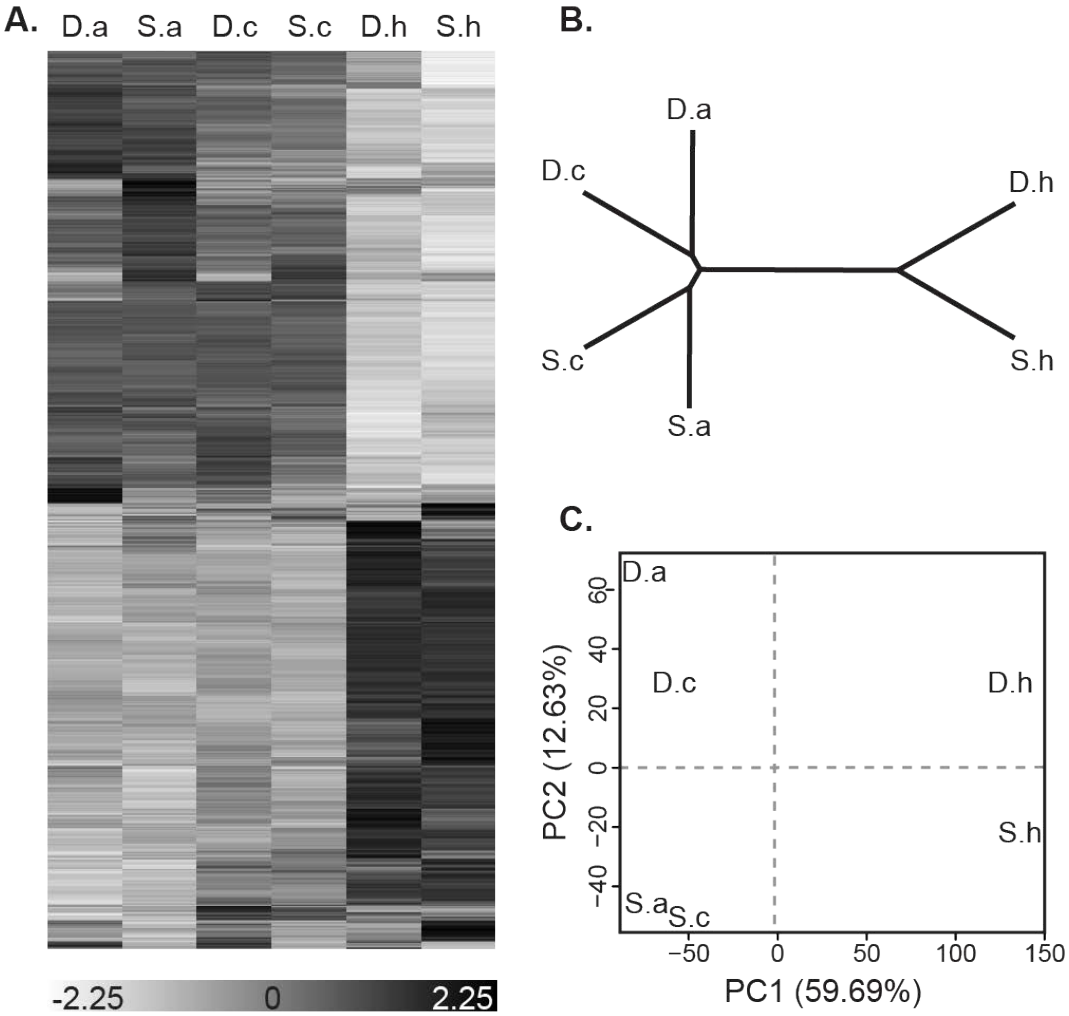

Figure 4-2 *Acropora palmata* microarray results. Samples are coded by a leading uppercase letter (transcriptional phenotype: D = Dynamic, S = Static) and trailing lowercase letter (temperature treatment: c = cold, a = ambient, h = hot). **(a)** Heatmap of transcription profiles for all probes differentially expressed in response to temperature or symbiont photochemical phenotype shown as scaled expression coefficients (standard scores above or below the probe mean). **(b)** Unrooted hierarchical clustering of samples as calculated in R using the hclust function and complete linkage agglomeration method. **(c)** Principle components 1 and 2 (x- and y-axis, respectively) of sample gene expression as calculated in R using the prcomp function and a covariance matrix.

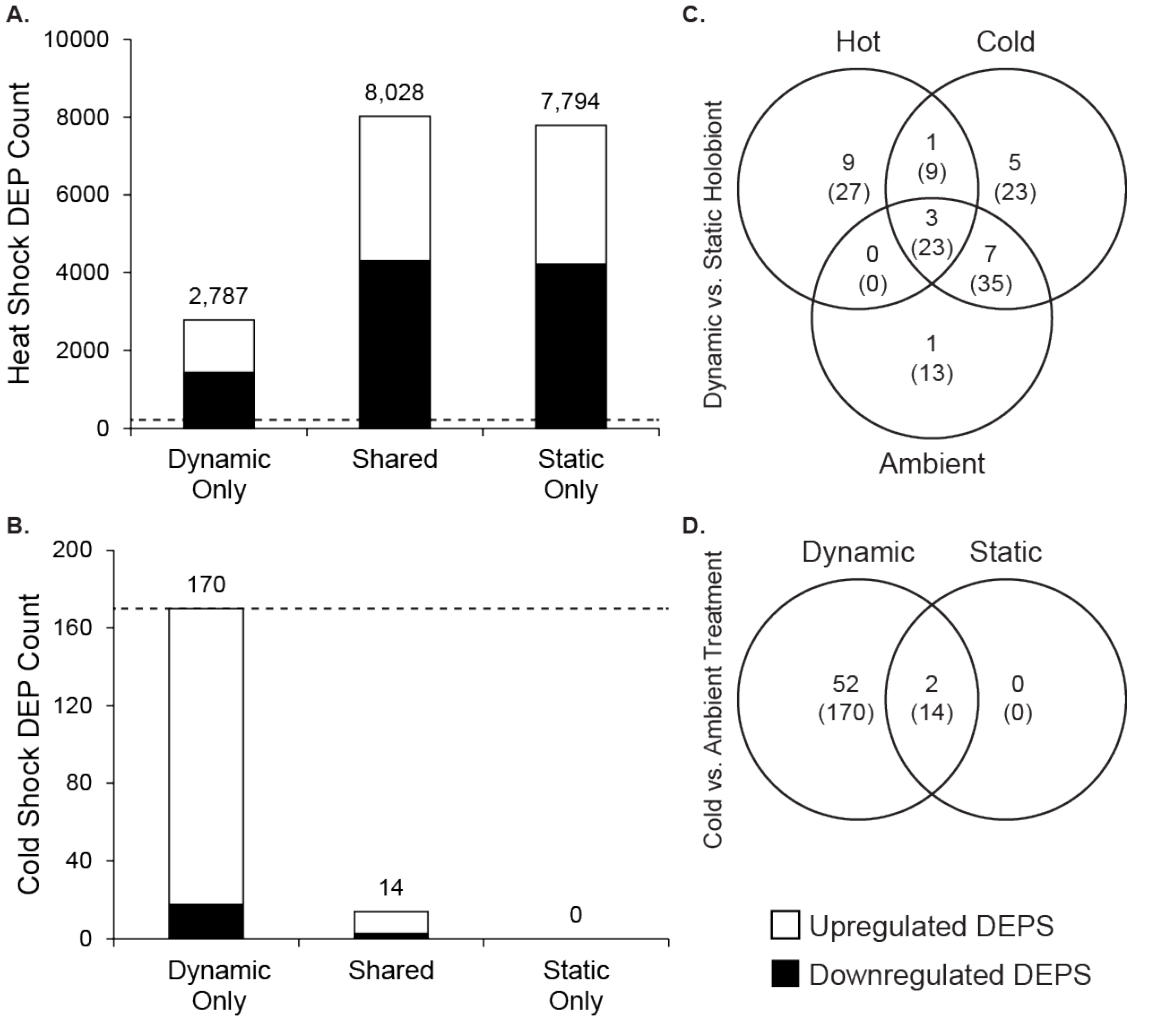

Figure 4-3 The total number of differentially expressed probes (DEPs) in response to **(a)** heat shock and **(b)** cold shock that were unique or shared among dynamic or static hosts. Note the drastically different scales between **(a)** and **(b)**; the dotted line indicates a common point of reference at a count value of 170. White shading indicates the proportion of upregulated probes, while black shading indicates the proportion of downregulated probes. Venn diagrams depict overlap in the number of annotated, nonredundant, differentially expressed genes as well as overlap in the total number of differentially expressed probes (in parentheses) that were significant for **(c)** Static versus Dynamic contrasts and **(d)** cold vs. ambient temperature contrasts.

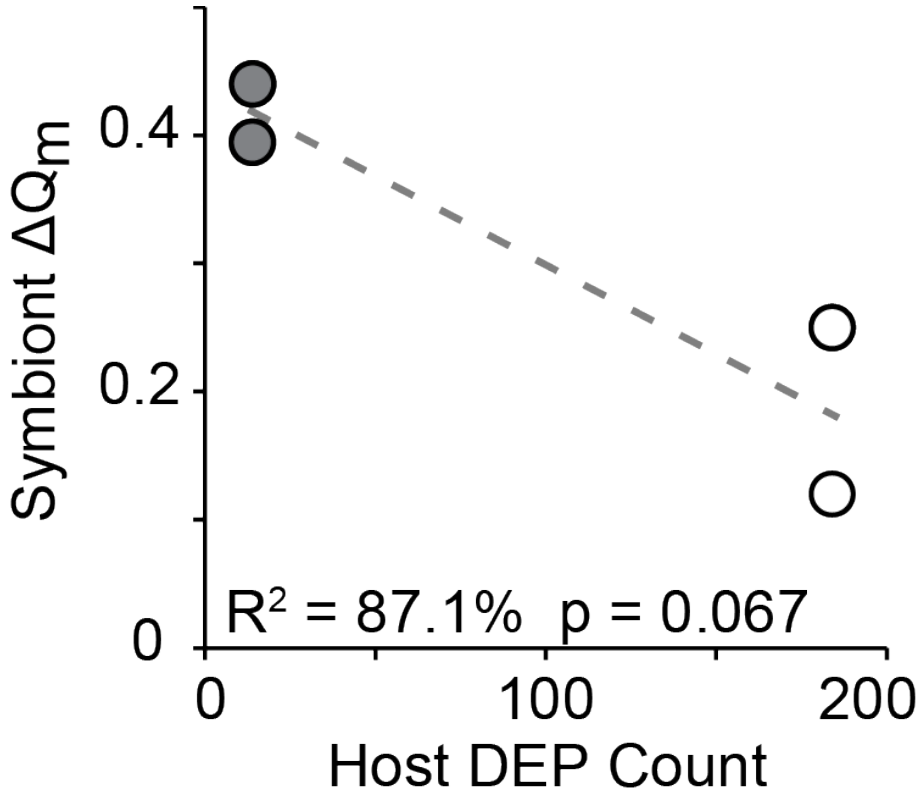
Symbiont ΔQm
0.4
0.2
0
R² = 87.1% p = 0.067
0
100
200
Host DEP Count

Figure 4-4 Correlation between the photochemistry (ΔQ_m) of a single *Symbiodinium* *'fitti'* strain and gene expression of four *Acropora palmata* genotypes (number of differentially expressed probes). White fills indicate Dynamic host gene expression phenotypes, while gray fills represent Static host gene expression phenotypes. When regressed, $R^2 = 0.87$ and $p = 0.067$.

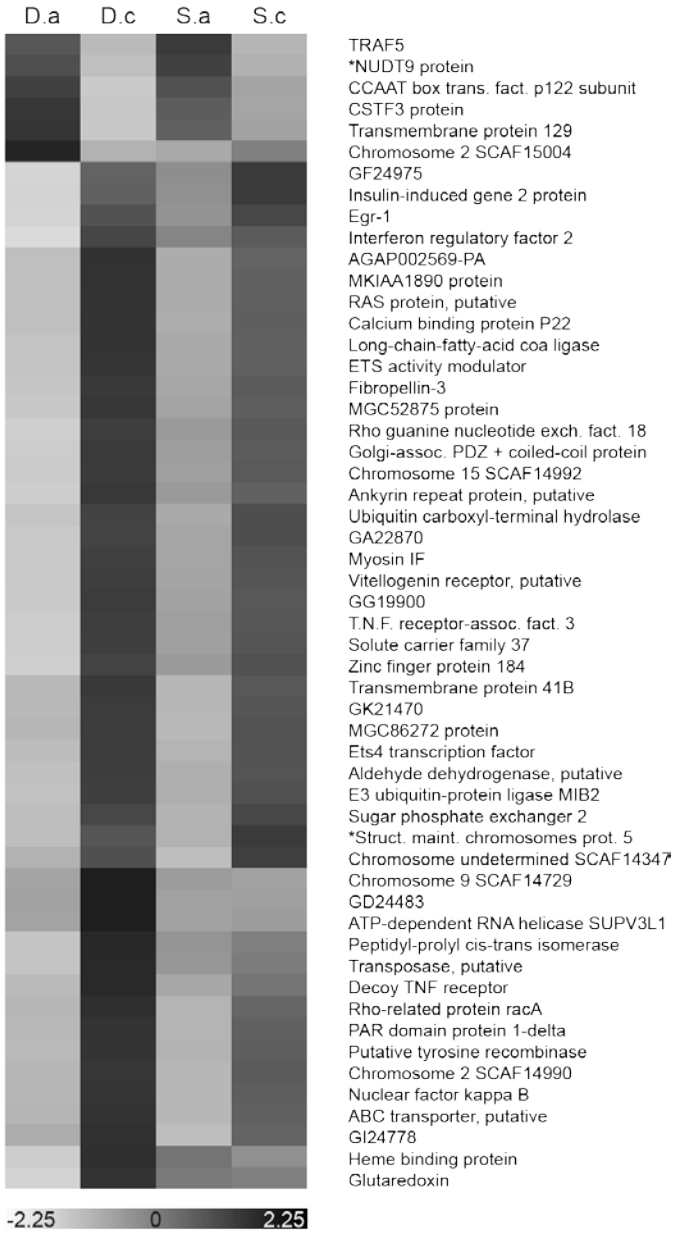

Figure 4-5 Heatmap of transcription profiles for all 54 annotated genes with significant differential expression in Dynamic hosts responding to cold stress shown as scaled expression coefficients (standard scores above or below the gene mean). Samples are coded by a leading uppercase letter (transcriptional phenotype: D = Dynamic, S = Static) and trailing lowercase letter (temperature treatment: c = cold, a = ambient). Asterisks precede genes that also showed significant expression differences in Static hosts (n = 2).

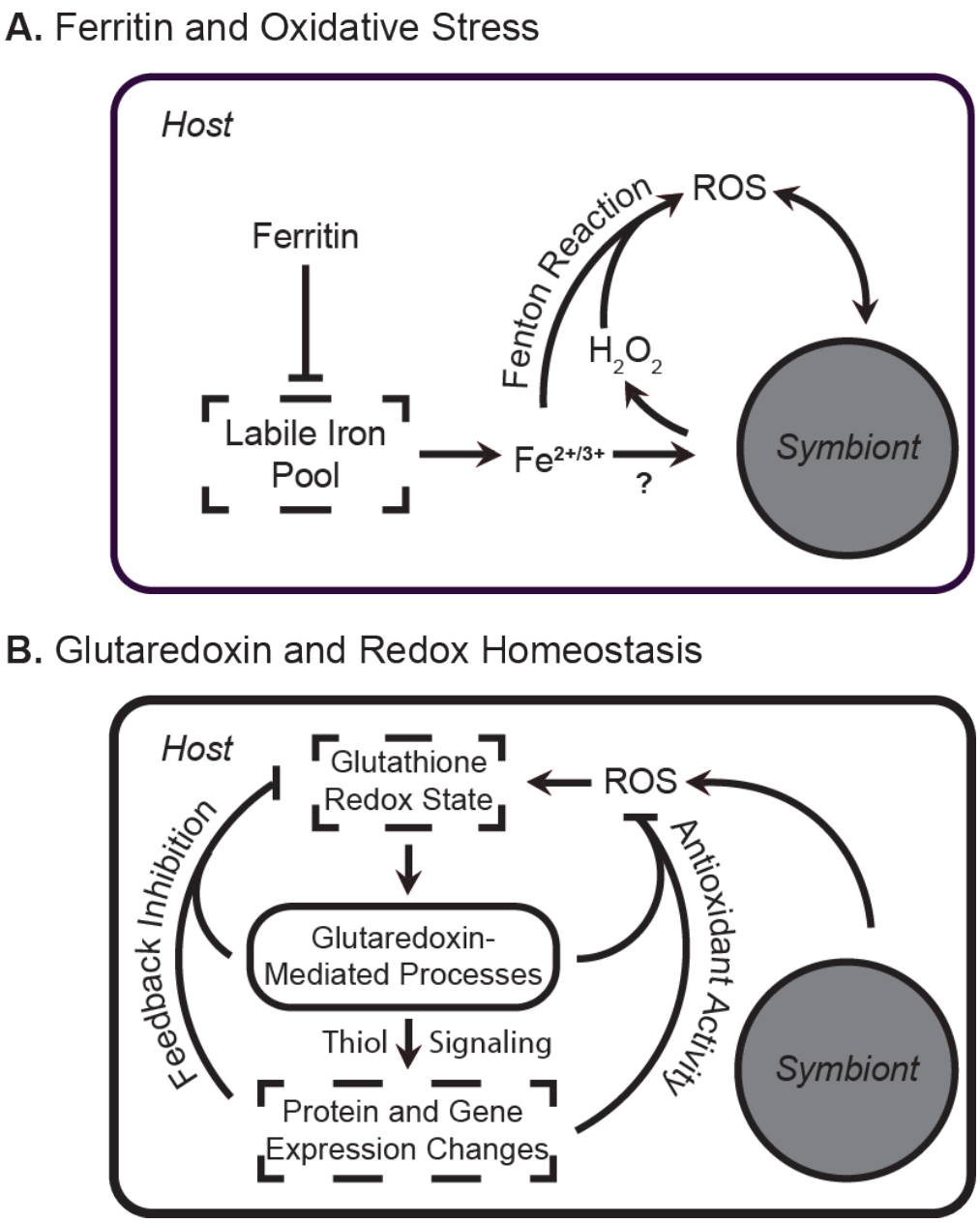

Figure 4-6 Mechanistic models for molecular interactions between partners related to **(a)** ferritin and oxidative stress and **(b)** glutaredoxin and redox homeostasis (see text). Lines connect interacting molecules or processes. Terminal arrows indicate activation or enhancement, terminal straight lines indicate inhibition. Terms in dashed squares represent targets of positive or negative regulation.

Table 4-S1 Multi-locus genotypes for *Acropora palmata* and *Symbiodinium 'fitti'* in holobionts from La Bocana Chica Reef, Puerto Morelos, Mexico. The six focal colonies are highlighted and labeled with letter names corresponding to the text. For these colonies, all hosts are unique but associate with the same symbiont strain. Asterisks indicate amplification failure in some colonies not used in the experiment.

Table 4-S1 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g45b).

Table 4-S2 Annotated genes differentially expressed between cold and ambient treatments within the two host transcription phenotypes (Dynamic and Static). *Expression Pattern* indicates which treatment showed higher expression levels. X indicates membership in functional categories of interest.

Table 4-S2 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g45b).

Table 4-S3 Annotated genes differentially expressed between Dynamic and Static hosts within three temperatures (ambient, cold, and hot). *Expression Pattern* indicates which host type showed higher expression levels. X indicates membership in functional categories of interest.

Table 4-S3 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g45b).

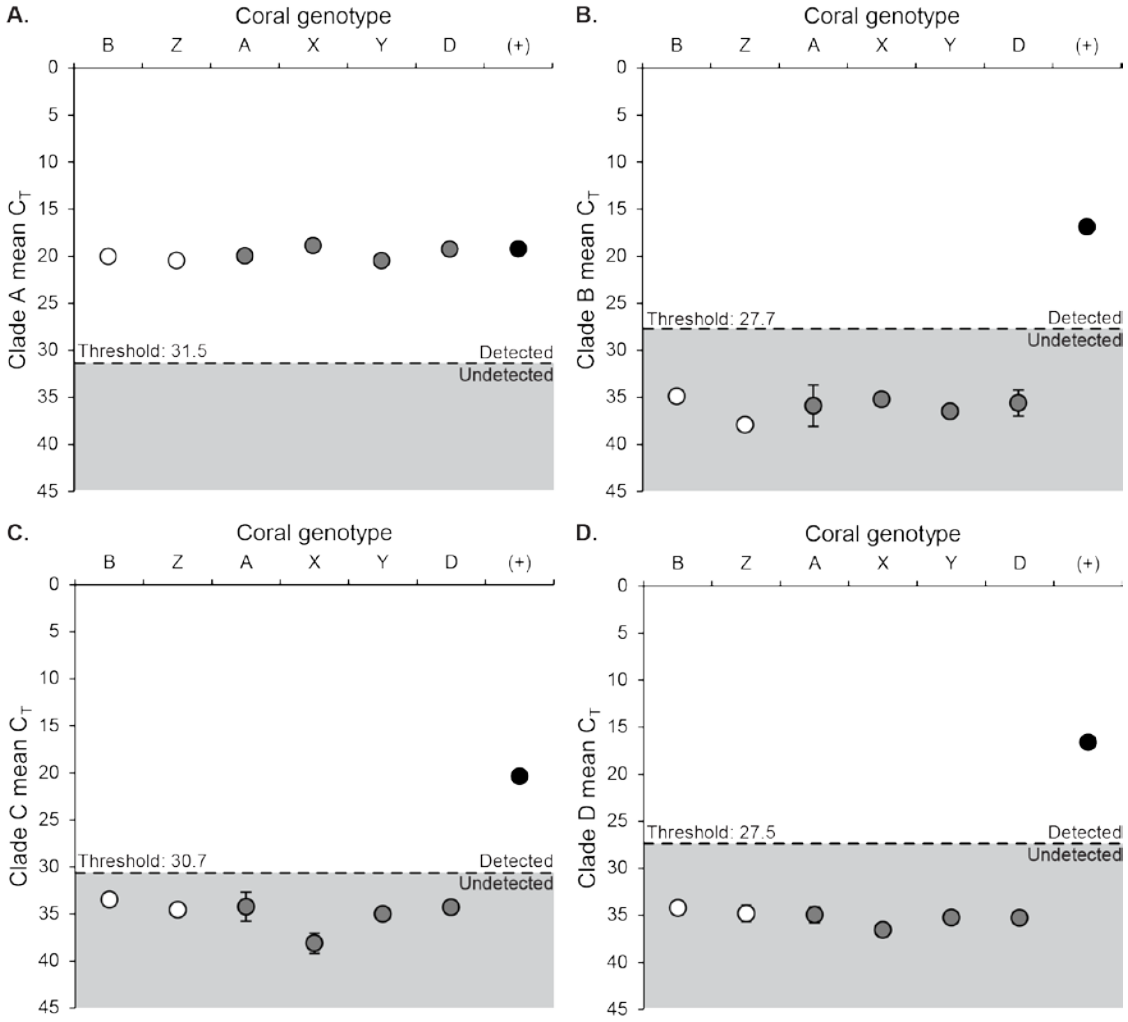

Figure 4-S1 rt-PCR screening for background symbiont strains in each host colony as in McGinley et al. (2012) using rDNA-based SYBR assays from Correa et al. (2009). Depicted are results for clades A **(a)**, B **(b)**, C **(c)**, and D **(d)**. Only clade A symbionts were detected. Thresholds for detection were determined by serial ten-fold dilution of DNA from monocultured *Symbiodinium* (1-0.0001 ng/µL); clade A: rt272 (Pacific A3); clade B: mac703 (*S. minutum*); clade C: rt152 (*S. goreaui*); clade D: A001 (*S. trenchii*). Each assay included a positive culture control and a no-template control. Each reaction was run in triplicate. Melting curves were used as in Correa et al. (2009) to confirm that "no detection" products were likely primer dimer (T_m <

80°C) and “detection” products were likely target template (T_m > 80°C). Dilution series analysis was conservatively restricted to C_T values with standard deviations within 5% of the mean across all three replicates. In addition to higher standard deviations, most excluded C_T values also melted at low temperature, indicating products were primer dimer. Had all dilution C_T values been included to extend the limit of detection of each assay, experimental C_T values still would have been above the threshold (indicating no detection). Error bars represent standard deviations based on three replicates. Where not visible, standard deviations were smaller than the point icon, except for clade B (genotypes Z, X, and Y) and clade C (genotype Z), where only one replicate had a detectable amplification signal (and therefore no standard deviation).

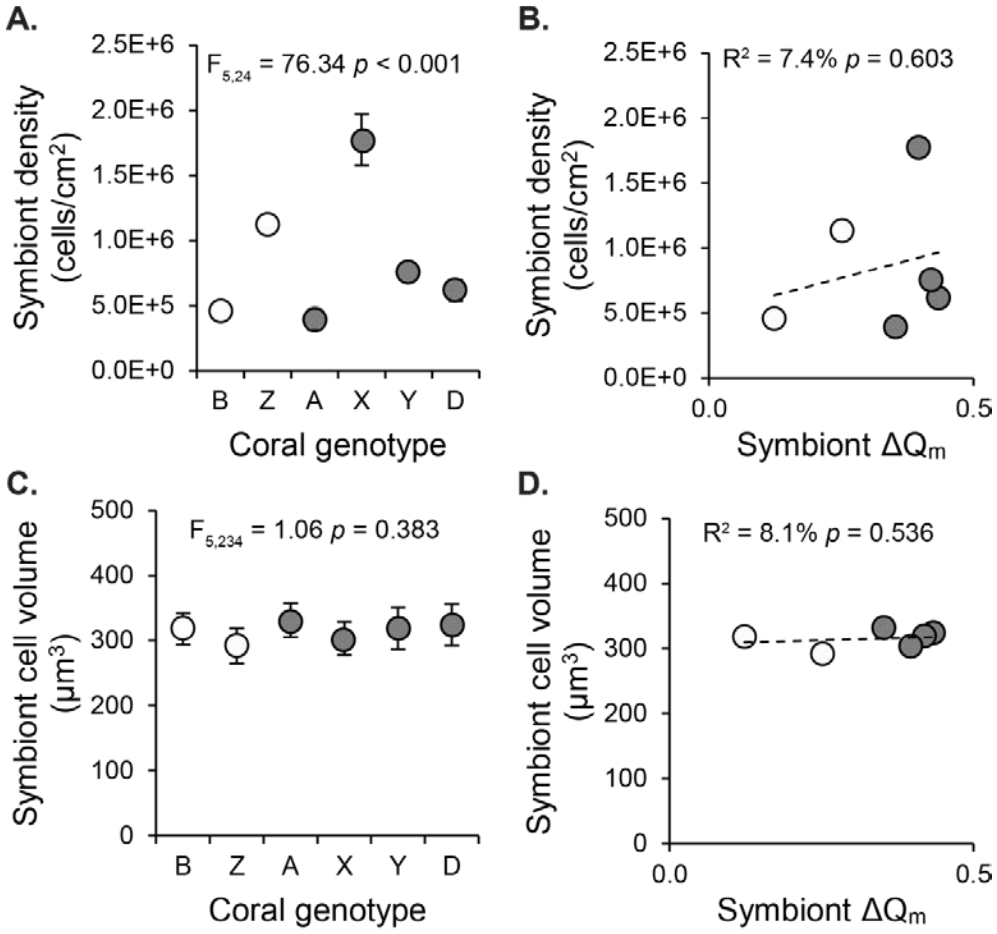

Figure 4-S2 Phenotypes of a clonal *Symbiodinium 'fitti'* strain found in six genetically distinct *Acropora palmata* host backgrounds. **(a)** Average symbiont density. **(b)** Regression of symbiont density against ΔQ_m. **(c)** Average symbiont cell volume. **(d)** Regression of symbiont cell volume against ΔQ_m. For **(a-b)**, error bars represent 95% confidence intervals for 5 replicate hemocytometer cell counts taken from a single $1cm^2$ tissue plug per colony. To normalize for ANOVA, density was natural logarithm transformed (untransformed data plotted). For **(c-d)**, error bars represent 95% confidence intervals calculated as $4\pi(abc)\bullet 3^{-1}$, where *a* is half the cell's longest diameter and *b* and *c* are taken as half the perpendicular diameter (n=40 cells per colony). To normalize for ANOVA, volume was square root transformed (untransformed data plotted). White fills indicate small ΔQ_m phenotypes, while gray fills represent large ΔQ_m phenotypes.

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Chapter 5

SYMBIODINIUM TRANSCRIPTION VARIES EXTENSIVELY AMONG INDIVIDUALS AND SPECIES WITHIN CLADE B.

Abstract

Symbiodinium spp. are dinoflagellates that often associate with reef-building corals and other marine invertebrates. While genetic data is able to differentiate at least eight highly divergent clades, hundreds of subclades, and an unknown number of species, the extent to which fine-scale genetic variation in this group translates into physiological differences is debated. To date, *Symbiodinium* gene expression has only been compared between representatives from different clades—the equivalent of contrasting genera or families. Here we applied deep-transcriptome sequencing in a comparative *Symbiodinium* framework incorporating data from species and individuals therein. Analysis of clonal strains from the four Clade B species *S. aenigmatum, S. minutum, S. pseudominutum,* and *S. psygmophilum* revealed extensive and distinct gene expression differences at the species and genotype level. These species span the two major phylogenetic radiations within Clade B and encompass two distinct ecologies. Transcriptional variation among individual cell lines within a species was primarily metabolic, whereas species-specific expression differences at orthologous sequences were consistently enriched for photosynthesis-related genes. Gene content at symbiosis-related loci segregated species by ecological niche. This study represents the first assessment of transcriptional variation within and between *Symbiodinium* species belonging to a single clade. Our data reveal that physiological differences as demonstrated by expression variation exist at the level of individuals within species and might be selected in coral-algal symbioses. Our findings expand the genomic

resources available for *Symbiodinium* and lay a foundation for future comparative genomic work with this group.

Introduction

Photosynthetic, unicellular dinoflagellates of the genus *Symbiodinium* play an essential role in the productivity of coral reefs (Muscatine and Porter 1977; Muscatine 1990; Yellowlees et al. 2008), which support tremendous marine biodiversity and ecological goods and services (Moberg and Folke 1999). Though capable of surviving independently in the water column, many *Symbiodinium* associate with corals and other cnidarians (Trench 1993; Baker 2003; Coffroth and Santos 2005). *Symbiodinium* transfer a large portion of their photosynthate to the host, providing energy for growth. Under stressful conditions, the association can break down in a process called 'coral bleaching,' with negative consequences for the host (Brown 1997; Fitt et al. 2001). Climate change is predicted to drive more frequent and intense bleaching events (Hoegh-Guldberg 1999). A large body of recent work focuses on how climate-related stressors might affect reef symbioses in the future and strives to partition the stress response of the holobiont into host versus symbiont components. Given that relatively few physiological phenotypes can be measured in corals, the community has embraced techniques that allow for detailed examination of organismal responses at the molecular level. Accordingly, the last decade has seen a surge of studies focused on coral host transcription in various contexts (Meyer and Weis 2012).

Genomics-empowered dinoflagellate and *Symbiodinium* research, on the other hand, is still in its infancy (Leggat et al. 2011a; Lin 2011). The first expressed sequence tag library for a *Symbiodinium* was published in 2007 (Leggat et al. 2007). With the incorporation of next-generation sequencing technology, genomic resources for this group have expanded greatly (Table 5-1). The first draft genome was released in 2013 (Shoguchi et al. 2013), with the

complete chloroplast genome following shortly thereafter (Barbrook et al. 2014). Multiple mRNA transcriptomes are available (Bayer et al. 2012; Ladner et al. 2012; Baumgarten et al. 2013), representing the four major clades known to associate with scleractinian corals (Clades A-D). Recent efforts have expanded in important new directions, such as the description of *Symbiodinium* microRNAs (Baumgarten et al. 2013), the comparison of orthologous genes among clades (Voolstra et al. 2009; Ladner et al. 2012; Barshis et al. 2014), and the development of the *Aiptasia-Symbiodinium* model system (Weis et al. 2008; Sunagawa et al. 2009; Lehnert et al. 2012; Xiang et al. 2013; Lehnert et al. 2014).

Evidence from gene-specific studies suggests that nuclear-encoded protein levels are modulated mainly by post-transcriptional processes in dinoflagellates generally (Morse et al. 1989; Fagan et al. 1999) and *Symbiodinium* specifically (Boldt et al. 2009; Leggat et al. 2011b; Rosic et al. 2011). It is now understood that dinoflagellates also exhibit some measure of transcriptional regulation, though exposure to different conditions causes few expression changes (Erdner and Anderson 2006; Moustafa et al. 2010). Compared to their hosts, the number and magnitude of expression changes among *Symbiodinium* exposed to thermal stress are relatively small (Leggat et al. 2011b; but see Baumgarten et al. 2013). Similar results were obtained recently with much higher coverage of the transcriptome (Barshis et al. 2014). No temperature stress expression changes were detected among *Symbiodinium* from Clades C or D *in hospite*, even though the host adjusted hundreds of genes (Barshis et al. 2013; Barshis et al. 2014). Interestingly, though the stress response was small within a given clade, a large number of transcriptional differences were maintained among clades regardless of temperature treatment (Barshis et al. 2014). This suggests that differences in steady-state, homeostatic expression profiles among lineages may strongly reflect evolutionary divergence and/or functional differences among species. If lineage-specific expression extends to the subcladal level—between species within clades or among clonal lines within species—it will be critical to recognize and incorporate this source of variation into future *Symbiodinium* genomics studies.

At present, whole transcriptome data are available for only one *Symbiodinium* species per clade (Table 5-1). For a more comprehensive understanding of physiological variation on ecologically relevant orders (i.e. at the population and species level), it is imperative to extend these expression analyses to the level of species and individuals within clades or subclades. Clade B is an ideal candidate for further characterization because of the recent description of several ecologically distinct species within the group (LaJeunesse et al. 2012; Parkinson et al. unpublished data), the existence of a draft genome, and the availability of multiple isoclonal cell cultures. The comparison of unique cultured genotypes is important because dinoflagellate transcription can vary from individual to individual (for example, among toxic and non-toxic strains; Yang et al. 2010). The extent of variation among individuals within a single *Symbiodinium* species is currently unknown. In the few studies that likely incorporated multiple symbiont genotypes (e.g. Ladner et al. 2012; Barshis et al. 2014), *Symbiodinium* were typed only at the *ITS2* and *cp23S* markers, which are not sufficient to provide individual-level resolution. To truly contextualize *Symbiodinium* gene expression data, baseline differences among individuals must also be quantified for future reference.

In this study, we analyzed steady-state gene expression among four species representing the two major evolutionary radiations within Clade B. For each radiation, two species with different ecologies were studied: either those commonly found as host-stable endosymbionts ('endosymbiotic' ecology) or those of cryptic ecological importance with a putatively free-living lifestyle ('enigmatic' ecology). Where available, we incorporated biological replication in the form of unique genotypes characterized by distinct alleles at several microsatellite loci. We found that despite an overall similarity in gene content and expression among Clade B species with diverse ecologies and evolutionary histories, each species showed a small number of diagnostic, lineage-specific expression differences, mainly in chloroplast genes. Differences among individuals within species were also detected, restricted mainly to fatty acid metabolism in *S. minutum* and photosynthesis or sulfate assimilation in *S. psygmophilum*. The genomic

resources developed herein should assist in the design and interpretation of future comparative transcriptional analyses among *Symbiodinium*.

Methods

Culturing

Isoclonal cultures of Clade B *Symbiodinium* were acquired from the Robert K. Trench and Buffalo Undersea Reef Research collections. This study included four strains of *S. minutum* (mac703, Mf1.05b, rt002, and rt351), four strains of *S. psygmophilum* (HIAp, Mf10.14b.02, PurPFlex, and rt141), one strain of *S. pseudominutum* (rt146), and one strain of *S. aenigmatum* (mac04-487), for the analysis of ten individual genomes. Most strains are available from the Provasoli-Guillard National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, or from the authors upon request. Within *S. minutum* and *S. psygmophilum*, strains were confirmed to represent unique genotypes based on repeat length variation at the microsatellite locus Sym15 (Pettay and LaJeunesse 2007) and haplotype differences in the *psbA* non-coding region (LaJeunesse and Thornhill 2011). Clade B is subdivided into two major radiations. *S. minutum* (ITS2 type B1[1]) and *S. pseudominutum* belong to the B1 radiation (sensu LaJeunesse 2005); the former commonly associates with the anemone, *Aiptasia*, and the latter is ecologically enigmatic—having been isolated from the background symbiont population of a cnidarian host but never having been detected as the numerically dominant symbiont in that host. *S. psygmophilum* (ITS2 type B2) and *S. aenigmatum* belong to the B19 radiation; the former is found as a common endosymbiont, but the ecology of the latter is enigmatic.

Single cells were originally isolated from host tissues by Schoenberg and Trench (Schoenberg and Trench 1980) using modified methods of McLaughlin and Zahl (1959) or by Mary Alice Coffroth following the methods of Santos et al. (2001). To establish initial crude

cultures, several drops of a heavy suspension of symbiont cells were transferred into nutrient-enriched filtered seawater (Provasoli 1968) and then spread onto semi-solid agar (0.8%) containing this medium. Vegetative cells from viable colonies on agar were then transferred to liquid medium ASP-8A (Ahles 1967). To generate isoclonal lines, only individual motile cells were transferred to fresh medium. An additional transfer to new media was made to synchronize all cultures. Final cultures were grown in 50 mL volumes in Erlenmeyer flasks for two weeks up to concentrations ~$1e^6$ cells mL^{-1}. Cultures were maintained in incubators at 26ºC under Philips fluorescent tubes (Koninklijke Philips Electronics, Amsterdam, the Netherlands) delivering 80–120 µmol quanta $m^{-2} s^{-1}$ photosynthetically active radiation (PAR) on a 14:10 (light: dark) photoperiod. All cultures grew together under identical conditions until processed simultaneously for RNA extraction.

RNA isolation and sequencing

At the seventh hour of the light photoperiod on the last day of the second week of growth, all target cultures were transferred to 50 mL tubes and centrifuged at 3000 RCF. The media was decanted and all pellets were flash frozen in liquid nitrogen within 1 minute of each other. Pellets were ground with a pre-chilled mortar and pestle and transferred into 1.5 mL tubes. Nucleic acids were extracted with TriReagent (Thermo Fisher Scientific, Waltham, MA) and RNA isolated and cleaned with the RNeasy Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocols.

Total RNA isolations were shipped on dry ice to the KAUST Bioscience Core Lab, where they were quality-checked using a Bioanalyzer (Agilent, Santa Clara, CA) and NanoDrop (ThermoScientific, Wilmington, DE) prior to library preparation. For Illumina sequencing, 2 × 150 bp paired-end reads were generated from oligo(dT) selected total RNA using the Illumina TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) according to manufacturer's protocols. Each pair ideally yielded a 180 bp overlapping contiguous sequence. mRNA sequencing libraries for each of the 10 samples were multiplexed in equal quantities and run in 1 lane on the Illumina

HiSeq 2000 platform, producing a total of 142 million paired-end reads. All raw RNA-seq data are available for download at the Reef Genomics website (http://www.reefgenomics.com).

Assembly and annotation

Adapters and low quality nucleotides (<20 Phred score in ASCII 33 format) were removed from raw reads with Trimmomatic (Bolger et al. 2014). Reads were error corrected with AllPaths-LG (Gnerre et al. 2011; Ribeiro et al. 2012). Quality-controlled reads combined from all samples on a per-species basis were assembled using the Trinity package (Grabherr et al. 2011) with minimum k-mer coverage of 2 and minimum contig length of 250 to generate one reference assembly per species (four total). For each sample, reads were mapped back to the appropriate assembly with Bowtie2 (Langmead and Salzberg 2012), and quantified by summing counts of all transcripts per gene (locus) with the program eXpress (Roberts and Pachter 2013), producing effective read counts and FPKM values (Fragments Per Kilobase of transcript per Million mapped reads).

Each reference assembly was annotated by sequential searches of the longest transcript per gene (locus) against SwissProt, TrEMBL, and NCBI nr sequence databases (UniProt Consortium 2013; Pruitt et al. 2014) using BLASTx (Altschul et al. 1990) and the October 2013 releases. Only hits with e-values $<1e^{-5}$ were retained. All genes remaining unannotated after BLASTx against the first database were passed onto the next database and so on. Gene Ontology categories were assigned through the BLASTx hit to SwissProt or TrEMBL databases and subsequent mapping to the UniProt-GOA project (Dimmer et al. 2012). The assembled and annotated transcriptome sequences for each species are available for download at the Reef Genomics website (http://www.reefgenomics.com).

The recently published *S. minutum* draft genome (Shoguchi et al. 2013) was derived from strain Mf1.05b, which was also included in this study. To compare our sequencing results to this resource, we aligned our Mf1.05b Illumina assembly to the exome of the draft genome to confirm that a high percentage of sequences matched.

To assess how comparable our Clade B species assemblies were in terms of gene content independent of expression, complete assemblies were uploaded into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). IPA compares user-provided gene lists to reference canonical pathways in the manually-curated Ingenuity Knowledge Base. It generates a ratio of genes present vs. total genes belonging to a pathway and testing for the probability of significant enrichment for that pathway in the *Symbiodinium* transcriptome with Fisher's Exact Test. The Ingenuity Knowledge Base is designed mainly for model organism data, and so results should be interpreted in the context of pathways that are well annotated and highly conserved across eukaryotes. We were less concerned about pathway identity and more interested in whether representation within a pathway was similar across Clade B species. Thus, we compared ratio values for all transcriptomes at all pathways determined to be significantly enriched in the data set at $p_{adj} < 0.05$.

As an additional metric of comparison across Clade B species, we mined all transcriptomes for repeats and flanking regions using the software SciRoKo (Kofler et al. 2007). We considered repeat motifs between 2 and 6 bp. To further develop genomic resources for the target Clade B species, we identified sequences with flankers amenable to primer design. Further work will be required to screen potential markers against cultures and wild-collected material to identify variable sites (Parkinson et al. unpublished data).

Ortholog identification and differential expression between species, lineages, and ecologies

To test for differential expression between the four species, it was necessary to identify a set of comparable orthologous genes. Open reading frames were predicted for each transcriptome with Transdecoder (Haas et al. 2013). Orthologous genes were identified via reciprocal BLASTp of open reading frames pairwise for each species within the program InParanoid (Remm et al. 2001), retaining hits with bitscores >300. Multiparanoid (Alexeyenko et al. 2006) was then used to identify orthologs that occurred exactly four times (once in each species).

Most current software designed to analyze differential expression for RNA-seq data assumes raw read counts among samples mapped to a common transcriptome, and therefore only accepts integer values as input. To compare expression at orthologous genes across species, it was necessary to normalize read counts by transcript length using FPKM to account for species-specific sequence length differences. This normalization produced many decimal expression values that were still informative. For example, a gene with an FPKM of 0.8 in one individual and 0.2 in another is still expressed four times greater in the first individual than in the second. Rather than lose the information associated with genes below an integer cut-off, we decided to scale FPKM by a common factor such that the lowest expressed gene's value equaled 1. Thus, a scaled FPKM of 50 means the gene is expressed 50 times higher than the lowest expressed gene retained in the data set. This way, all orthologs could be compared in the scaled FPKM space.

Scaled FPKM data were then used as input for the R package EdgeR (Robinson et al. 2010), which accommodates data sets with unequal replication when performing comparisons among treatments. No additional normalization procedures were carried out within the program. Significant differential expression was determined by pairwise comparisons among species with FDR <0.1. To test by lineage, all *S. minutum* and *S. pseudominutum* samples were grouped as "B1" and all *S. psygmophilum* and *S. aenigmatum* samples were grouped as "B19." To test by ecology, all *S. pseudominutum* and *S. aenigmatum* samples were grouped as 'enigmatic,' and all *S. minutum* and *S. psygmophilum* samples were grouped as 'endosymbiotic.'

A number of cnidarian genes were recently proposed as potentially important in symbiosis maintenance (Meyer and Weis 2012). To assess whether there were differences in the number of these genes among *Symbiodinium* (particularly in enigmatic vs. endosymbiotic species), we searched each transcriptome file for the number of lines (genes) matching a given input string from the list of candidate genes and compared across the four Clade B species. We converted the ordinal gene content information into interval data via optimal scaling for use in a

categorical principle component analysis (PCA) implemented in IBM SPSS Statistics v.22 (IBM, Inc., Armonk, NY).

Differential expression within species

For both *S. minutum* and *S. psygmophilum*, isoclonal cultures of four individual genotypes each were available, providing two opportunities to test for differential expression within a species. Each species was analyzed separately with the R/Bioconductor package DESeq (Anders and Huber 2010). Raw read counts were normalized with the geometric mean method. Due to a lack of replicates for each individual, dispersion was calculated across all four individuals per species under the assumption that a majority of genes were not differentially expressed, appropriate for *Symbiodinium* of the same species under steady-state conditions. The dispersion estimate from this procedure is generally more conservative than when calculated using replicates. Significance of differentially expressed genes (DEGs) was determined by pairwise comparisons among individuals based on the negative binomial distribution with a false discovery rate adjusted p-value (FDR) <0.1.

Visualization and functional analyses

Each list of differentially expressed genes was visualized as a heatmap by converting expression data (scaled FPKM for between species, geometric mean normalized read counts for within species) to standardized expression above or below the gene mean and plotting the output in MeV (Saeed et al. 2003). Lists were tested for Gene Ontology (GO) term functional enrichment with the R/Bioconductor package topGO (Alexa and Rahnenfuhrer 2010), using the default "weight01" Alexa algorithm with a cutoff of p <0.05. Additionally, lists of the top 200 highest expressed genes in each transcriptome were tested for enrichment. GO term functional enrichment analysis tests for over-representation of genes related to a particular biological process within a set of DEGs. It does so by comparing the observed number of those genes against the number expected to be drawn by chance given the length of the DEG list and the background distribution of all genes in the transcriptome.

Results

Transcriptome assemblies

From ten samples run on one multiplexed lane of Illumina sequencing we generated ten high quality transcriptome data sets (Table 5-2). Total reads per sample ranged from 8.7-23.7 million (mean: 14.3 million); the percent of reads per sample surviving quality control ranged from 88.1-89.0% (mean: 88.5%). For each of the four species of interest, we generated a single reference assembly from either a combination of all samples of a given species (in the cases of *S. minutum* and *S. psygmophilum*) or from the single representative sample (in the cases of *S. aenigmatum* and *S. pseudominutum*). Across species, transcriptome statistics were quite similar.

The number of loci (the longest transcript for a related set of splice variants of a gene) per transcriptome ranged from 45.3-51.2 k (mean: 48.7 k). The number of predicted open reading frames ranged from 38.9-42.9 k (mean: 41.3 k; Table 5-3). The N50 statistic (the value for which all loci of equal or longer length encompass half of the total length of all loci) ranged from 1,355-1,579 bp (mean: 1,515 bp), while the average locus length ranged from 984-1,128 bp (mean: 1,078 bp). The percentage of loci that could be successfully annotated ranged from 44.9-47.8% (mean: 46.5%). After mapping, coverage for at least 75% of loci ranged from a low of 4× (an outlier) to a high of 12× (mean: 7×). When BLASTed against the draft *S. minutum* genome's exome, our Mf1.05b assembly had an overall alignment rate of 73%.

After uploading the four reference assemblies into IPA, 19 relevant canonical pathways with significant gene set representation in all species were identified. When the ratio of genes observed to total associated genes per pathway were plotted side by side (Figure 5-1), each pathway was evenly represented in the transcriptomes generated for each Clade B *Symbiodinium* species. The four species were also roughly equivalent in terms of their proportions of repeat motifs (Figure 5-2). Repeat numbers ranged from 829-1,141 per species. Tri- and hexanucleotides were the most common motifs.

Top 200 highly expressed genes

For each sample we identified the top 200 most highly expressed genes and analyzed them for enrichment of GO terms (Table 5-S1). Though all species shared enrichment among highly expressed genes for the biological processes nucleosome assembly, translation, protein-chromophore linkage, glycolysis, and photosynthesis, *S. minutum* was uniquely enriched for hydrogen peroxide catabolism, long-chain fatty-acid metabolism, glutamine metabolism, and response to cold, while *S. psygmophilum* was uniquely enriched for fatty acid beta-oxidation, photorespiration, and cell wall catabolism, and *S. aenigmatum* was uniquely enriched for gluconeogenesis, immune response signaling pathway, negative regulation of apoptosis, and positive regulation of cell proliferation. Only *S. minutum* and *S. psygmophilum* were enriched for nitrate assimilation and pyruvate metabolism. *S. pseudominutum* had the least unique enrichment profile.

Between species comparisons

The number of predicted open reading frames per species reference assembly ranged from 38.9-42.9 k (mean: 41.3 k; Table 5-3). The total number of orthologous sequences shared among all species after filtering out paralogs and low quality matches was 19,359. For the restricted set of orthologs, FPKM values were scaled relative to the lowest expressed gene, and each species was tested against all others pairwise for differential expression. A total of 452 unique DEGs (184 annotated) were detected among all contrasts. The numbers of DEGs for each contrast were as follows: *S. psygmophilum*-*S. pseudominutum*: 45 (19 annotated); *S. pseudominutum*-*S. minutum*: 53 (23 annotated); *S. aenigmatum*-*S. pseudominutum*: 56 (28 annotated); *S. psygmophilum*-*S. aenigmatum*: 169 (69 annotated); *S. aenigmatum*-*S. minutum*: 208 (91 annotated); *S. psygmophilum*-*S. minutum*: 256 (103 annotated). DEG numbers are summarized in Figure 5-4; heatmaps for annotated genes in each contrast are presented in Figure 5-S1. Samples were visualized in a multidimensional scaling (MDS) plot using expression

profile fold changes (Figure 5-6), showing clustering based on non-differentially expressed or differentially expressed genes.

The full list of GO terms enriched in the DEG list for each species contrast can be found in Table 5-S3. Highlights are listed below. B1/B19 refers to phylogenetic radiation, whereas endosymbiotic/enigmatic refers to symbiont ecology. The *S. psygmophilum*-*S. pseudominutum* contrast (a B19 endosymbiotic vs. a B1 enigmatic) was enriched for processes including photosynthesis, response to cold, and transmembrane transport. The *S. pseudominutum*-*S. minutum* contrast (a B1 enigmatic vs. a B1 endosymbiotic) was enriched for photosynthesis and apoptosis. The *S. aenigmatum*-*S. pseudominutum* contrast (a B19 enigmatic vs. a B1 enigmatic) was enriched for photosynthesis and heat acclimation. The *S. psygmophilum*-*S. aenigmatum* contrast (a B19 endosymbiotic vs. a B19 enigmatic) was enriched for photosynthesis and mitosis. The *S. aenigmatum*-*S. minutum* contrast (a B19 enigmatic vs. a B1 endosymbiotic) was enriched for stress response (notably not for photosynthesis). The *S. psygmophilum*-*S. minutum* contrast (a B19 endosymbiotic vs. a B1 endosymbiotic) was enriched for photosynthesis, phagocytosis, and cell signaling.

When testing by lineage, the contrast between B1 and B19 radiation samples resulted in the same DEG list as the contrast between *S. minutum* and *S. psygmophilum*. When testing by ecology, the contrast between enigmatic and endosymbiotic samples resulted in a DEG list with only four additional genes that were not detected when testing either *S. pseudominutum* or *S. aenigmatum* against either *S. minutum* or *S. psygmophilum* (Figure 5-S2). Of the four, only one was annotated (the pre-mRNA splicing factor SLU7).

A total of 57 unique strings derived from candidate symbiosis-related gene descriptions were queried against the annotation files for each species (Table 5-S2). Only 36 returned hits. Of these, only “o-methyltransferase,” “abc transporter,” “carbonic anhydrase,” “caspase,” and “glutathione s-transferase” showed a range greater than five hits. “O-methyltransferase” had an average of 43.5 vs. 63 hits in the enigmatic-endosymbiotic contrast, whereas the difference

between B1 and B19 was only 55 vs. 51.5. Similarly, “caspase” and “glutathione s-transferase” had fewer hits in enigmatic compared to endosymbiotic species, with no average difference among lineages. In terms of expression differences among enigmatic and endosymbiotic species at these genes, glutathione S-transferase was significantly downregulated in *S. aenigmatum* and *S. pseudominutum* relative to either *S. minutum* or *S. psygmophilum*. An abc transport and a carbonic anhydrase were also downregulated in *S. aenigmatum* (but not *S. pseudominutum*). An aquaporin was upregulated in *S. aenigmatum.* When gene content data were analyzed in a categorical PCA, the first dimension segregated species by ecology and explained 69% of variance; the second dimension segregated species by phylogenetic lineage and explained 29% of the variance (Figure 5-7).

Within species comparisons

When compared against each other pairwise in the program DESeq, the four *S. minutum* individuals differentially expressed an average of 126 genes per contrast (34 annotated) with a mean of 2.8 significant contrasts for each of the 126 genes (out of 6 pairwise comparisons; Figure 5-3a). Among the four *S. psygmophilum* individuals, an average of 111 genes per contrast were differentially expressed (31 annotated) with a mean of 2.3 significant contrasts for each of the 111 genes (Figure 5-3b). In *S. minutum*, the most enriched biological processes were malonyl-CoA biosynthesis, long-chain fatty acid biosynthesis and metabolism, carbohydrate metabolism, ATP and GTP catabolism, and regulation of mitosis, cell growth, and RNA splicing (Table 5-S3a). In *S. psygmophilum*, the top categories were photosystem II electron transport, oxidation-reduction, sulfate assimilation, hydrogen sulfide biosynthesis, and DNA replication (Table 5-S3b).

Discussion

The unprecedented fine-scale resolution of this study revealed expression variation in key physiological functions among closely related *Symbiodinium* cell lines, information essential for

future physiological and ecological studies of *Symbiodinium in hospite* and in culture. The species and cell lines within *Symbiodinium* Clade B investigated here maintain transcriptional differences reflective of their life styles and phylogenetic histories. The B1 radiation was characterized by 135 DEGs in comparison to the B19 species while the endosymbiotic species had 23 DEGs in comparison to the enigmatic, putatively non-symbiotic species.

Transcriptome assemblies

The ten transcriptomes were of high quality, and yielded four reference assemblies with remarkably similar characteristics (Table 5-2). Our results matched well with previously published data for *S. minutum* transcriptome assembly size (48.7 k vs. 48.9 k; Bayer et al. 2012) and annotation (46.5% vs. 44.6%; Baumgarten et al. 2013), as well as the predicted number of protein-coding genes from the *S. minutum* genome (41.3 k vs. 41.9 k; Shoguchi et al. 2013). Our assembly of the *S. minutum* strain Mf1.05b matched a large percentage of the draft exome based on the same culture (73%), with most differences likely accounted for by restricting acceptable loci to those with lengths >250bp. Moreover, pathway analysis revealed each species to have nearly equal representation across several conserved eukaryotic gene sets (Figure 5-1), and repeat region analysis resulted in equivalent microsatellite frequencies (Figure 5-2). Thus, the Clade B *Symbiodinium* transcriptomes were relatively homogenous across species and Illumina sequencing captured most of the available gene content resulting in expression coverage sufficient for comparative analyses of differential expression.

Top 200 highly expressed genes

When compared to their entire transcriptomes, all *Symbiodinium* strains in this study were enriched in their most highly expressed genes for transcripts associated with the ribosome, nucleosome, cell wall, chloroplast, thylakoid membrane, photosystems I and II, respiratory chain, and ATP synthase (Table 5-S1). Given that *Symbiodinium* derive most of their nutrition from photosynthesis (but see Jeong et al. 2012), it is not surprising that a large number of chloroplast genes are expressed under steady state conditions. In culture, *Symbiodinium* develop thicker cell

walls than *in hospite* (Colley and Trench 1983); high expression of cell wall genes may reflect the fact that these cultures were maintained independent of a host.

Between species comparisons

By comparing species within a single clade, we were able to greatly expand on the number of orthologs available for global expression profiling of *Symbiodinium*. We identified ~20k orthologs in our study of Clade B (Table 5-3); far more than the ~5k identified in another pair of studies with similar methods contrasting Clades C and D (Ladner et al. 2012; Barshis et al. 2014). Overall, steady-state gene expression is quite similar amongst Clade B *Symbiodinium*. Of the nearly 20k orthologs shared across all species, at most only ~.01% were differentially expressed under steady-state conditions (Figure 5-4; Figure 5-S1), supporting the contention that only a small fraction of genes are under transcriptional control in *Symbiodinium* (Boldt et al. 2009; Leggat et al. 2011b; Rosic et al. 2011) and other dinoflagellates (Morse et al. 1989; Fagan et al. 1999). Nuclear genes are permanently super-coiled in *Symbiodinium* (Blank and Trench 1985) and therefore perhaps more difficult to regulate through traditional transcriptional means. The plastid minicircles on the other hand may be subject to alternative transcriptional control (Dang and Green 2010), perhaps accounting for the large representation differentially expressed plastid genes in this and other studies (Baumgarten et al. 2013).

The majority of transcripts provided almost no species-level signal. When clustered based on non-significant gene expression, 8 of 10 samples spanning 3 species grouped closely (Figure 5-6a), with only the *S. minutum* strain mac703 and *S. aenigmatum* strain mac04-87 separating from the main cluster. However, when plotting just the few (452) significant DEGs, all species segregated well, matching expectations based on species identity (Figure 5-6b). The shift in clustering that takes place when considering only DEGs suggests that a small number of genes may have a large influence on the differences among ecologically distinct *Symbiodinium* (see below). Along the primary multi-dimensional scaling (MDS) axis, the two enigmatic species appeared to have expression profiles intermediate between the two endosymbiotic species,

suggesting shared molecular characteristics among *Symbiodinium* that do not generally establish dominance in cnidarian hosts despite divergent evolutionary ancestry. These enigmatic species may have more similar ecological habitats, which may account for some degree of shared expression. *S. aenigmatum* was the most transcriptionally unique species when considering the first and second MDS axis.

Not surprisingly, the largest number of DEGs was detected when comparing *S. minutum* and *S. psygmophilum*, the contrast with the greatest power to detect differences owing to the four biological replicates within each species (Figure 5-4; Figure 5-S1). *S. minutum* is a predominantly tropical species, whereas *S. psygmophilum* is a predominantly temperate species; some expression differences likely correlate with these distinct ecologies even though both species are 'endosymbiotic.' The next three contrasts with the largest number of significant genes all involved *S. aenigmatum*, likely either one of the oldest or one of the most rapidly evolving lineages within Clade B (LaJeunesse 2005; Parkinson et al. unpublished data). The three contrasts with the smallest number of DEGs all involved *S. pseudominutum*, which had the most intermediate expression profile. Hierarchical clustering of DEGs based on scaled FPKM distances yielded three major groups: an *S. minutum* cluster, and *S. psygmophilum* cluster, and a combined cluster for the two enigmatic species (*S. aenigmatum* and *S. pseudominutum*), which each only had one strain represented in the study (Figure 5-5). K-means clustering supported the same three groups.

Combining species by lineage (B1 or B19) or by ecology (enigmatic or endosymbiotic) for comparing expression provided almost no additional information over the two-species comparisons. The B1-B19 contrast was equivalent to the *S. minutum*-*S. psygmophilum* contrast in terms of identity of DEGs, meaning that the *S. minutum*-*S. psygmophilum* contrast either captured all the major differences between lineages, or that adding just one more sample to each group even though they were different species did not affect gene-wise variation sufficiently to alter the statistical outcome. Similarly, the enigmatic-endosymbiotic contrast only recovered four unique

genes that had not been identified in two-species contrasts. Only one was annotated (a general mRNA splicing factor). It is possible that including additional species and/or more replication among the enigmatic species in future gene expression comparisons might lead to a different result.

Photosynthesis gene enrichment

Expression differences among Clade B species are consistently enriched for photosynthesis genes (Table 5-S2). This observation is not surprising for several reasons. First, they are highly expressed, and therefore differences are more easily detected. Second, a number of them are encoded on minicircles (Koumandou et al. 2004), which have different transcriptional mechanisms than nuclear encoded genes (Dang and Green 2010). Finally, light availability is a main axis of niche differentiation for *Symbiodinium* (Iglesias-Prieto and Trench 1997). In biogeographic surveys of marine mutualisms, depth is often a key factor explaining the distribution of *Symbiodinium* molecular and functional diversity (e.g. Rowan and Knowlton 1995; LaJeunesse 2002; Frade et al. 2008). Ecological specialization for unique light niches may be facilitated mechanistically by transcriptional variation in plastid minicircles, which may then be reinforced by coevolution with particular hosts.

Although we would expect photosynthesis genes to be regulated by light intensity in *Symbiodinium* as it is in other photosynthetic organisms (Escoubas et al. 1995; Pfannschmidt 2003), only small changes in *psaA* expression (encoding the P_{700} protein of photosystem I) and *psbA* expression (encoding the D1 protein of photosystem II) have been detected during low-light to high-light transitions (McGinley et al. 2013). Over-representation of plastid genes in the species contrast DEG lists cannot be attributed to light intensity differences because all cultures were reared under identical light conditions. Instead, we likely observed homeostatic differences in expression maintained by distinct species.

These differences may relate to inherent variation in the circadian rhythm among species (Van Dolah et al. 2007; Sorek and Levy 2012) or to functional variation in photosynthetic

processes. For example, distinct *Symbiodinium* species show unique patterns of *psaA* and *psbA* expression during thermal stress (McGinley et al. 2012), with thermally sensitive types downregulating these genes and thermally tolerant types remaining stable. The rt-013 Clade B culture used by McGinley et al. (2012) is a strain of *S. pseudominutum* that had previously exhibited thermal sensitivity in terms of PSII photochemistry disruption during thermal stress (Robison and Warner 2006). Interestingly, this individual did not show the same signature of downregulation as the other thermally sensitive culture (type A13, which also exhibits an enigmatic, possibly opportunistic lifestyle).

Other genes

Differential expression among species is not restricted to photosynthesis genes, however. For example, we used the list of candidate symbiosis genes proposed by Meyer and Weis (2012) to look for differences in gene copy number and expression among enigmatic and endosymbiotic strains, where one might most expect to find disparity. With the caveat that these candidate genes were derived from host tissues and that endosymbiotic strains growing in culture independent of a host likely don't have the same symbiosis gene expression patterns as they might *in hospite*, we nevertheless identified a small number of differences (Table 5-S3). The primary PCA dimension clearly separated species by symbiosis ecology, and accounted for 69% of the variance (Figure 5-7). Component loading vectors showed that the pattern was driven by a higher number of genes related to catalase, glutamate dehydrogenase, glutamine synthetase, glutathione S-transferase, glutathione peroxidase, O-methyltransferase, peroxiredoxin, and vitamin K epoxide reductase in endosymbiotic species.

On average, enigmatic species possessed 20 fewer copies of O-methyltransferase in their transcriptomes than endosymbiotic species, though no expression changes were detected for this gene. Glutathione S-transferase, on the other hand, had fewer copies and lower expression in enigmatic species, while carbonic anhydrase and glutathione reductase were differentially expressed in at least one enigmatic species, despite overall similarity in copy numbers among

species. These examples highlight the fact that ecological differentiation among *Symbiodinium* may stem from both differences in gene content and from differential expression of genes in shared pathways. However, the method of gene detection described here was susceptible to false signals due to the unequal representation of individuals in each group. For example, more peroxiredoxin genes were recovered on average in endosymbiotic species simply because there were eight transcriptomes to search compared to just two for enigmatic species. Due to variation among individuals, some endosymbiotic genotypes expressed fewer peroxiredoxin genes than their enigmatic counterparts. Complete genome sequencing will be crucial to reliably quantify gene presence or absence for comparative purposes, but these results provide preliminary evidence that at least some symbiosis gene copy numbers vary among *Symbiodinium* species with distinct symbiosis ecologies.

Within species comparisons

Comparison of four clonal cell lines within a single species recovered ~100 differentially expressed genes (Figure 5-3). Interestingly, the biological processes enriched in the set of differentially expressed genes among *S. minutum* did not overlap with those of *S. psygmophilum*, except for basic molecular processes like nucleic acid synthesis (Table 5-S4). This raises two important points. First, transcriptional variation among individuals differs from species to species. Second, the extent of variation among individuals may be much greater than previously acknowledged. Though 100 out of 50 k genes represents a small fraction of the transcriptome, such differences may be important. For example, in *S. minutum*, strain-level differences included an abc transporter and a glutathione reductase, which are sometimes differentially expressed among symbiotic vs. nonsymbiotic hosts (Meyer and Weis 2012), and may be linked to variation in symbiosis quality. There were also inherent differences in the expression of long chain fatty acid CoA ligase, long chain acyl-CoA synthetase, and acetyl-CoA carboxylase, indicating that certain strains regulate fatty acid metabolism differently even under identical conditions. These differences may scale up *in hospite* or under environmental stress, potentially contributing to

performance differences among holobionts. Similar fine-scale variation has been observed among maize genotypes with distinct flavonoid content (Casati and Walbot 2003) and among dinoflagellate genotypes with distinct toxicities (Yang et al. 2010).

Future prospects in Symbiodinium *genomics studies using field-collected samples*

The three previous comparisons of *Symbiodinium* orthologs to date have all relied on at least one sample derived from field-collected fragments of host tissue. By using isoclonal cultures exclusively, we removed potential host effects on symbiont transcription, though at the expense of learning more about how *Symbiodinium* interact with their hosts on a molecular level. Ongoing studies with clonal hosts and symbionts in the model *Aiptasia-Symbiodinium* system will certainly address this knowledge gap in the future (Lehnert et al. 2014). In the meantime, most *Symbiodinium* transcriptomic studies will continue to incorporate field-collected rather than laboratory-reared samples, which makes sense given their ecological relevance. However, it will be important for investigators to consider all possible sources of variation that might be contributing to their results, including differences at the clade, species, and individual level.

We recommend that in the future, care should be taken when designing comparative expression studies to be certain that the focal *Symbiodinium* indeed belong to the same species and are relatively homogenous (that is, represent the majority of the symbiont population). *ITS2* typing alone may be insufficient; at least among Clade B, this marker can underestimate diversity, as multiple species share the same ancestral *ITS2* type (Finney et al. 2010; Parkinson et al. unpublished data). Where possible, more than one marker should be used, and relative proportions of each species should be calculated. The studies of Ladner et al. (2012) and Barshis et al. (2014) provide a good example for dealing with these issues. At this point, it is unclear how much ‘noise’ low-abundance or background symbionts may contribute to overall expression levels from a sample collected from host tissue, but we anticipate that if they represent only a small fraction of the cells, then their mRNA is unlikely to be detected absent an intermediate amplification step or sequencing to a high depth.

This study provides a large genomic resource. Transcriptome-wide scans of genes under selection, identification of single nucleotide polymorphisms, and phylogenetic and microsatellite marker development based on these data are underway. Subsequent experiments with the same cultures under different thermal, osmotic, and/or light conditions may reveal further expression variation during stress. It will also be useful to reexamine these strains' transcriptomes once placed in an experimental host.

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Figures

Table 5-1 Summary of the genomic resources available for *Symbiodinium*. *S. minutum* belongs specifically to the "B1[1]" ITS2 type (in the B1 radiation).

ITS2 Type	*Species Name*	*Source Material*	*Resource Type*	*Publication*
C3	NA	*Agaricia* sp. host tissue	Chloroplast Genome	Barbrook et al. 2013
A1	*S. microadriaticum*	Culture rt370	mRNA Transcriptome	Baumgarten et al. 2013
A1	*S. microadriaticum*	Culture rt370	smRNA Transcriptome	Baumgarten et al. 2013
B1[1]	*S. minutum*	Culture Mf1.05b	Draft Genome	Shoguchi et al. 2013
A1	*S. microadriaticum*	Culture CassKB8	mRNA Transcriptome	Bayer et al. 2012
B1[1]	*S. minutum*	Culture Mf1.05b	mRNA Transcriptome	Bayer et al. 2012
C3k	NA	*Acropora hyacinthus* host tissue	mRNA Transcriptome	Ladner et al. 2012
D2	NA	*Acropora hyacinthus* host tissue	mRNA Transcriptome	Ladner et al. 2012
A1	*S. microadriaticum*	Culture CassKB8	EST Library	Voolstra et al. 2009
C3	NA	*Acropora aspera* host tissue	EST Library	Leggat et al. 2007

Table 5-2 Sequencing and assembly quality for four species and ten culture of Clade B *Symbiodinium*. QC = quality control; ORF = open reading frame.

Species	*Strain*	*Total Read Count*	*Survived QC (%)*	*Assembled Base Pair Count*	*Loci Count*	*Predicted ORF Count*
S. minutum	rt351	8.68E+6	88.68	57.2E+6	51,199	42,929
S. minutum	mac703	10.9E+6	89.03			
S. minutum	rt002	12.4E+6	88.00			
S. minutum	Mf1.05b	19.3E+6	88.40			
S. psygmophilum	Mf10.14b.02	11.1E+6	88.63	57.2E+6	50,745	42,740
S. psygmophilum	PurPflex	11.7E+6	88.55			
S. psygmophilum	HIAp	13.4E+6	88.14			
S. psygmophilum	rt141	19.5E+6	88.47			
S.aenigmatum	mac04-487	11.9E+6	88.10	44.6E+6	45,343	38,923
S. pseudominutum	rt146	23.7E+6	88.83	51.3E+6	47,411	40,716

Species	*Strain*	*Loci Annotated (%)*	*Longest Locus Length (bp)*	*Mean Locus Length (bp)*	*N50*	*GC Content (%)*
S. minutum	rt351	47.3	37,483	1,118	1,579	51.33
S. minutum	mac703					
S. minutum	rt002					
S. minutum	Mf1.05b					
S. psygmophilum	Mf10.14b.02	47.7	31,367	1,128	1,618	51.37
S. psygmophilum	PurPflex					
S. psygmophilum	HIAp					
S. psygmophilum	rt141					
S.aenigmatum	mac04-487	44.9	24,202	984	1,355	51.39
S. pseudominutum	rt146	46	31,393	1,081	1,508	51.51

Table 5-3 Orthologous open reading frames for each between-species comparison.

	S. minutum	*S. pseudominutum*	*S. psygmophilum*	*S. aenigmatum*
S. minutum				
S. pseudominutum	30,891			
S. psygmophilum	31,389	28,744		
S. aenigmatum	24,959	25,627	25,091	

Total that occur exactly four times with bitscore >300: **19,359**

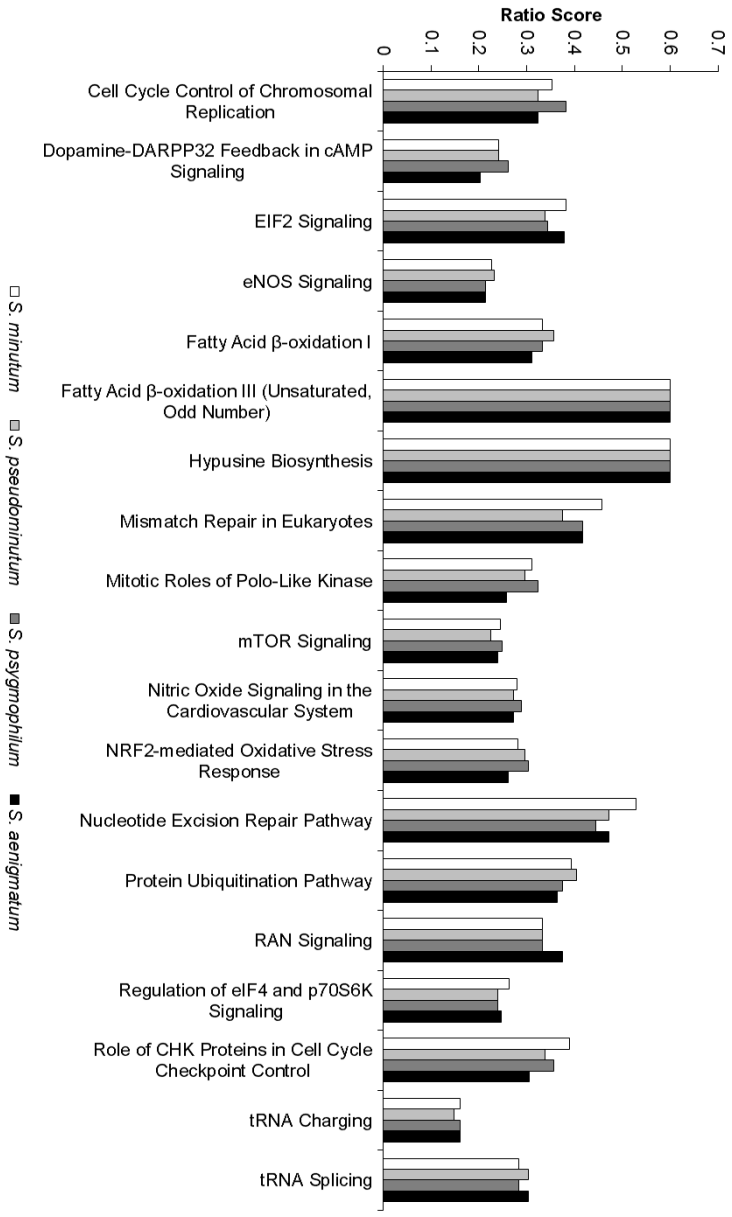


Figure 5-1 Relevant pathways from the Ingenuity Knowledge Base that were significantly enriched (Fisher's Exact Test; $p<0.05$) in *Symbiodinium* Clade B transcriptome assemblies. The ratio score depicts the number of genes in the assembly that belong to the pathway divided by the total number of genes in the pathway.

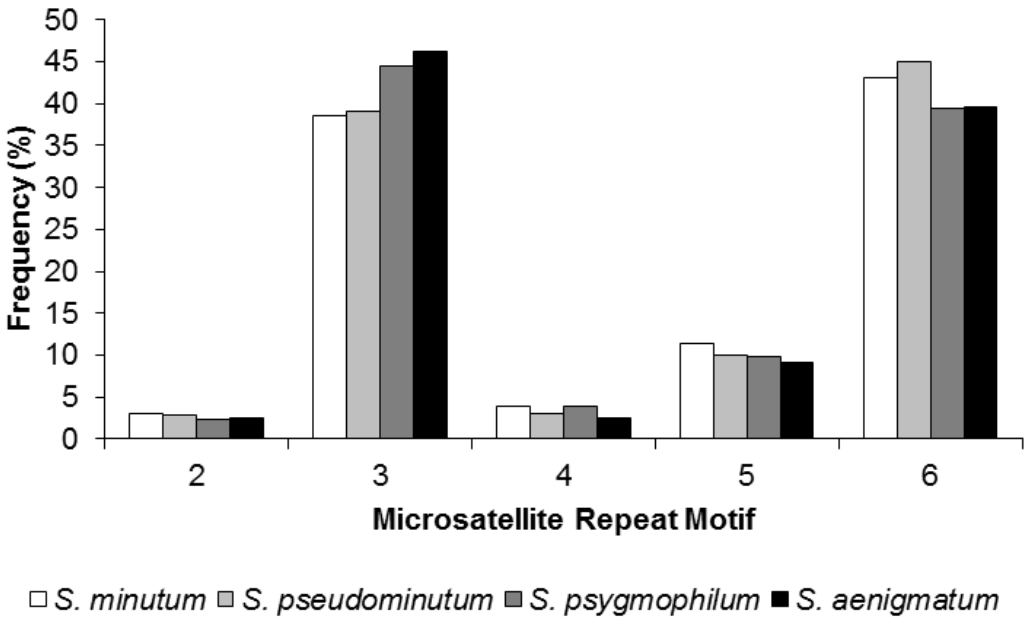
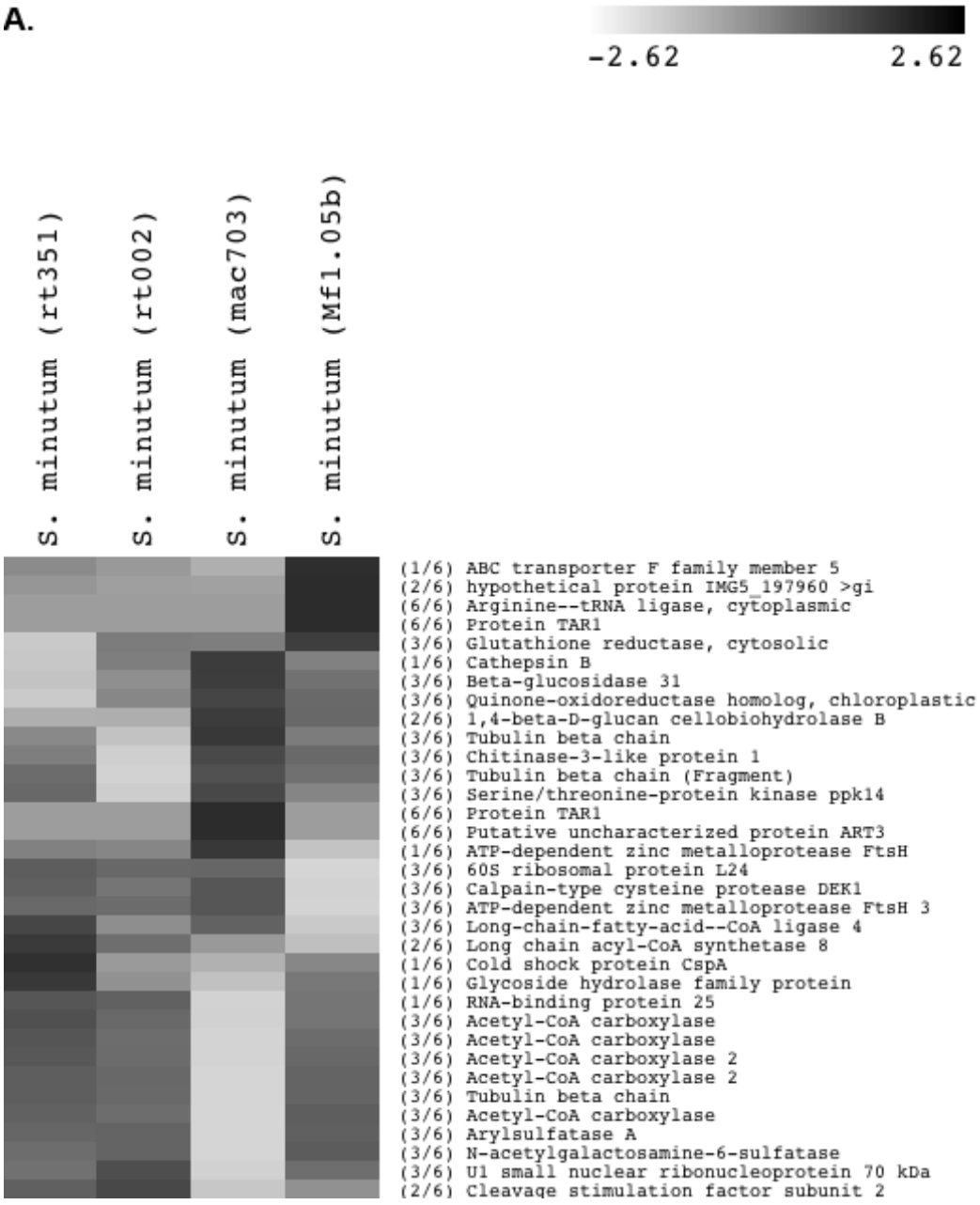


Figure 5-2 Microsatellite motif frequency in each Clade B *Symbiodinium* species. Trinucleotide and hexanucleotide motifs were most common in all species. Species were similar in their motif frequencies, with an overall average coefficient of variation of 11% across all motifs.

A.


-2.62
2.62
S. minutum (rt351)
S. minutum (rt002)
S. minutum (mac703)
S. minutum (Mf1.05b)
(1/6) ABC transporter F family member 5
(2/6) hypothetical protein IMG5_197960 >gi
(6/6) Arginine--tRNA ligase, cytoplasmic
(6/6) Protein TAR1
(3/6) Glutathione reductase, cytosolic
(1/6) Cathepsin B
(3/6) Beta-glucosidase 31
(3/6) Quinone-oxidoreductase homolog, chloroplastic
(2/6) 1,4-beta-D-glucan cellobiohydrolase B
(3/6) Tubulin beta chain
(3/6) Chitinase-3-like protein 1
(3/6) Tubulin beta chain (Fragment)
(3/6) Serine/threonine-protein kinase ppk14
(6/6) Protein TAR1
(6/6) Putative uncharacterized protein ART3
(1/6) ATP-dependent zinc metalloprotease FtsH
(3/6) 60S ribosomal protein L24
(3/6) Calpain-type cysteine protease DEK1
(3/6) ATP-dependent zinc metalloprotease FtsH 3
(3/6) Long-chain-fatty-acid--CoA ligase 4
(2/6) Long chain acyl-CoA synthetase 8
(1/6) Cold shock protein CspA
(1/6) Glycoside hydrolase family protein
(1/6) RNA-binding protein 25
(3/6) Acetyl-CoA carboxylase
(3/6) Acetyl-CoA carboxylase
(3/6) Acetyl-CoA carboxylase 2
(3/6) Acetyl-CoA carboxylase 2
(3/6) Tubulin beta chain
(3/6) Acetyl-CoA carboxylase
(3/6) Arylsulfatase A
(3/6) N-acetylgalactosamine-6-sulfatase
(3/6) U1 small nuclear ribonucleoprotein 70 kDa
(2/6) Cleavage stimulation factor subunit 2

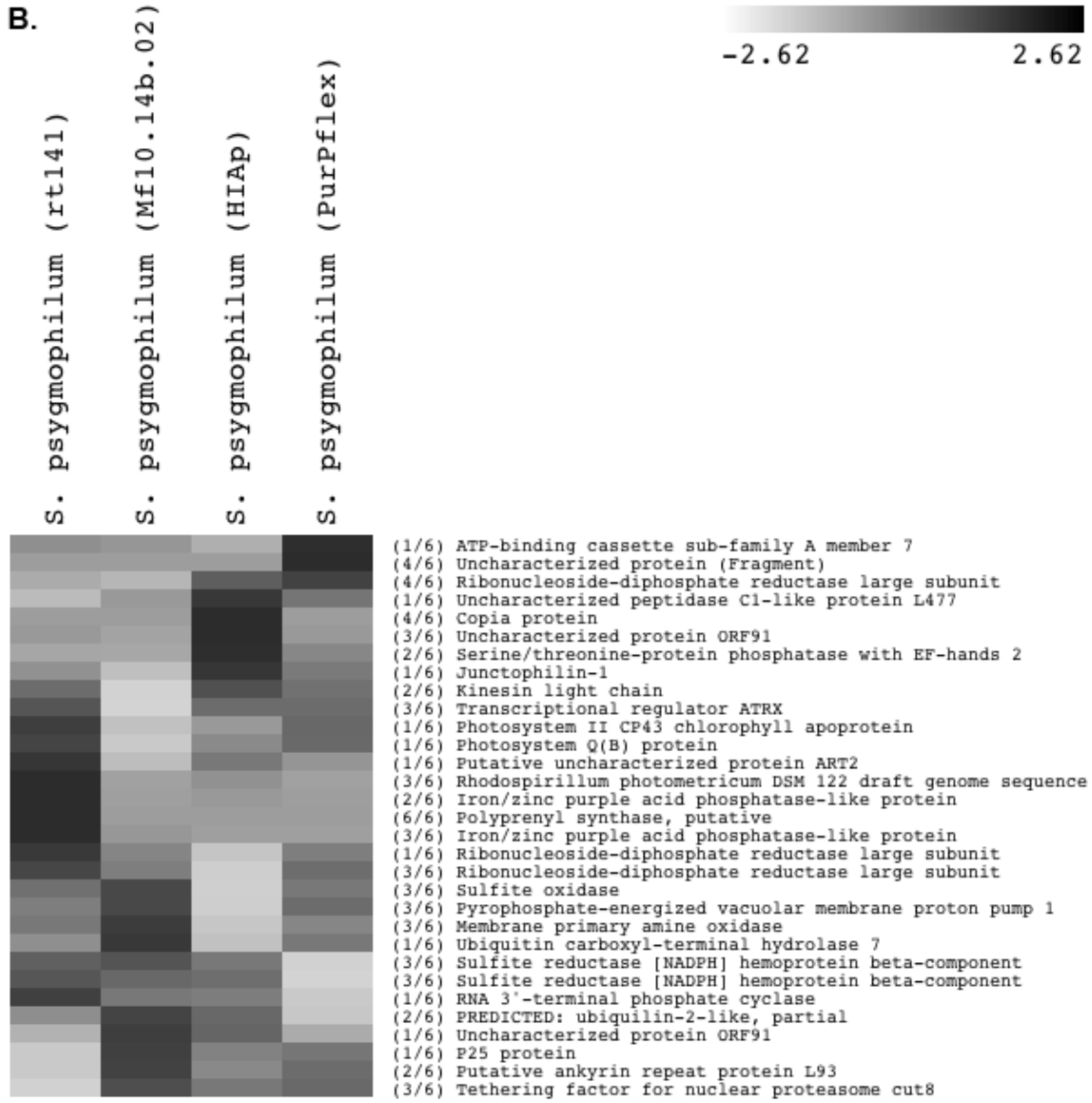


Figure 5-3 Heatmaps of geometric mean normalized expression (counts) for annotated differentially expressed genes among individual clonal cell lines within **(a)** *S. minutum* and **(b)** *S. psygmophilum*. Fractions in parentheses indicate the number of pairwise contrasts (out of 6) for which a given gene was significantly differentially expressed.

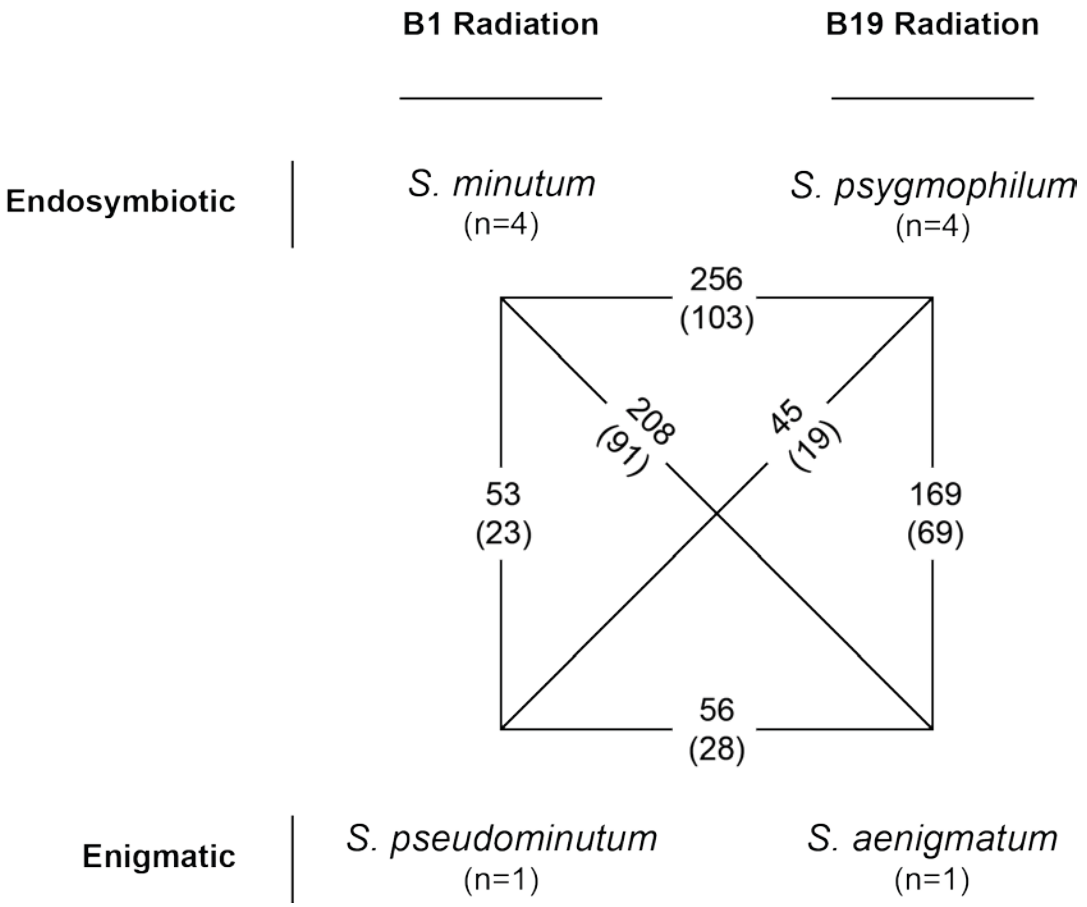


Figure 5-4 Diagram depicting the numbers of differentially expressed genes (DEGs) between Clade B *Symbiodinium* species. The numbers are placed on the lines connecting the two species being contrasted. The top number indicates total DEGs; the bottom number in parenthesis indicates annotated DEGs. Also depicted are the phylogenetic and ecological memberships of each species, and the number of individual clonal cell lines per species (cultures) used in the study.

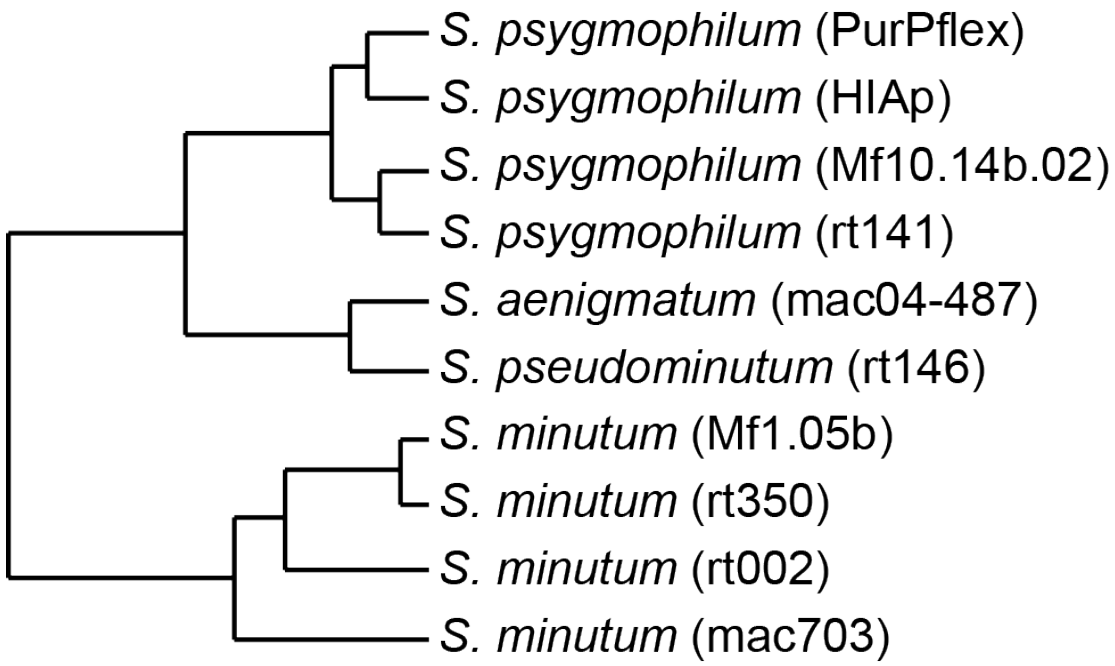


Figure 5-5 Hierarchical clustering of strains based on scaled FPKM expression value distances. K-means clustering supported the same three major groups: *S. minutum* only, *S. psygmophilum* only, and *S. aenigmatum* and *S. pseudominutum* together.

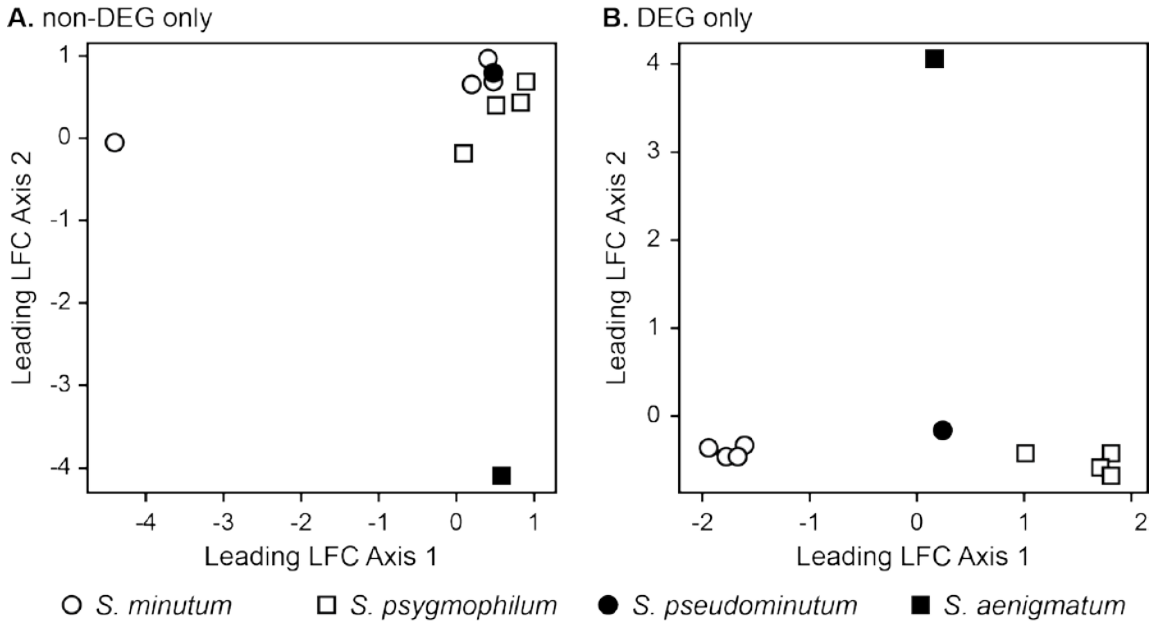


Figure 5-6 Multidimensional scaling plots depicting sample clustering based on the primary and secondary leading log-fold change (LFC) axes for **(a)** non-differentially expressed genes and **(b)** differentially expressed gene values. White fill = endosymbiotic; black fill = enigmatic; circles = B1 radiation, squares = B19 radiation.

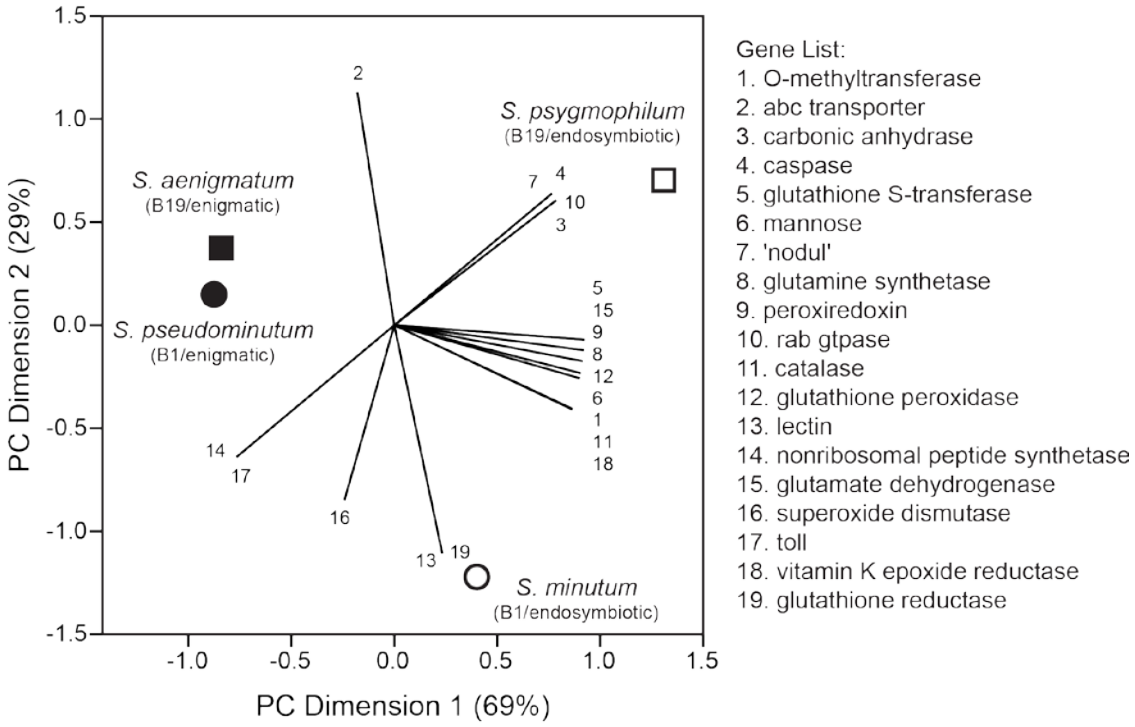


Figure 5-7 Categorical principle component analysis of symbiosis gene copy numbers among Clade B *Symbiodinium* species. The first axis separates species by ecology; the second separates them by phylogenetic lineage. White fill = endosymbiotic; black fill = enigmatic; circles = B1 radiation, squares = B19 radiation.

Table 5-S1 Gene Ontology (GO) terms enriched in the top 200 most highly expressed genes for each strain in the study.

Table 5-S1 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g55k).

Table 5-S2 Gene Ontology (GO) terms enriched in each pairwise contrast of differential expression between species within *Symbiodinium* Clade B.

Table 5-S2 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g55k).

Table 5-S3 List of positive hit symbiosis gene strings derived from Meyer and Weis (2012) along with gene copy number per species and means for different ecologies and lineages.

Table 5-S3 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g55k).

Table 5-S4 Gene Ontology (GO) terms enriched in contrasts of differential expression within species. Note that Revigo (Supek et al. 2011) was used to reduce redundant terms.

Table 5-S4 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g55k).

Figure 5-S1 Heatmaps of normalized expression for annotated DEGs among species between contrasts of (A.) *S. psygmophilum-S. pseudominutum,* (B.) *S. pseudominutum-S. minutum,* (C.) *S. aenigmatum-S. pseudominutum,* (D.) *S. psygmophilum-S. aenigmatum,* (E.) *S. aenigmatum-S. minutum,* and (D.) *S. psygmophilum-S. minutum*.

Figure 5-S1 can be accessed in the Penn State ScholarSphere database (https://scholarsphere.psu.edu/files/sf268g55k).

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Appendix

All supplementary tables, figures, and data associated with the chapters of this dissertation can be accessed in the Penn State ScholarSphere database.

Chapter 1: not applicable

Chapter 2: not applicable

Chapter 3: https://scholarsphere.psu.edu/files/sf268g52r

Chapter 4: https://scholarsphere.psu.edu/files/sf268g45b

Chapter 5: https://scholarsphere.psu.edu/files/sf268g55k

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