Ultrastructural, Immunohistochemical, and Biochemical Variation Amongst *Symbiodinium* Isolates

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 3, 2013

Keywords: Symbiodinium; symbiosis; coral reef; Aiptasia pallida; symbiosome; cell-cell recogntion

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Abstract

Symbiodinium spp. reside intracellularly in a complex symbiosome (host and symbiont-derived) in a specific host-symbiont associations. *Symbiodinium* is a diverse genus with variation equivalent to that of other dinoflagellate orders. First, this investigation examines antigenic variation in the algal mucilage secretions at the host-symbiont interface. Cultured *Symbiodinium* from a variety of clades were labeled with one or the other of two antibodies that bind to components of the symbiont mucilage (PC3 antibody, developed using a clade B alga cultured from *Aiptasia pallida*; BF10 antibody, developed using a clade F alga cultured from *Briareum* sp.). PC3 antigen was found in cultured *Symbiodinium* from clades A and B, but not clades C, D, E and F. The correlation between labeling and clade may account for some of the specificity between host and symbiont in the field. Within clades A and B there was variation in the amount of label present. BF10 antigen was more specific and only found in cultures of the same cp23S-rDNA strain the antibody was created against. Since the mucilage forms the host-symbiont interface, variation in its molecular composition is likely to be the source of signals involved in recognition and specificity. Next, I examined variation in the ultrastructural morphology amongst 5 *Symbiodinium* isolates (2 from clade A, and 1 from clade B, 1 from clade C, and a free-living isolate from clade E) to create a baseline standard for future ultrastructural work and to determine if there is any ultrastructural basis for taxonomic description of species within this genus. Ultrastructural examinations were performed using High Pressure Rapid Freezing. All 5 isolates studied exhibited the standard dinokaryon nucleus, a peripheral multi-lobed chloroplast

with a single stalked pyrenoid surrounded by a starch cap, a dinoflagellate-type mitochondria with tubular cristae, and various other cellular inclusions. Additional small electron dense inclusions were typically positioned near the cell periphery and may be related to mucocysts sometimes seen in dinoflagellates. Some isolates (CCMP421 and Y109) had a few cells that exhibited large electron dense structures containing calcium oxalate crystals. Cell size amongst different isolates was significantly different and consistent with previous studies on the same or similar isolates. Examination of the ultrastructure of 5 isolates of *Symbiodinium* revealed that there is very little variation in morphological characters. Therefore, it is unlikely that isolates or species of *Symbiodinium* can be usefully differentiated on the basis of morphology alone. Finally, in order to continue examining *Symbiodinium* isolates for variation that may impact the association with a specific host, I investigated the variation of storage products (lipid droplets and starch granules) within 5 isolates. Amongst the 5 isolates examined, the two isolates from clade A contained the least amount of both starch and lipid. However, another small isolate from clade B contained some of the largest amounts of both starch and lipid. A clade F isolate also contained many storage products while the only free-living isolate examined, a clade E isolate, contained more lipid and starch than the clade A symbionts but less than the clade B and F isolates. This variation in storage products amongst isolates grown under the same culture regime reveals physiological differences that may relate to an individual isolate's suitability as a symbiotic partner under certain environmental conditions such as those arising from global climate change. Further biochemical examination of excreted exudate for the presence of carbohydrate and protein components found that in all 5 isolates, more carbohydrate compounds were secreted into the culture medium than protein. Examination of the composition of PC3 antigen, a component of the exudate of some *Symbiodinium* isolates, found that the antigen is

composed, at least in part, of carbohydrate structures. The presence of carbohydrate components in the exudate compounds that are associated with the cell surface is consistent with the hypothesis that initiation and perhaps maintenance of the host-symbiont relationship is mediated by a glycan/lectin interaction.

Acknowledgments

I would like to thank Dr. Stephen C. Kempf for acting as my major professor and the chair of my dissertation committee and for teaching me much about the art of electron microscopy. I would also like to thank the members of my committee, Dr. Eduardus Duin, Dr. Anthony Moss, Dr. Scott Santos, and Dr. Christine Sundermann for their guidance throughout my graduate career at Auburn. Dr. Michael Miller (AU AMIL), Dr. Roger Bridgman (AU Hybridoma Lab), and Dr. John Dennis (AU Vet) were also vital to my graduate research at Auburn University. Thanks also go to Dr. William Fitt and Dr. Beth Richardson at the University of Georgia for providing use of equipment at UGA. I would also like to thank Ivey Ellis, Shanna Hanes, Katy Prince, Limin Gong, Nate Kirk, Kevin Kocot, and Spyridon Mourtzinis for their assistance and advice in this research. My husband, David, also deserves a huge amount of gratitude for his support during my graduate career. My incredibly large family has also been very supportive in my pursuit of this degree. I would like to thank my grandfather, Vincent Mazzillo for teaching me to be tenacious; my grandmother, Jean Barr for teaching me that I can do anything I put my mind to; my grandfather, Donald Barr for teaching me to be "always good". Jean Mazzillo, Vince Mazzillo, Drew Mazzillo, Meredith Teat Mazzillo, Amanda Mazzillo, Coker Mays, Julia Mays, Beth Mays Woodrow, Seth Woodrow, Ryleigh Woodrow, Reagan Woodrow, Mia Smith, Donald Smith, Seralyn Smith, Katherine Smith, Robert Dotzey, Keith Jacobson, and Noelle Easterday have also been a constant source of strength, support and love.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

2XCTAB	cetyltrimethylammonium bromide
5.8S	a segment of ribosomal RNA
ANOVA	Analysis of Variance
BF10	antibody created against the mucilage from a clade F *Symbiodinium*
cp23S	chloroplast 23S rRNA gene
ELISA	enzyme linked immunosorbant assay
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
MFSW	millepore filtered sea water
np-H_2O	nanopure filtered water
nr28S	nuclear ribosomal large subunit
nr18S	nuclear ribosomal small subunit
PBS	phosphate buffered saline
PC3	antibody created against the mucilage from a clade B *Symbiodinium*
RFLP	restriction fragment length polymorphism

I. A REVIEW OF *SYMBIODINIUM* SYMBIOSES WITH AN EMPHASIS ON INITIATION AND MAINTENANCE OF THE CNIDARIAN-*SYMBIODINIUM* ASSOCIATION.

ABSTRACT

Endosymbioses are relationships where a symbiont lives inside a host organism. These can be situations where the symbiont lives within a host cavity like the gut, between the cells of host tissues or within the cells themselves. These associations can be vital to the lives of both partners who are joined in symbiosis. *Symbiodinium* sp. is a common endosymbiont of various invertebrate species that are best known for their associations with cnidarians where they reside in the cytoplasm of host endodermal cells. These dinoflagellates form a diverse genus that was originally thought to be a single pandemic species. The Cnidarian-*Symbiodinium* partnership is vital not only to the host cnidarian, but also to the health of the entire coral reef ecosystem. The physical and nutritional basis for the coral reef ecosystem relies on the photosynthetically fixed carbon that is translocated from *Symbiodinium* to its cnidarian host. This association is diverse with a huge genetic variation in symbiont (similar to other dinoflagellate orders), and many potential host organisms. Despite the diversity in members, the association between *Symbiodinium* and its cnidarian host is usually specific with an association between one host species and one or a few symbiont types. The specificity in this relationship is conferred in a number of different ways. First, some hosts transfer their symbionts directly to their eggs or larvae (vertical transmission). If the larvae are aposymbiotic (have no symbionts) after leaving the parent and obtain the symbionts from the environment (horizontal transmission), specificity can be conferred a number of ways. During infection the host may feed only on material that is likely to contain the preferred symbiont isolate leading to a specific association. Just after

infection, specificity may be conferred by a cell-cell recognition process that determines acceptance or rejection of the symbiont. Specificity in the association may be achieved later *via* a process in which the symbiont better suited to the host environment outcompetes other symbiont types. Over the last 50 years there has been an increase in coral bleaching events. This massive loss of symbionts from host tissues often leads to the death of the host and thus the loss of reef habitat. While the exact cellular mechanisms behind the breakdown of the symbiosis during bleaching are not fully understood, it is clear that both the hosts and symbionts show varying degrees of susceptibility to thermal stress (the major cause of most bleaching events). Once both 1) the process of infection and 2) the mechanisms resulting in bleaching are understood, better management decisions can hopefully be made to ameliorate the devastation of the reef environment that is likely to occur as sea temperatures rise due to global warming.

ENDOSYMBIOSIS

Symbioses are relationships between at least two different species that can range from parasitism to mutualism (Lewin, 1982). A parasitism is characterized by one member of the symbiosis benefiting (the parasite) while the other is harmed (the host). A mutualism is a relationship in which both members benefit (Lewin, 1982). While these definitions seem straightforward, recent research into different symbioses tends to suggest that many associations are mutualistic under certain conditions and parasitic if the conditions (environmental, health of one member, etc.) change (Lewin, 1982).

An endosymbiosis is a specialized symbiotic relationship in which one member resides within or between the cells of the other. Endosymbioses have specialized terminology, the smaller member of the endosymbiosis residing inside the host (either intracellulary or in

intercellular space) is called the symbiont. The larger member is the host (Lewin, 1982). In cases where the symbiont resides intracellularly, it is generally encased in at least one layer of host membrane. This membrane is called the symbiosome membrane (Roth and Stacey, 1989).

A good example of an endosymbiotic relationship where one member resides intercellularly is between humans and our symbiotic gut bacteria. In most cases this is a mutualism, the symbiont provides the host with certain molecules such as vitamins while the host provides nutrients and an optimal environment for growth (Backhed *et al.*, 2005). A well-studied example of intracellular endosymbiosis is between bacteria in the genus *Rhizobia* and legumes. Legumes are known as nitrogen-fixing plants; however the endosymbiont *Rhizobia* actually fixes the nitrogen. *Rhizobia* live in special anaerobic compartments (an enzyme required for nitrogen fixation, nitrogenase, is oxygen sensitive) in the root nodules of legumes providing the plant with fixed nitrogen. The *Rhizobia* in turn obtain carbon compounds from the host and live in a protected environment (Young and Johnston, 1989; Sturz, 1997; Oke and Long, 1999).

Since the endosymbiont is residing within the host, there is the potential for an immune reaction against the symbiont. For example, the human gut hosts mutualistic bacteria, but can be plagued by disease-causing bacteria such as *Mycobacterium paratuberculosis, Listeria monocytogenes,* and certain strains of *Escherichia coli*; these pathogens can be controlled by the host maintaining a mutualism with symbiotic bacteria that bolster the immune system (Damaskos and Kolios, 2008). In endosymbioses there is usually a high degree of specificity between host and symbiont (Sturz, 1997). In the root nodules of legumes, some species of *Rhizobium* thrive in certain legumes and not in others (Sturz, 1997). Niche partitioning also maintains some endosymbionts in the root nodules and others only in the foliage or stem (Sturz, 1997).

Here I am interested in the endosymbiosis between dinoflagellates of the genus *Symbiodinium* and cnidarian hosts. This association forms the foundation, both physically and nutritionally of the coral reef ecosystem (Richmond and Wolanski, 2011). This symbiosis is an area of current interest due to its recognized importance in providing for extreme biodiversity and the fact that the effects of global climate change have put this association, and therefore the many species living in coral reef ecosystems, at risk (Rodrigues and Grottoli, 2007).

SYMBIODINIUM SYMBIOSIS: THE MEMBERS

Dinoflagellates of the genus *Symbiodinium* occur in association with many invertebrate species in the marine environment. In the symbiosis between *Symbiodinium* and cnidarians, the symbionts provide the host with photosynthetically fixed carbon (e.g., Davy and Cook, 2001; Goodson *et al.*, 2001; Muller-Parker and Davy, 2001). Various studies have concluded that the photosyntheate can be transferred as a variety of different compounds, most commonly glycerol (Muscatine, 1967) or glucose and succinate/fumarate (Whitehead and Douglas, 2003). In return, the host waste products (N, CO_2, etc) and UV protection (Lesser, 1989; Muscatine, 1990) are of benefit to the symbiont, as is the protected intracellular environment where the symbionts are maintained.

SYMBIODINIUM SP. LIFECYCLE, MORPHOLOGY, AND TAXONOMY

'Zooxanthellae' was a blanket name historically used to refer to dinoflagellates and some diatoms (Brandt, 1881). In this symbiosis it has most often described a group of single-celled photosynthetic symbiotic dinoflagellates of the genus *Symbiodinium*. Initially, these gymnodinoid symbionts were described morphologically as a single species, *Symbiodinium*

microadriaticum Freudenthal (1962). This species was initially assumed to be pandemic based on ultrastructural studies (Taylor, 1968; Taylor, 1974).

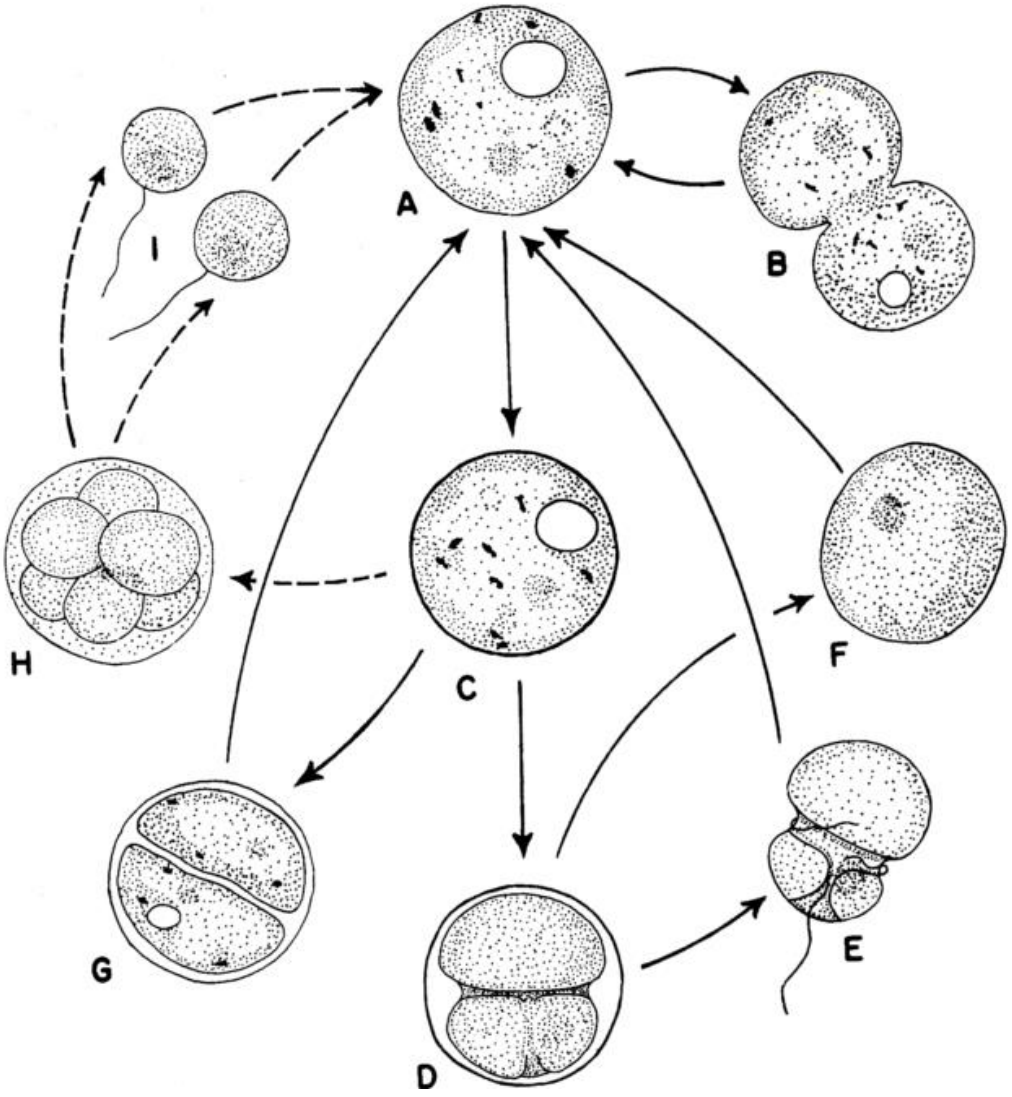

Figure 1. Symbiodinium microadriaticum life cycle taken from Freudenthal, 1962. A. Vegetative cell. B. Vegetative cell dividing. C. Vegetative cyst. D. Zoospore. E. Motile zoospore. F. Non-motile aplanospore. G. Vegetative cyst division to form two subspecies. H. Gametogenesis? I. Release of gametes?

The initial described life cycle was based on cultured *Symbiodinium*, and is presumed to be similar to what is seen *in hospite* and in free-living dinoflagellates in the genus *Symbiodinium*. The dominant stage is the vegetative phase (Figure 1A) characterized by a single, thin-walled

autotrophic cell with a visible chloroplast and an unobtrusive nucleus. Over time, the cell loses some chloroplast volume and the accumulation body grows. Eventually, if this cell continues to grow without dividing it will lose chloroplast volume and die (Freudenthal, 1962). However, the vegetative phase generally undergoes cell division (Figure 1B), producing two daughter cells each with half the parent cytoplasm and organelles, with the exception of the accumulation body which is transferred whole to one of the daughter cells. The vegetative cell may also form a cyst with a thickened cell wall (Figure 1C) that can become a motile zoospore (Figure 1D and 1E) or non-motile aplanospore (Figure 1F). While no sexual reproduction has been observed, it is hypothesized that the vegetative cyst may also produce gametes (Freudenthal, 1962).

The motile zoospore (gymnodinoid cell) seen in culture (Figure 1D and 1E) has few food reserves and chloroplasts (compared to the vegetative cyst), but does contain longitudinal and transverse flagella for movement (Freudenthal, 1962). The motile zoospore becomes the dominant life cycle stage at dawn, reaching a maximum at midday (Yacobovitch *et al.*, 2004). At the end of this diel ecdysis cycle, they become sessile and settle. These zoospores were not observed reproducing sexually (Freudenthal, 1962). The vegetative cyst may also divide to form 2 daughter vegetative cells or autospores (Figure 1G). Upon emerging from the cyst stage, the daughters are normal independent vegetative cells (Freudenthal, 1962).

While life cycle studies have not identified gametogenesis or gametes (Freudenthal, 1962; Schoenberg and Trench, 1980b), information gathered using molecular techniques has indicated that *Symbiodinium* is haploid in the vegetative life stage, both in culture and in the host (Santos and Coffroth, 2003). At many loci, *Symbiodinium* had only one allele indicating haploidy in the group. Additionally, the data differed from what would be expected under Hardy-Weinberg equilibrium, further indicating that *Symbiodinium* are haploid (Santos and

Coffroth, 2003). While it appears that during most of its life cycle *Symbiodinium* is haploid, there is evidence for sexual reproduction of some kind. Allozyme (isoenzyme), RAPD, and DNA fingerprinting analyses show high levels of variation in both cultured and field sampled populations (Schoenberg and Trench, 1980a; Goulet and Coffroth, 1997) that suggests sexual recombination (Baillie *et al.*, 1998; Baillie *et al.*, 2000a; LaJeunesse, 2001). Further evidence examining isoenzyme similarity and phylogenetic similarity found that the two are not correlated, providing evidence for recombination of alleles by sexual reproduction of some kind (Tibayrenc *et al.*, 1991; Baillie *et al.*, 2000b; LaJeunesse, 2001).

Taxonomically, the species is difficult to place. It possesses a gymnodinoid form, which may place it in the genus *Gymnodinium*, if both morphology and life cycle are considered, the species does not fit in any described dinoflagellate genus. As stated above, novel genus *Symbiodinium* was created by Freudenthal (1962) and assumed a pandemic type species *Symbiodinium microadriaticum* Freudenthal (Taylor, 1974). However, symbionts from different hosts differed morphologically (Trench, 1979) and in specificity with their host (Kinzie *et al.*, 1977; Kinzie and Chee, 1979). In 1980, further work by Schoenberg and Trench examined isoenzymes, soluble protein patterns, morphology, specificity, and infectivity to examine variation amongst *Symbiodinium* (1980a; 1980b; 1980c). The symbionts used in these studies were isolated from a variety of hosts including Anthozoans (including Actinaria, Alcyonaria, Hexactiniida, Scleractinia, and Zoanthidea), Scyphozoans, and one molluscan sample (Bivalvia). The isoenzyme data arranged the 17 isolates into 12 ‘strains’. Soluble protein patterns mostly agreed with isoenzyme ‘strain’ identity, though soluble protein suggested greater difference between ‘strains’ (Schoenberg and Trench, 1980a).

Morphological studies using light microscopy revealed that different 'strains' had different average diameters (Schoenberg and Trench, 1980a). Aposymbiotic *Aiptasia tagetes* were reinfected with the 'strains' studied here and diameter of each specific *Symbiodinium* 'strain' was maintained, suggesting size is a function of *Symbiodinium* 'strain' and not of host (Schoenberg and Trench, 1980b). Different outer membranes were seen *in situ* using the electron microscope including a layer of membranous vesicles associated with the plasma membrane, the inner pellicle membrane, the pellicle (later referred to as the cell wall), and an outer pellicle membrane (Schoenberg and Trench, 1980b).

'Strains' used to successfully infect aposymbiotic *Aiptasia tagetes* that were similar in isoenzyme and soluble protein analysis to the original *A. tagetes* symbiont formed dense stable relationships more quickly (Schoenberg and Trench, 1980c) suggesting specificity in the host-symbiont relationship. The hypothesis was put forth that specificity was dependent on recognition during infection (Schoenberg and Trench, 1980c). Chromosome number and volume of symbionts from different hosts also found marked differences between 'strains' and were not due to differences in ploidy (Blank and Trench, 1985). These results support the idea that there are multiple genetically distinct species, within the genus *Symbiodinium*.

Molecular investigations of the *Symbiodinium* small ribosomal subunit RNA from symbiotic, non-cultured algae identified variation in restriction enzyme polymorphisms. From 16 cnidarian hosts, 6 different ssRNAs were sequenced (Rowan and Powers, 1991b). These preliminary studies also showed that some hosts contained only 1 type while other hosts showed a mixture of symbiont types (Rowan and Powers, 1991b). Again using small ribosomal subunit RNA, high levels of diversity were found within the previously supposed pandemic group called

Symbiodinium that were comparable to the diversity among different orders of non-symbiotic dinoflagellates (Rowan and Powers, 1992; LaJeunesse, 2001; Coffroth and Santos, 2005).

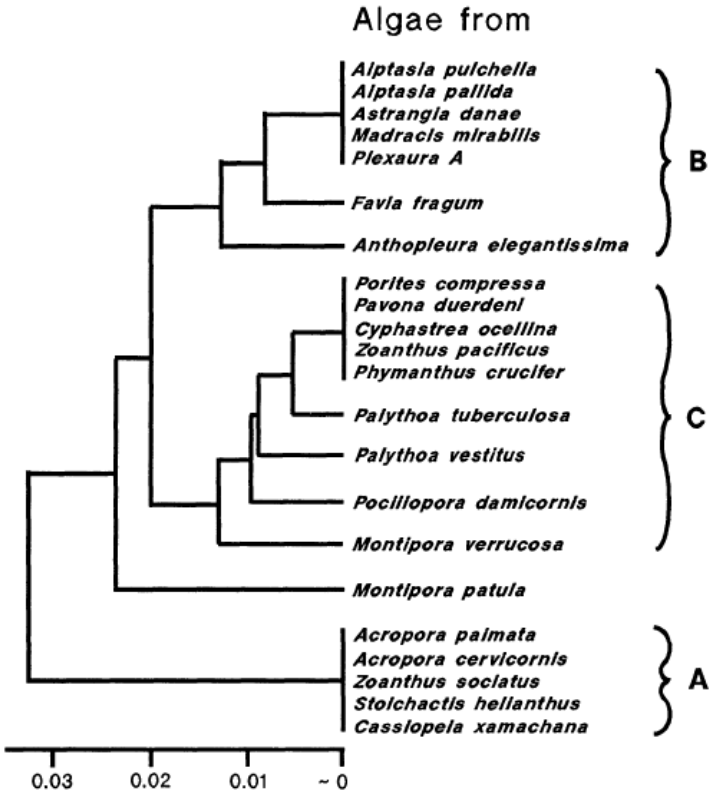

Figure 2. Phylogenetic relationships of zooxanthellae from different host species taken from Rowan and Powers (1991a). Genetic distances were found using ssRNA sequences, groups A, B and C correspond to the RFLPs (group C includes RFLPs D1 and D2).

Subsequent investigations (McNally *et al.*, 1994; LaJeunesse, 2001) into *Symbiodinium* diversity revealed similar high levels of difference. Examination of a variety of different 'strains' (from Schoenberg and Trench, 1980a-c and Blank and Trench, 1985) showed that these symbiotic dinoflagellates grouped with the genus *Gymnodinium* (McNally *et al.*, 1994).

Interestingly, some strains from distant locations appear closely related (e.g. Indo-Pacific strain from the bivalve *Corculum cardissa* and Caribbean strain from the zoanthid *Zoanthus sociatus*), while other geographically close 'strains' appear genetically distant (e.g. Pacific strain from the bivalve *C. cardissa* and Hawaiian strain from the anemone *Aiptasia pulchella*) (McNally *et al.*, 1994). Another study using the internal transcribed spacer (ITS) regions of 47 *Symbiodinium* cultures (some novel cultures and some previously described by Freudenthal, 1962; Trench and Blank, 1987; Trench and Thinh, 1995) identified 17 distinct 'types' or 'strains' (LaJeunesse, 2001). Some of the 'strains' cultured from distant locations appear related, indicating a global distribution of these 'strains' of *Symbiodinium* (LaJeunesse, 2001). To distinguish 'strains', it was necessary to develop a reliable method. Initially, 'strains' were placed in 3 clades A, B, and C (Figure 2; Rowan and Powers, 1991a) identified by differences in restriction fragment length polymorphism (RFLP) patterns and small ribosomal subunit RNA sequences (Rowan and Powers, 1991a; Carlos *et al.*, 1999). Subsequent work has revealed 9 recognized clades with multiple 'strains' in each clade (Rowan and Powers, 1991b; Carlos *et al.*, 1999; LaJeunesse and Trench, 2000; LaJeunesse, 2001; Pochon *et al.*, 2001; Pochon *et al.*, 2004; Stat *et al.*, 2006; Pochon and Gates, 2010).

Chloroplast and mitochondrial genomes have also been probed to elucidate differences between clades. The first *Symbiodinium* phylogeny to use an organellar genome used chloroplast large subunit (23S)-rDNA sequences (Santos *et al.*, 2002). In sequences from 35 dinoflagellate cultures (from a variety of hosts and locations) the topology of the cp 23S-rDNA Domain V phylogeny was not statistically different from nuclear rDNA topologies and supported the major *Symbiodinium* clades (Santos *et al.*, 2002). Domain V of the cp23S-rDNA evolves 9-30 times faster than V1-V4 regions of nuclear small subunit (18S) rDNA, 1-7 times faster than D1-D3

regions of nuclear large subunit (28S) rDNA, and 0.27-2.25 times faster than the ITS-rDNA (Santos *et al.*, 2002) and could be an extremely useful sequence in exploring differences between closely related *Symbiodinium* (Santos *et al*., 2002).

Use of the mitochondrial genome of *Symbiodinium* and the cytochrome oxidase subunit I (*cox*1) (Takabayashi *et al*., 2004) supported the division between clade A and clades B, C, D, E, and F. Relationships within clades B, C, D, E, and F however, were not as well resolved using *cox*1 as they are when using nrDNA or cp23S-rDNA (Takabayashi *et al*., 2004). Relationships between the major clades are congruent with those determined from nrDNA and cp23S-rDNA; the relationships within the major clades are robust therefore, any further studies are likely to find variation within these clades, but the major clade divisions should be upheld (Takabayashi *et al*., 2004).

Using the chloroplast genome for future studies may allow for fine genetic differences to be understood since the chloroplast genome has higher variability than the nuclear or mitochondrial genomes (Barbrook *et al.*, 2005). The chloroplast *psbA* gene produced similar trees to the n28S-rDNA. Additionally, the *psbA* minicircle and either a longer fragment or other minicircles may provide sufficient variation to determine relationships within phylotypes (Barbrook *et al.*, 2005). The diversity in what was once considered a single, pandemic species is still being examined using a variety of DNA sequences (Santos and Coffroth, 2003; Santos and Coffroth, 2005; Barbrooik *et al.*, 2006; Hunter *et al.*, 2007; Thornhill *et al.*, 2007; Pochon and Gates, 2010).

HOSTS

While most hosts of *Symbiodinium* sp. are cnidarians, there are also hosts in other invertebrate groups. Various radiolarians (Phylum Radiolaria), foraminiferans (Phylum Foraminifera), sponges (Phylum Porifera), nudibranchs (Phylum Mollusca, Class Gastropoda), clams (Phylum Mollusca, Class Bivalvia), and a few Acoelomorph species (Phylum Acoelomorpha) are the major examples of such associations (Kempf, 1984; Hill, 1996; Hoegh-Guldberg and Hinde, 1986a; Carlos *et al.*, 2000; Gast and Caron 2001; Ruppert *et al.*, 2004; Barneah *et al.*, 2007a).

PROTISTA. Foraminifera are amoeboid protists with pseudopods and a calcium carbonate shell (Hallock *et al*., 2003). They contribute to the primary production of the oceans where they are able to increase in number due to the carbon fixation of their algal symbionts. Since these are single-celled organisms, the symbionts reside intracellularly. Small subunit ribosomal DNA was used to determine foraminifera symbiont diversity which included dinoflagellates (including *Symbiodinium* sp.), prasinophytes, and prymnesiophytes (Gast and Caron, 1996; Pawlowski *et al*., 2001).

Radiolarians are single-celled amoeboid protists that produce a mineral skeleton. They have a rapid turn-over rate and are found throughout the oceans (sometimes to such an extent that their skeletal remains cover vast portions of the ocean floor) (Gast and Caron, 2001). Radiolarians have a wide variety of siliceous skeleton size and structure. Like foraminiferans, they associate with a variety of symbionts including dinoflagellates, prasinophytes, and prymnesiophytes. Radiolarians have also been shown to include *Symbiondinium* sp. amongst their endosymbiotic assemblage (Gast and Caron, 2001).

PORIFERA. Sponges may maintain symbionts either inter- or intra-cellularly (Taylor *et al.*, 2007). Like cnidarians, sponges use the photosynthetically fixed carbon compounds from the algae for metabolic processes (Carlos *et al.*, 1999). A boring sponge, *Anthosigmella varians*, has been shown to have enhanced growth and boring ability that is correlated with higher symbiont concentrations. This indicates that the sponge uses the photosynthetically fixed carbon from *Symbiodinium* for energy (Hill, 1996).

PLATYHELMINTHES. Acoelomorphs are small flatworms lacking an epithelium lined gut that resemble coelenterate planula larvae. A few acoelomorph symbiotic associations have been well-studied in the past including a prasinophyte, a diatom, and a dinoflagellate (*Amphidinium*) (Barneah *et al*., 2007a). The symbiosis of these basal bilaterians with *Symbiodinium* sp. has only begun to be examined (Barneah *et al*., 2007a; Ogunlana *et al*., 2005). Current research identifying *Symbiodinium* sp. as a symbiont of acoelomorphs shows a high degree of specificity with *Waminoa* sp. hosting a single strain of *Symbiodinium* (Barneah *et al*., 2007a). Further studies with *Waminoa brickneri* have shown maternal transmission of the algal endosymbionts at the oocyte stage (Barneah *et al*., 2007b).

MOLLUSCA. Symbiotic nudibranch species possess symbionts within host-derived 'carrier' cells in or associated with the digestive gland (Kempf, 1984). Nudibranch symbioses range from simple temporary to complex long-term associations (Kempf, 1984; Hoegh-Guldberg and Hinde, 1986a). A simple, temporary symbiosis begins with feeding and lasts only a few days before symbionts are expelled in fecal matter (Kempf, 1991). In such associations, constant feeding on a symbiotic prey maintains a continuous presence of symbionts in the host tissues. A complex long-term relationship includes host morphology that supports the association (e.g. increased cerrata surface area or number, positioning symbionts near irradiance, and specialized

internal structure to maintain the symbionts) and allows the host to survive periods of starvation (Kempf, 1984; Hoegh-Guldberg and Hinde, 1986b). Nudibranchs derive nutrition from symbionts and survive longer in the light than dark while starved (Kempf, 1984; Hoegh-Guldberg and Hinde, 1986b). Nudibranch hosts may have large lipid deposits in the symbiont containing cells, suggesting nutrient transfer from *Symbiodinium* to the host (Kempf, 1984; Rudman, 1991). Many nudibranchs acquire symbionts when they feed and feed only on certain prey species (housing specific *Symbiodinium* strains), resulting in specific associations between the nudibranch and *Symbiodinium* (Rudman, 1991; Rudman and Berguist, 2007). In some cases, the nudibranch host does not feed on *Symbiodinium* containing prey so any specificity is due to the *Symbiodinium* (Kempf, 1984).

Unlike symbioses between *Symbiodinium* and coelenterate hosts, where some hosts associate almost exclusively with one clade**,** most bivalve hosts associate with more than one symbiont, sometimes simultaneously. In a study examining 6 clam species (*Tridacna gigas, T. squamosa, T. crocea, Hippopus hippopus, H. porcellanus,* and *Corculum cardissa*), each associated with at least 4 *Symbiodinium* strains (Carlos *et al.*, 2000). Clam symbionts vary in location dependent on host species and can be located in the haemal sinuses of the siphon (*T. gigas*; Carlos *et al.*, 2000), the gonads, or the digestive gland (*Strombus tricornis*; Berner *et al.*, 1986; Garcia-Ramos and Banaszak, 2007). However, unlike most other bivalve hosts, *T. gigas* also maintains algal symbionts extracellularly near the gape. The symbionts may also be defecated when the population becomes too dense for the host (Trench *et al.*, 1981).

CNIDARIA. The phylum cnidaria is radially symmetric and has an oral-aboral axis (Ruppert *et al.*, 2004). The basic body plan in the phylum Cnidaria includes two body forms, polyp and medusa. In general, these forms are composed of a gastrovascular cavity opening to

the external environment *via* a mouth surrounded by tentacles (Ruppert *et al.*, 2004). The polyp consists of a stalk attached to substrate at the pedal disc and an oral disc opposite. The oral disc is effectively the feeding apparatus and has tentacles projecting from the edge. These forms are generally sessile, benthic organisms and are the prominent and/or only life stage in the Anthozoa and the Staurozoa (Ruppert *et al.*, 2004). The medusa, on the other hand, is shaped like a bell with the exumbrella (outer non-oral side of the umbrella disk) forming the aboral surface. The oral disc lies opposite the aboral surface in the subumbrella with tentacles surrounding it. Medusa are generally buoyant and have a thick, gelatinous connective tissue (mesoglea) separating the epidermis and gastrodermis. The medusa is the dominant form in the Hydrozoa, Scyphozoa, and Cubozoa (Ruppert *et al.*, 2004).

The body wall of cnidarians is composed of two tissue layers, an epidermis and gastrodermis with a layer of extracellular material, the mesoglea (and occasionally wandering cells sometimes with a tissue-like organization), between the two cellular layers (Ruppert *et al.*, 2004). Both the epidermis and gastrodermis contain specialized cells (e.g., cnidocytes, muscle, nerve, glandular, and ciliated cells; Schmid *et al.*, 1999; Ruppert *et al.*, 2004). Cnidarians include a cell type specific to the phyla called a cnidocyte, a specialized cell the animal uses to sting and immobilize its prey (Ruppert *et al.*, 2004). As such, cnidarians are general, filter-feeding carnivores that catch and disable prey using their cnidocytes. Cnidocytes discharge their nematocysts when stimulated by a mechanical or chemical stimulus that indicates a prey organism is nearby (Holstein and Tardent, 1984; Watson and Hessinger, 1989).

SYMBIOTIC CNIDARIANS. The major classes with symbiotic members are Anthozoa, Scyphozoa, and Hydrozoa (Ruppert *et al.*, 2004). The host in which *Symbiodinium* was initially described was *Cassiopea xamachana*, an upside-down jelly in the class Scyphozoa (Freudenthal,

1962; Fitt and Trench, 1983). The symbionts are phagocytosed by the gastrodermal cells similar to the manner food is endocytosed. Lysosomes do not fuse with a symbiont containing vacuole, possibly because the symbiont prevents this fusion (Fitt and Trench, 1983).

Corals that house *Symbiodinium* generally live in warm, nutrient-poor waters and form reefs, anemones often live in similar habitats, but do not secrete a calcium carbonate skeleton (Davy and Cook, 2001). Corals receive much of the carbon used in respiration from their *Symbiodinium* assemblage (Verde and McCloskey, 1998). The symbiont fixes carbon and donates photosynthetically fixed carbon to the host, this carbon supports the metabolism and reproduction of the host and aids in building the calcium carbonate skeleton (Verde and McCloskey, 1998). Cnidarian hosts donate their waste products to *Symbiodinium* (such as CO_2 and inorganic nutrients- Muscatine, 1990; Yellowlees *et al*., 2008). As a result of this nutritional exchange, symbiotic corals are especially good reef builders due to the carbon transferred from the symbionts (Lang *et al.*, 1992).

NUTRITIONAL ASPECTS OF THE SYMBIOSIS

The cnidarian-*Symbiodinium* symbiosis is based on nutritional transfer of photosynthetically fixed carbon from symbiont to host. Some studies have shown that the transfer of photosynthetically fixed carbon can provide up to 100% of the hosts metabolic carbon needs in a healthy coral (Muscatine *et al.*, 1981). Studies have examined symbioses to determine what carbon compound and how much photosynthetically fixed carbon is transferred. While there is variation between associations when using the same method, by examining many hosts the average amount of photosynthetic carbon and the most common molecules transferred in the cnidarian-*Symbiodinium* symbiosis can be estimated.

Within hosts in the genus *Cassiopea*, *Symbiodinium* provides 5-10% of net photosynthate to the host (*C. andromedea*; Hofmann and Kremer, 1981) providing approximately 169% of the hosts metabolic demand (*C. xamachana*; Verde and McCloskey, 1998). An early study using ^{14}C found that 45-50% of photosynthetically fixed carbon was transferred to *Anthopleura elegantissima* while only 20 to 25% was transferred in *Palythoa townsleyi* (Trench, 1971a). Another method for determining the amount of photosynthetic carbon used by the host is the CZAR (contribution of translocated *Symbiodinium* carbon to animal daily respiratory requirements) defined by the equation $CZAR=(P_z * T)/R_a$ where P_z is the gross amount of C fixed by the *Symbiodinium*, T is the percent that is translocated to the host, and R_a is the amount of C respired by the animal (Muscatine and McClosky, 1981). Two experiments using this methodology with *Pocillopora damicornis* and *Fungia scutaria* found that 63% and 70% of metabolic needs, respectively, were supplied by carbon from the symbiont (Muscatine and McClosky, 1981). The CZAR of *Stylophora pistillata* in light was 143%, while in shade it was only 58% (Muscatine *et al.*, 1984). Transfer to *Anemonia sulcata* under normal conditions provided 110% of the needs of the host (Stambler and Dubinsky, 1987); providing sufficient energy for host respiration and some additional energy for growth or reproduction.

Further studies on the transfer of photosynthetically fixed carbon using ^{13}C found high levels of transfer. In *Acropora pulchra* transfer of >86% of the total photosynthetically fixed carbon to host tissues was seen over a 20 day period (Tanaka *et al.*, 2006). This is in agreement with some earlier CZAR studies that suggested a supply of 100% of the host's needs (Muscatine *et al.*, 1984; Stambler and Dubinsky, 1987). The percent of photosynthetically fixed carbon translocated varies greatly amongst host species (the usual average reported is 25%-60%; Trench, 1993) making it almost impossible to assign an average translocation percentage across

species. Most researchers that attempt to collect and analyze this data report a range of efficiencies or focus on the diversity of translocation efficiency seen in the *Symbiodinium*/invertebrate symbioses (Davy and Cook, 2001; Grottoli *et al.*, 2006; Tanaka *et al.*, 2006; Loram *et al.*, 2007; Gordan and Leggat, 2010).

Comparing fed to starved anemones found the same rate of translocation of photosynthetically fixed carbon (16%) regardless of host nutrition status in *Aiptasia pallida* (Davy and Cook, 2001). Another variable is the type of symbiont isolate (clade/strain/species) within the host. Determining the impact of different *Symbiodinium* isolates on translocation rates in the giant sea anemone, *Condylactis gigantea*, found that both clade A and clade B symbionts released between 30-40% of their fixed carbon to the animal tissues; however, hosts with clade A symbionts showed higher incorporation into the lipid and amino acids fractions than hosts with clade B algae (Loram *et al.*, 2007). In *Acropora millepora*, symbionts of type C1 transferred more carbon than hosts with symbionts of clade D (Cantin *et al.*, 2009).

Determining which carbon compound is translocated to the host involves determining what compounds the alga secretes containing photosynthetically fixed carbon. The symbionts of both corals and *Tridacna crocea* produced primarily glycerol in the presence of host tissue (Muscatine, 1967). While incubated with ^{14}C in culture, glycerol again was the major product with alanine, glucose, fumaric acid, succinic acid, and glycolic acid released in smaller quantities (Trench, 1971b). These compounds were secreted in increased quantities in the presence of host homogenates (Trench, 1971c). In *Anemonia viridis*, ^{14}C labeled glucose and succinate/fumarate given to the host produced the same signature as anemones incubated with $NaH^{14}CO2$ in the light suggesting that glucose and succinate/fumarate are transferred from *Symbiodinium* to *A. viridis* (Whitehead and Douglas, 2003).

ULTRASTRUCTURAL ASPECTS OF THE SYMBIOSIS

Early ultrastructural description of the endosymbiont from Anemonia sulcata (described as being "akin" to *Symbiodinium microadriaticum* but likely another isolate from the genus *Symbiodinium*) described the thick cellulosic wall surrounding a single lobed chloroplast with a single stalked pyrenoid. Cells also contained a large vacuole termed an accumulation body because it appears to accumulate waste materials (Taylor, 1968). The nucleus is approximately spherical, large and the chromosomes have the typical dinoflagellate appearance (not undergoing decondensation even during resting stages; Dodge, 1966). Further ultrastructural studies have described *Symbiodinium* from several other hosts including *Cassiopeia* sp., *Condylactis* sp. (Kevin *et al*., 1969), Tridacnidae (Taylor, 1969), *Ragactis lucida, Montipora verrucosa, Zoanthus sociatus* (Trench and Blank, 1987), *Aiptasia pallida,* and *Phyllactis flosculifera* (Wakefield *et al.*, 2000). The anthozoan hosts house their symbionts within symbiosome membranes in the gastrodermal cells (Wakefield and Kempf, 2001). The symbiosome membrane consists of the host membrane that covers the symbiont *in situ* derived from the original phagocytic vacuole from infection. Beneath this outer layer, there are many other membranous layers observed within the host that may result from a repeated ecdysis cycle within the host symbiosome membrane (Wakefield *et al.*, 2000; Wakefield and Kempf, 2001).

INITIATION OF THE CNIDARIAN-*SYMBIODINIUM* SYMBIOSIS

The *Symbiodinium*-cnidarian symbiosis can be initiated in two ways. Vertical transmission of the symbiont can occur directly from the parent, with *Symbiodinium* included in the forming oocyte or larva by the parental polyp (Fautin, 2002). Horizontal transmission can occur once the larva is released from the parent and is either a planktotrophic larva or a settled

polyp. In horizontal transmission, larvae acquire symbionts from the environment, usually during feeding (Fautin, 2002).

In experimentally manipulated *Cassiopeia xamachana*, during vertical transmission the host experiences higher levels of asexual reproduction (and therefore high host fitness) indicating the symbionts are mutualistic; whereas, during horizontal transmission the host experiences lower levels of asexual reproduction and thus lower fitness. This indicates that the horizontally transmited *Symbiodinium* strains infecting the host initially behave more like parasites (Sachs and Wilcox, 2006). In most species (including *C. xamachana* which uses vertical transmission in nature) one mode of transmission dominates or is the only possibility. In these species (ex. brooding corals that undergo vertical transmission; Magalon *et al*. 2007) there is little opportunity to determine how a change of transmission mode would affect the mutualistic or parasitic nature of the symbiosis.

VERTICAL TRANSMISSION. Vertical transmission occurs in several ways. Maternal tissues can transfer symbionts to the egg before fertilization (Davy and Turner, 2003; Barneah *et al*., 2007b**),** the parent can brood larvae internally and transfer symbionts to the larvae before dispersal (Benayahu *et al*., 1988), or anything in between. While the exact process of the transfer may not be known in individual cases, in many of the brooding corals vertical transmission is assumed based on genetic identification of symbionts from a variety of individuals (Thornhill *et al*., 2006; Magalon *et al*., 2007). Regardless of the specific mechanism behind a particular vertical transmission event, the type of symbiont present in the offspring is the same as in the parent.

Transmission in *Anthopleura ballii* occurs early in development when the ova become infected with *Symbiodinium* just prior to spawning. The ova are then fertilized and undergo

cleavage (Davy and Turner, 2003). Development continues normally with the symbionts sequestered to one side of the embryo (Davy and Turner, 2003). This species may have adopted this form of vertical transmission that occurs early in development because it generally thrives in areas where *Symbiodinium* are scarce (the temperate zone) (Davy and Turner, 2003). This is an excellent example of the early initiation of vertical transmission being intimately tied to an organism's development.

Other examples of vertical transfer *via* ova containing *Symbiodinium* sp. are found in *Porites cylindrica*, *Montipora digitata*, *Pocillopora verrucosa* (Hirose and Hidaka, 2006), and *P. damicornis* (Harii *et al.*, 2002). In the case of the *Pocillopora* species, symbionts are sequestered in one part of the ovum, embryo, and the larva. The blastomeres containing *Symbiodinium* become endodermal tissues of the host (Hirose *et al*., 2000). However, in *Porites cylindrica* and *Montipora digitata* the symbionts are equally distributed throughout the cytoplasm of the oocyte and during early cleavage only becoming sequestered to the endoderm during gastrulation when blastomeres containing *Symbiodinium* drop into the blastocoel and develop into the gastrodermis (Hirose and Hidaka, 2006).

In all these cases the symbionts must become part of the endodermal tissues at some point in development (Hirose and Hidaka, 2006). In *Euphyllia glabrescens*, *Symbiodinium* are distributed evenly throughout the oocyte, and later throughout the epidermis and gastrodermis (Huang *et al*., 2008). Following planulation, symbionts are redistributed to the gastrodermis and by metamorphosis the symbionts are found only in the gastrodermis (Huang *et al*., 2008). This indicates that *Symbiodinium* infection may interact with host development.

HORIZONTAL TRANSMISSION. Horizontal transmission can happen a variety of different ways contingent on the host life stage when infection occurs. Horizontal infection is

considered open since offspring can potentially obtain any *Symbiodinium* type present in the water column. This is seen commonly in broadcast spawning scleractinian corals (van Oppen, 2004). There are a few different proposed mechanisms by which horizontal transmission can result in a specific host-symbiont association. These include 1) preferential feeding seen in the planulae of *Aiptasia tagetes* and *Anthopleura elegantissima* on material that includes conspecific components (such as symbionts and associated host membranous material expelled by other hosts; Riggs *et al.*, 1988; Schwarz *et al.*, 2002) 2) chemical attraction of the motile symbiont to the host (Kinzie, 1974; Pasternak *et al*., 2006), 3) phototaxis of the motile symbiont to the host (host pre-symbotic larvae of *Fungia scutaria* show an increased production of light from luminescent green fluorescent protein (GFP) which symbionts swarm toward; Hollingsworth *et al*., 2004; Hollingsworth *et al.*, 2005) and 4) uptake of a number of *Symbiodinium* types followed by selection of only one or a few isolates (Dunn and Weis, 2009). Depending on the host-symbiont association, interactions between some or all of these mechanisms could result in a stable association between host and symbiont.

In typical horizontal transmission *Symbiodinium* is phagocytosed into the endodermal cells during feeding. One study in *Aiptasia tagetes* fed the planula larvae one of four diets; crab, *Artemia* flakes, *Symbiodinium*, the unicellular alga *Isochrysis*, or a combination of these (Riggs *et al.*, 1988). All the food items were eaten and throughout the study (35 days), the planulae retained symbionts that were ingested as part of their diet. While this study did not examine phagocytosis of symbionts or preference in food choice, it did indicate that planula could and did ingest symbionts and maintain them during the study.

In *Heteroxenia fuscencens*, aposymbiotic planula larvae are released from the parent, settle rapidly (within 7-8 hours), and within about 3 days are symbiotic (Benayahu *et al.*, 1989).

Symbiodinium are acquired during metamorphosis, along with the appearance of tentacles, and are initially near the mouth. This is consistent with the host becoming symbiotic *via* feeding (Benayahu *et al.*, 1989). *Fungia scutaria* also releases aposymbiotic larva that acquire symbionts during feeding. When trying to reinfect these larvae with *Symbiodinium* from *Fungia scutaria, Montipora verrucosa, Porites compressa,* and *Pocillopora damicornis,* the homologous *Symbiodinium* strain (from the parent species) infected the aposymbiotic larvae more easily (Weis *et al.*, 2001), indicating specificity between host and symbiont.

Aposymbiotic *Anthopleura elegantissima* obtain symbionts while feeding as a planula larva. During feeding trials, planulae of *Anthopleura elegantissima* did not feed when presented with just *Symbiodinium* (Schwarz *et al.*, 2002). However, if macerated animal tissue (of various species) was added, a feeding response was elicited and larvae ingested any available particulate matter. After ingestion, animal tissue is digested, but symbionts are endocytosed and remain undigested while held inside vesicles in the endodermal cells (Schwarz *et al.*, 2002). Feeding on material that contains macerated animal tissue is not unique and has also been documented in *Aiptasia tagetes* (Riggs *et al.*, 1988), and *Fungia scutaria* (Weis *et al.*, 2001). One hypothesis is that the initiation of feeding in the presence of macerated host tissue could show a preference for symbiont containing material (Schwarz *et al.*, 2002), supporting the theory of specificity early in uptake for food likely to contain a preferred symbiont isolate. This may be the case since, in nature, when symbiont populations within a host get too large the host will expel some material containing living *Symbiodinium* and host membrane remnants (Steele, 1977; Sachs and Wilcox, 2006).

Another method for obtaining a specific symbiont-host relationship could rely on symbionts seeking out the appropriate host, possibly through chemical cues (Kinzie, 1974;

Pasternak *et al.*, 2006). Aposymbiotic planulae of *Pseudopterogorgia bipinnata* observed during experimental infection had symbionts swimming either towards the mouth or near the polyp suggesting the host is somehow attracting symbionts (Kinzie, 1974). In a study with motile *Symbiodinium* isolated from *Cassiopeia xamachana*, taxis assays to various host compounds showed attraction to compounds containing nitrogen (ammonium, nitrate, urea, and amino acids), while no response was observed to phosphate, sulfate, sugars, glycerol, or vitamins (Fitt, 1985). High concentrations of ammonia didn't attract *Symbiodinium*, but more dilute concentrations led to some attraction. This suggests that symbionts are attracted to concentrations of nitrogen-containing compounds excreted by hosts under natural conditions (Fitt, 1985). There is no data to suggest that attraction to nitrogen compounds may convey specificity on the system (e.g., a host attracts only the alga of its preferred association) and it seems much more likely that this is a general response to nitrogen-containing compounds which all hosts excrete as waste. In the soft coral *Heteroxenia fuscescens*, *Symbiodinium* swimming became slower and directed to the source of host attractants (in this case, attractants were chemical substances associated with the juvenile polyp, but not with the adult; Pasternak *et al*., 2004; Pasternak *et al.*, 2006).

Further studies investigated the potential for *Symbiodinium* phototaxis. Initially, researchers noted an upregulation of green fluorescent protein (GFP) in pre-symbiotic larvae of the mushroom coral (*Fungia scutaria*) that coincided with larval ability to accumulate *Symbiodinium* (3-5 days after fertilization) (Hollingsworth *et al.*, 2004; Hollingsworth *et al.*, 2005). Further investigation revealed *Symbiodinium* swarmed toward the light source under green light, but under UV and blue light the symbiont swam away from the light source (Hollingsworth *et al.*, 2005). The host secretion of more GFP in pre-symbiotic hosts combined

with increased phototaxis of the symbiont towards green light suggests that *Symbiodinium* may swim toward these pre-symbiotic planulae larvae. Chemotaxis and phototaxis to host attractants (chemical and light) suggest the symbiont is actively seeking a host. However, chemotaxis and phototaxis probably are not responsible for specificity as many types of *Symbiodinium* would likely be attracted to green light and nitrogenous compounds. Further research into the next steps in infection that result in a specific association are necessary.

SPECIFICITY

Since vertical transmission results in the offspring receiving the same symbiont isolate as the parent, these associations should be highly specific. In horizontal transmission, the host obtains symbionts from the environment. The variety of symbionts present in the water column may allow for variation during the uptake process and in the association. The two transmission methods could confer different levels of variability or specificity. The current evidence is far from conclusive, but seems to suggest high levels of specificity (in most cases) regardless of transmission type (Benayahu *et al*., 1988; Davy and Turner, 2003; van Oppen, 2004; Barneah *et al*., 2007b).

Examinations of Acroporid corals in Indonesia and the central Great Barrier Reef found that diversity of *Symbiodinium* in *Montipora* species (vertical transmitters) and *Acropora* species (horizontal transmitters) was not significantly different. Both genera had symbionts mostly from one clade (about ¾ of hosts within each genus hosted symbionts from one clade). Though the two coral genera hosted some symbionts from the same clade (C), these were relatively divergent from each other (5% sequence divergence) (van Oppen, 2004) suggesting that mode of uptake (either horizontal or vertical) does not affect specificity of the association (van Oppen, 2004).

Investigations on Red Sea soft coral symbionts found that hosts practicing vertical transmission associated exclusively with Clade A, while horizontal transmitting hosts contained Clade C (Barneah *et al*., 2004). Therefore, in the Red Sea, hosts with horizontal uptake developed specificity for Clade C symbionts; while Clade A symbionts co-evolved with vertical transmitting hosts (Barneah *et al*., 2004). Due to high genetic variability amongst Clade C symbionts it is likely that in the Red Sea some Clade C symbionts have a specific physiological adaptation to their host environment, resulting in highly specific associations (Barneah *et al*., 2004). Any contradictions between these two studies could indicate that specificity related to transmission type varies between different hosts or geographic regions.

This early evidence for specificity between host and symbiont may call into question the adaptive bleaching hypothesis. The adaptive bleaching hypothesis states that bleaching may allow hosts to change *Symbiodinium* strains to a strain more suitable to the extant environmental conditions (and less susceptible to future bleaching). In 2006, Goulet examined 442 coral species from 43 studies to determine whether the hosts could associate with multiple symbiont types. Presumably, the ability to change symbiont strain is linked to the ability for a host to associate with multiple strains, either at different depths, locations or microclimates within the host (Goulet, 2006). Using these criteria, only 23% of coral species associate with multiple clades and could change symbiont suggesting that most corals will not change symbionts under environmental stress and only hosts that already maintain symbionts not susceptible to high temperature bleaching would survive (Goulet, 2006).

A response to the Goulet review (2006) re-evaluated the data and found that 64% of the corals used in the study (198 out of 307 species; excluding species noted only to the genus level) were sampled 5 times or less. Scleractinian species sampled at least 10 times did associate with

multiple clades; while scleractinians sampled fewer times did not (Baker and Romanski, 2007). The octocorals were more specific to one clade than scleractinians; almost all of the scleractinian families associated with multiple clades (only the Rhizangiidae associated with only 1 clade) (Baker and Romanski, 2007). Many of the scleractinian corals examined are actually a dynamic community with the ability to associate with multiple symbionts (Baker and Romanski, 2007). Thus in many of the species Goulet (2006) considered, she may have missed the potential for association with multiple clades due to insufficient sampling. However, the original data set (Goulet, 2006) found that in 87.5% of cases diversity in symbiont hosting was seen with less than 5 samples (Goulet, 2007). It is still unclear whether this data supports a lack of flexibility (Goulet, 2006; Goulet, 2007; Goulet *et al*., 2008) or the ability for many scleractinians to change symbionts (Baker and Romanski, 2007).

Both scleractinian corals and octocorals are generally horizontal transmitters (Schwarz *et al.*, 1999; Koike *et al*., 2004) and horizontal transmission dominates (about 85% of species) across all cnidarians (Koike *et al*., 2004). The variation in association with multiple strains between the octocorals (most associating with 1 *Symbiodinium* isolate) and scleractinians (many species associating with multiple isolates) is inconsistent with the conclusion that mode of transmission affects the specificity of the relationship. While there appears to be a difference in specificity between octocorals and scleractinians however; there is no difference in transmission mode.

MECHANISMS OF SPECIFICITY. Once it was established that there was some level of specificity in these symbioses, investigators wanted to know how the specific relationships are established. Initial studies in the *Symbiodinium*-cnidarian association investigated what stable associations could be established. Investigations of *Symbiodinium* infection in *A. pallida* found

that infection with clade B symbionts was more successful (faster and with denser populations) than with clades C and A. This suggested a mechanism for recognition of the symbiont after phagocytosis or different rates of division among clades within the host (Belda-Baillie *et al.*, 2002). For *Cassiopeia xamachana*, the scyphistomae took up *Symbiodinium* freshly isolated from several host species; however, *Symbiodinium* originally extracted from *C. xamachana* established a denser, stable population twice as fast (14 vs. 27 days) as those from other hosts (Colley and Trench, 1983).

Infection in similar associations reveals some possibilities for the mechanisms involved in specificity between *Symbiodinium* and the cnidarian hosts. Symbiotic chlorellae showed decreased rates of infection in aposymbiotic *Paramecium bursaria* when the surface of chlorella was coated with antibodies or lectins. This suggests molecules on the surface of chlorellae are involved in recognition during the onset of symbiosis in this system (Reisser *et al.*, 1982). In the *Hydra viridis* association with chlorellae-like symbionts, reinfection was inhibited with lectin-treated symbionts (Meints and Pardy, 1980). These studies may suggest possible mechanisms for recognition in the *Symbiodinium*-cnidarian association. Examination of the glycoproteins on *Symbiodinium* during the initiation of symbiosis with *Aiptasia pallida* showed that infection decreased when symbionts were treated with lectins altering the surface coat (Lin *et al.*, 2000).

Investigation of the macromolecules associated with the cell wall of *Symbiodinium* found that the cell wall contained cellulose and proteins (Markell *et al.*, 1992). These proteins are hypothesized to be glycoproteins because other microalgae have a high content of glycoprotein associated with the cell wall (Miller *et al.*, 1974; Catt *et al.*, 1976). Subsequent investigation of the macromolecules exuded into culture media identified the amino acid and sugar composition

as components of large molecular weight glycoconjugates (Markell and Trench, 1993). Glycoconjugates were released in varying amounts by different *Symbiodinium* isolates, but all essential amino acids along with neutral sugars (glucose, galactose, fucose, ribose, and mannose) were present in the exudate (Markell and Trench, 1993). A more recent study examined glycoproteins in initiation of this association by using either enzymes to presumably destroy recognized glycoprotein moieties or lectins to mask carbohydrate moieties. These treatments were found to hinder the establishment of the symbiosis (Lin *et al*., 2000).

In addition to studies examining the surface coat, several studies have focused on the molecules in the phagosomes and how they affect retention or digestion of the symbionts. One of these is Rab7, part of the Rab family which is a group of GTPase regulators of vesicular trafficking (Zerial and McBride, 2001). *Aiptasia pulchella* Rab protein (ApRab7) is homologous to other Rab7 proteins found in the human, mouse, and worm genomes (Chen *et al*., 2003). Live photosynthetically active symbionts were included in phagosomes without ApRab7, while heat-killed or photosynthetically-impaired symbionts were housed in phagosomes with ApRab7 (Chen *et al*., 2003). Further investigation showed the Rab5 homolog (ApRab5) was present in early endocytic and phagocytic compartments that promote symbiont survival (Chen *et al*., 2004). ApRab5 was mostly excluded from phagosomes containing dead algae (Chen *et al*., 2004). This could suggest that live *Symbiodinium* retain ApRab5 in the symbiosome membrane so they may persist in the host cell and exclude ApRab7 for the same purpose (Chen *et al*., 2003; Chen *et al*., 2004). ApRab11 showed similar patterns to ApRab7 and was included in phagosomes of heat-killed *Symbiodinium*, photosynthetically inhibited *Symbiodinium*, or latex beads and actively excluded from phagosomes that contained live *Symbiodinium* (Chen *et al*.,

2005). These proteins could act as one mechanism in the *Symbiodinium*-cnidarian symbiosis to establish and maintain the association.

If multiple symbionts are phagocytosed in horizontal transmitting hosts or after a bleaching event, some of the *Symbiodinium* isolates will be maintained while others are digested or egested. This process is referred to as "the winnowing" (Nyholm and McFall-Ngai, 2004). In *Fungia scutaria*, both homologous and heterologous symbionts are phagocytosed into host cells, but shortly thereafter homologous algae were found to localize between the oral and aboral ends while heterologous algae did not localize in any specific place (Rodriguez-Lanetty *et al*., 2004; Rodriguez-Lanetty *et al.*, 2006). In *Acropora tenuis* and *A. millepora*, juveniles become initially colonized by both heterologous and homologous algae, but by 3.5 years of age the polyps were hosting homologous symbionts (Abrego *et al.*, 2009b). Further investigation in *A. tenuis* found that ITS-1 type C1 symbionts initially dominated infections, but after 96 h, type D symbionts (the homologous type) began to dominate, suggesting that the winnowing process begins shortly after infection (Bay *et al.*, 2011). On the cellular level, *Fungia scutaria* exhibited an increase in caspase activation and apoptosis when infected with a heterologous symbiont, suggesting that apoptosis may be involved in the post-phagocytic winnowing (Dunn and Weis, 2009).

With the high levels of specificity in the cnidarian-*Symbiodinium* symbiosis, there are many possible mechanisms that may play a part in establishing the relationship between host and symbiont. Specificity may be due in part to the origin of symbionts (either from parent or from environment; Benayahu *et al*., 1988; Davy and Turner, 2003; van Oppen, 2004; Barneah *et al*., 2007b). Recognition may also occur at the symbiosome membrane because of glycoprotein coats secreted by the alga (Weis *et al.*, 2001). Other studies suggest the symbiont may be responsible for maintaining the association by halting host digestion (Chen *et al.*, 2003; Chen *et al.*, 2004;

Chen *et al.*, 2005) or that a post-phagocytic winnowing process may establish the stable relationship (Dunn and Weis, 2009). An alternative mechanism, the "Test Tube Hypothesis", suggests algae are ingested unselectively to the digestive vacuole and then subjected to a selection process similar to the selection process that would occur in the media in a test tube (Huss *et al*., 1994). The algae preadapted to live in the host environment will be maintained and reproduce faster and more effectively than other symbionts (Huss *et al*., 1994). At this point, there is evidence for each of these mechanisms and each or combinations of 2 or more may be important in many *Symbiodinium* symbioses.

BREAKDOWN OF THE SYMBIOSIS

Symbiodinium symbioses can break down in a process which leaves the invertebrate host devoid of symbionts. The breakdown of the *Symbiodinium*-cnidarian symbiosis resulting in the loss of *Symbiodinium* from the host or loss of pigments from the symbionts is called bleaching. Thus far a number of causative agents have been implicated in bleaching, among these are changes in temperature, ultraviolet radiation, disease, and pollution (Jokiel and Coles, 1977; Glynn, 1991; Hoegh-Guldberg, 1999; Hughes *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007). Due to recent rises in global temperature, the bleaching agents that are best understood and most often seen are those of temperature and ultraviolet radiation (either alone or in concert). Over the last 50 years the frequency and scale of bleaching events has increased rapidly (Hughes *et al.*, 2003). Using this information to create predictive models indicates that reefs in some areas (Micronesia and Western Polynesia in particular) could be extremely vulnerable to catastrophic collapse over the next 30-50 years (Donner *et al.*, 2005). Increases of just 1°C predict an increase in bleaching

occurrence involving 50% to 82% of reefs while a more dramatic temperature increase of 2ºC would increase the occurrence of bleaching to 97% of all reefs (Berkelmans *et al.*, 2004).

The precise cellular causes of bleaching and which partner in the relationship initiates the event are beginning to be understood. The process is a response to stress by the association (Perez *et al.*, 2001; Perez and Weis, 2006; Weis, 2008). The translocation of energetic nutrients from *Symbiodinium* to the host has been shown to decrease in bleaching situations (high irradiance and temperature; Porter *et al.*, 1989). Furthermore, study at the cellular level has suggested a variety of mechanisms including host cell detachment (Gates *et al.*, 1992), apoptosis, and autophagy of host cells (Hanes and Kempf, 2013). These cellular responses appear to be related to the increase in reactive oxygen species (ROS) produced by damage to the photosynthetic membranes of the symbiont (Tchernov *et al.*, 2004) and to mitochondrial membranes in both the host and symbiont (Weis, 2008) during thermal bleaching. In a healthy, non-stressed host, these reactive oxygen species would be managed by enzymes such as catalase and superoxide dismutase; however, these ameliorative mechanisms are not sufficient during bleaching episodes (Richier *et al.*, 2005; Lesser, 2006; Merle *et al.*, 2007; Weis, 2008). One reactive oxygen species, nitric oxide, has been well studied and is produced as a response to stress in the model anemone, *Aiptasia pallida* (Perez and Weis, 2006). Nitric oxide may act not just as a cytotoxin but also as a signaling molecule in host innate immunity (Fang, 2004; Pacher *et al.*, 2007). While there is still much to be learned about the cellular processes behind bleaching, it is clear that thermally induced bleaching is a threat to coral reefs.

While the cellular bleaching process is not completely understood, there has been research investigating variation in susceptibility to bleaching. Examination of host species reveals that heavier bodied corals have better survival rates than those with more delicate

branching structure (Loya *et al.*, 2001; McClanahan, 2004). Additionally, studies examining the thermal tolerance of *Symbiodinium* in culture found that there is variation among isolates in regard to their response to elevated temperatures (Warner *et al.*, 1996; Iglesias-Prieto and Trench, 1997; Rowan *et al.*, 1997; Kinzie *et al.*, 2001; Perez *et al.*, 2001; Bhagooli and Hidaka, 2004). Often these studies found that clade D *Symbiodinium* were more tolerant to high temperatures (Baker *et al.*, 2004; Chen *et al.*, 2003; Rowan, 2004) while clade A symbionts were often identified as being more susceptible to damage from high temperatures (Diaz-Almeyda *et al.*, 2011; Steinke *et al.*, 2011). Likewise, in the field clade D dominated sites that routinely received elevated temperatures, indicating that clade D symbionts are better adapted to high temperature (Fabricius *et al.*, 2004). Clade D also dominated reefs that had just undergone a bleaching event indicating either that clade D symbionts did not bleach from their hosts or that clade D symbionts readily re-colonized bleached hosts (Glynn *et al.*, 2001; Jones *et al.*, 2008; Oliver and Palumbi, 2011). Even though thermal stress acts on the holobiont (symbiont, host, and any associated bacteria), it is important to understand how both the host and symbiont impact the thermal tolerance and therefore the bleaching susceptibility of the coral as a whole.

There is some evidence that non-fatal bleaching episodes may be an adaptive mechanism to change symbionts to a more robust association (Buddemeier and Fautin, 1993; Baker *et al.*, 2004; Stat and Gates, 2011). A study of 3 genera (*Acropora, Pocillopora,* and *Porites*) on the Great Barrier Reef that encompassed the 1998 and 2002 bleaching events found that both *Pocillopora* and *Acropora* showed an increase in tolerance compared with what was expected in the 2002 bleaching event. This increase in tolerance may be in part due to selective mortality (of more susceptible individuals) during the 1998 bleaching but also may indicate some capacity for acclimatization and hope for coral reefs (Maynard *et al.*, 2008). Information on the

susceptibility and tolerance of hosts and symbionts could be used to practice better reef management (transplanting more robust corals to reefs) which could in turn protect the coral reef ecosystem from collapse. Understanding both the bleaching process and the potential stability of a variety of host/symbiont associations should improve future reef management and appropriate responses to bleaching events.

II. IMMUNOHISTOCHEMICAL VARIATION AMONGST *SYMBIODINIUM* ISOLATES.

ABSTRACT

Symbiodinium (Freudenthal) reside intracellularly in a complex symbiosome (host and symbiont-derived) within cnidarian hosts in a specific host-symbiont association. *Symbiodinium* is a diverse genus with variation equivalent to other dinoflagellate orders. In this paper, our investigation into specificity examines antigenic variation in the algal mucilage secretions at the host-symbiont interface. Cultured *Symbiodinium* from a variety of clades were labeled with one of two antibodies to symbiont mucilage (PC3, developed using a clade B alga cultured from *Aiptasia pallida*; BF10, developed using a clade F alga cultured from *Briareum* sp.). The labeling was visualized with a fluorescent marker and examined with epifluorescence and confocal microscopy. PC3 antigen was found in cultured *Symbiodinium* from clades A and B, but not clades C, D, E and F. The correlation between labeling and clade may account for some of the specificity between host and symbiont in the field. Within clades A and B there was variation in the amount of label present. BF10 antigen was more specific and only found in cultures of the same cp23S-rDNA strain the antibody was created against. These results indicate that the mucilage secretions do vary both qualitatively and quantitatively amongst *Symbiodinium* strains. Since the mucilage forms the host-symbiont interface, variation in its molecular composition is likely to be the source of signals involved in recognition and specificity.

INTRODUCTION

Coral reef ecosystems are among the most diverse habitats in the world. Reefs provide benefits that include: ecotourism, fish hatchery and/or nursery for many commercial fisheries, species habitat, and coastal protection (Richmond and Wolanski, 2011). These diverse

ecosystems are built upon the relationship between *Symbiodinium* sp. and their host cnidarians. Hermatypic corals are the structural foundation of the reef in large part due to the photosynthetically fixed carbon compounds donated by *Symbiodinium* (Muscatine and Porter, 1977; Hallock, 2001). Unfortunately, the coral reef ecosystems are threatened world-wide by symbiont loss (commonly referred to as coral bleaching) that occurs due to increased sea surface temperature in addition to other stress-related factors resulting from human activities (Jokiel and Coles, 1977; Hoegh-Guldberg, 1999).

Symbiodinium associate with a variety of hosts as endosymbionts. In symbioses with cnidarians, the symbionts reside intracellularly (Colley and Trench, 1983). The cnidarian/*Symbiodinium* symbiotic association can be initiated in one of two ways: either by vertical transmission (obtaining symbionts from the parent) or horizontal transmission (obtaining symbionts from the surrounding environment). *Symbiodinium* are acquired *via* horizontal transmission in 85% of invertebrate hosts (Schwarz *et al.*, 2002).

Association between *Symbiodinium* and the host cnidarian is based on nutrient exchange. The symbiont produces photosynthate that is transferred to the host and can potentially provide enough carbon based nutrients to meet a host's energetic demands (Falkowski *et al.*, 1984; Muscatine *et al.*, 1984; Muscatine, 1990). The symbiont benefits from a protected environment and host waste products. These include nitrogen compounds such as ammonium and nitrate that are typically limiting on reefs (Muscatine and Porter, 1977; Muller-Parker and D'Elia, 1997), as well as CO_2 (Pearse, 1970). The fact that this symbiosis exists in hermatypic corals allows for the energetic demands of higher calcification rates necessary for reef building (Pearse and Muscatine, 1971; Barnes and Chalker, 1990), as well as better conservation of nitrogen

compounds within the reef environment (Lewis and Smith, 1971; Burris, 1983; Ambariyanto and Hoegh-Goldberg, 1996).

Due to their nearly identical morphology, symbiotic algae in corals were originally thought to be the single species *Symbiodinium microadriaticum* (Freudenthal, 1962); however, molecular methods have revealed that the genus actually is a complex of (at least) 9 clades (Rowan and Powers, 1991b; Carlos *et al.*, 1999; LaJeunesse and Trench, 2000; LaJeunesse, 2001; Pochon *et al.*, 2001; Pochon *et al.*, 2004; Pochon and Gates, 2010). The variation within this one genus is equivalent to the diversity amongst other dinoflagellate orders (Rowan and Powers, 1992). Variation in thermal tolerance of *Symbiodinium* isolates is based on observational studies of hosts in different habitats (Oliver and Palumbi, 2011) or before and after bleaching (Glynn *et al.*, 2001; Baker *et al.*, 2004; Jones *et al.*, 2008; LaJeunesse *et al.*, 2010). Studies manipulating either host habitat in transplantation experiments or temperature regimes of cultured symbionts also indicate that certain symbiont isolates survive more effectively than other strains in thermally stressful environments and are therefore thermally tolerant (Iglesias-Prieto and Trench, 1997; Rowan, 2004; Berkelmans and van Oppen, 2006). In particular, strains A1, A2, D, and F2 are accepted as being thermally tolerant (Rowan, 2004; Baker *et al.*, 2004; Robison and Warner, 2006; Steinke *et al.*, 2011).

Previous studies have revealed that the wide variation in both host and symbiont species generates potential for specificity or plasticity within the associations. Previous studies have examined a variety of associations and found varying levels of specificity between partners. Many studies of cnidarians have found host species typically paired with the same symbiont strain (Schoenberg and Trench 1980a, b, c; Rowan and Powers 1991a, b; Goulet and Coffroth, 2003a, b; Santos *et al.*, 2003; Goulet and Coffroth, 2004; Baker *et al.*, 1997). This specificity

exists both in host species that display vertical as well as horizontal transmission (van Oppen, 2004). Host species that can associate with more than one symbiont strain tend to show variation in their associated strains between colonies in different habitats (Rowan and Knowlton, 1995; Rowan *et al.* 1997) or within colonies at different depths or even parts of a given colony exposed to different light regimes (van Oppen *et al.*, 2001; Ulstrup and van Oppen, 2003). Host species that in the field can associate with several symbiont strains may have varied mortality during a bleaching event; hosts containing more tolerant symbionts show reduced mortality compared to hosts with thermally susceptible symbionts (Jones *et al.*, 2008; LaJeunesse *et al.*, 2010). Following a bleaching event, hosts with multiple strains may change to a more thermally tolerant, dominant, symbiont strain (Baker *et al.*, 2004; Berkelmans and van Oppen, 2006). Thus, the association between host and symbiont is often specific, but in some cases also allows for some variation depending on environmental conditions.

Within the cells of the host, the symbiont resides within a host derived (Wakefield and Kempf, 2001) vacuole membrane with several layers of additional membranous material called the symbiosome (Roth, 1989). In the association between *Symbiodinium* and cnidarians, the symbiosome is composed of both host and symbiont components (Wakefield and Kempf, 2001). The symbiont component consists of multiple layers of a "membranous material" that Wakefield and Kempf (2001) have suggested is composed of shed thecal plates from a reduced ecdysis cycle that occurs in situ. The host component consists of the phagosome membrane that resulted from the host cell endocytosing the symbiont (Colley and Trench, 1983; Fitt and Trench, 1983; Schwarz *et al.*, 1999; Wakefield and Kempf, 2001; Schwarz *et al.*, 2002). This membrane is sometimes referred to as the outer symbiosome membrane (Wakefield and Kempf, 2001).

The symbiont secreted layers of presumed shed thecal plates are intermingled with exudates produced by the symbiont (Wakefield and Kempf, 2001). Work by Markell *et al*. (1992) and Markell and Trench (1993) on cultured symbionts suggest that these exudates are largely composed of glycoproteins. Symbiont-derived glycoproteins that are water soluble in culture are secreted through the cell wall and are thought to form the symbiont component that is in direct contact with the host *via* the phagosome membrane (Wakefield and Kempf, 2001). The region of contact between host and symbiont exudate is the likely location of any recognition molecules involved in both initial uptake of the symbiont by the host and maintenance of the symbiosis. Lin *et al*. (2000) used lectins to mask the cell surface and proteolytic enzymes to remove cell surface glycoproteins and saw inhibition of symbiont uptake by the host in both types of treatment. This suggested that the affected glycoconjugates are used for cell-cell recognition in the initiation of this symbiosis (Lin *et al.*, 2000). The process of recognition probably contains multiple steps. Initially, there is selection for living symbionts with an intact outer layer while dead symbionts or those whose outer layer is compromised are discarded (Fitt and Trench, 1983; Lin *et al.*, 2000). However, there is also evidence that in nature the initial uptake of symbionts is dominated by "highly infectious" symbiont strains (Abrego *et al.*, 2009a) that may later be replaced by that hosts's preferred symbiont strain. The mucilaginous outer layer is secreted in situ by symbionts already taken up by the host tissue (Wakefield and Kempf, 2001). Wakefield and Kempf's observation suggests that this layer may be involved in later stages of the recognition process and that secretion of mucilage may be a factor in preventing the host from digesting or expelling the symbiont.

Cell-cell recognition processes have been identified in a number of other organisms, from the mammalian immune system (Takeda *et al.*, 2003) to predatory dinoflagellates (Jeong *et al.*,

1997; Martel, 2006; Breckels *et al.*, 2011). These processes have a few things in common: a molecule on each cell involved in the recognition and a mechanism for these molecules to come into contact with each other. These molecules can then bind and produce a cascade of events resulting in a variety of different outcomes. In predatory dinoflagellates, recognition results in the dinoflagellate either consuming its preferred prey or leaving the non-prey organism alone (Jeong *et al.*, 1997; Martel, 2006; Breckels *et al.*, 2011). In cnidarian/*Symbiodinium* associations, recognition (or lack thereof) results either in the symbiont remaining in the host gastrodermal cell or potential digestion and/or expulsion of the non-preferred partner. This study is directed toward an examination of variation in the composition of surface bound exudates of a number of strains of *Symbiodinium*. Such variation could be involved in recognition specificity and host/symbiont "communication" in cnidarian host-*Symbiodinium* symbioses.

MATERIALS AND METHODS

ALGAL CULTURE. *Symbiodinium* isolates, representing a range of host species, geographic locations and genetic diversity (Table 1), were cultured in F/2 medium (without silica to prevent the growth of diatoms; Guillard and Ryther, 1962) using a 12/12 hr light/dark regimen at Auburn University. Light intensity was maintained at ~180 μmol photons /m^2/s using Sylvania Cool White 20W fluorescent bulbs. Samples were collected from each culture prior to the lights coming on to ensure cells were non-motile (Yacobovitch *et al.*, 2004).

DEVELOPMENT OF MONOCLONAL ANTIBODIES BINDING TO MUCILAGE FROM SYMBIODINIUM *CULTURE*. Two antibodies that bind components of *Symbiodinium* mucilage were used in this study. The first, PC3, was previously developed by Wakefield (2001) against a component of the mucilage from *Symbiodinium* ITS2 "type" A4 of *Aiptasia pallida*

from the Florida Keys (Santos *et al.*, 2002; Santos, personal communication). The second, BF10, was generated (see below) for this study using a *Briareum* sp. clade F (chloroplast large subunit (cp23S)-ribosomal DNA genotype F178) alga originally collected from the Florida Keys. Aliquots were divided into separate 15 ml glass centrifuge tubes and either left (1) untreated or subjected to (2) fixation in 4% paraformaldehyde containing 0.2 M Millonig's phosphate buffer for 30 minutes followed by rinsing in 0.2 M Millonig's phosphate buffer (3X) and resuspension in 15-16 megaohm Nanopure water (np-H2O), (3) boiling for 1 minute in Millepore filtered sea water (MFSW), (4) three rounds of freezing and thawing by transferring the sample to a 1.5mL microcentrifuge tube, dropping the tube in liquid nitrogen, then re-warming the tube, or (5) resuspending the sample in 50% ethanol and homogenizing with a Virtis Handishear Homogenizer for 2 minutes followed by centrifugation and resuspension in np-H2O. All 5 types of samples were combined and used as the antigen to produce monoclonal antibodies. Antibodies were produced and screened using standard monoclonal techniques as described by Wakefield (2001). In brief, this symbiosome slurry was injected into BALB\C mice followed by a second injection boost after 3 weeks. Blood serum was tested for reaction to the symbiosome slurry antigens using an enzyme-linked immunosorbant assay (ELISA); the mouse exhibiting highest titer of antibody was sacrificed and splenocytes were isolated. Hybridoma cells were created using a standard polyethylene glycol fusion technique with the splenocytes and AG-8.653 myeloma cells 4:1. Antibody producing clones were screened by ELISA. Two antibody producing cell lines, BA6 and BF10, strongly labeled the mucilage of *Symbiodinium* cp23S-rDNA genotype F178. Hybridoma cells secreting these antibodies were cloned by limiting dilution and then recloned to assure that only this line of hybridoma cells was isolated. Antibody to be used in labeling was collected as spent culture medium, from the final cloned cell line,

aliquoted in 0.5mL amounts into 1.5 mL Eppendorf tubes, frozen, lyophilized, and frozen for future use.

Table 1. Symbiodinium cultures examined for presence of antigens PC3 and BF10.

Host Organism	**Culture ID**	**Collection Location**	**cp23S–rDNA designation**
Zoanthus sociatus	Zs	Jamaica	A188
Unknown Host	Y109	Japan	A191
Cassiopea sp.	CassKB8	Hawaii	A194
Cassiopea xamachana	Cx	Jamaica	A194
Cassiopea xamachana	FLCass	Florida	A194
Pseudoplexaura porosa	719	Panama	A194
Cassiopea sp.	CassMJ300	Hawaii	A198
Tridacna gigas	T	Hawaii	A198
Cassiopea sp.	CassEli	Hawaii	A198
Plexaura kuna	708	Panama	Undetermined (A)
Montastrea faveolata	Mf11.4	Florida	Undetermined (A)
Aiptasia pallida	FLAp4	Florida	A193
Aiptasia pallida	FLAp3	Florida	A193
Aiptasia pallida	FLAp210AB	Florida	B184
Plexaura kuna	706	Panama	B184
Aiptasia pulchella	Ap10	Japan	B184
Porites evermanni	Pe	Hawaii	B184
Zoanthus pacificus	Zp	Hawaii	B184
Plexaura kuna	13	Florida	B184
Plexaura kuna	702	Panama	B211
Briareum asbestinum	579	Florida	B223
Aiptasia pulchella	Ap	Hawaii	B224
Plexaura flexuosa	PurPFlex	Florida	B224
Montastrea faveolata	Mf11.5b.1	Florida	Undetermined (B)
Unknown anemone	Ap31	Japan	C180
Montastrea faveolata	Mf8.5Tb.2	Florida	F178[a]
Acropora sp.	A002	Japan	D206
Acropora sp.	A001	Japan	D206
Unknown	CCMP421	New Zealand	E202
Montipora verrucosa	Mv	Hawaii	F178
Briareum sp.	PtBr	Florida	F178

[a] This culture was classified as a clade C symbiont using the 18S RFLP (Santos, personal communication, 2012). Here, this strain was retested using the cp23S-rDNA sequence information and was reclassified as clade F178. Clade F and clade C are closely related and using the 18S RFLP they appear the same.

FIXATION AND IMMUNOLABELING FOR EPIFLUORESCENCE MICROSCOPY. Cultured *Symbiodinium* isolates (Table 1) were individually centrifuged and the culture medium removed. Pellets were resuspended in 4% paraformaldehyde in 0.2M Millonig's Phosphate Buffer and allowed to fix for 1 hour at room temperature. Samples were then rinsed once in 20mM PBS containing 0.1% sodium azide and 0.1% Triton X100 (PBS+) and then dehydrated and rehydrated through an ethanol series (np-H2O, 30% ethanol, 50% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, np-H2O) for 5 minutes in each solution. Following rehydration, samples were blocked in 5% heat-inactivated goat serum in PBS+ (blocking medium) for 10 minutes at room temperature. Blocking medium was removed by centrifugation and samples were incubated with spent medium containing primary antibody (either PC3 or BF10) diluted 1:1 in blocking medium for 1 hour at room temperature (a third sample of each strain was incubated with only blocking medium for this hour as a negative control). Samples were then washed in PBS+ 3 times for 5 minutes each, followed by incubation in secondary antibody (Alexa fluor 488 Goat anti-mouse IgM (µ chain) - Invitrogen, A-21042) diluted 1:300 in blocking medium for 30 minutes at room temperature in darkness (to prevent quenching). Samples were then rinsed 3 times in PBS+ for 5 minutes each and resuspended in a small amount of np-H2O as follows. Ten µl of labeled sample was added to 7 µl of suspended standard fluorescent beads (InSpeck Microscope Image Intensity Calibration Kit in Green, 2.5 µm, I-7219) and plated on slides using a Shandon Cytospin 2 to ensure that beads and cells would lie flat on the surface of the slides.

EPIFLUORESCENCE MICROSCOPY. Glycerol mounting media was used to mount cover slips on the slides which were then kept in pop up slide holders so that slides would remain covered before being viewed on the microscope. This would minimize any bleaching between taking the first micrograph and the last micrograph of a given run. A control slide was imaged at

the beginning of each run and then also imaged again at the end of the run to determine whether any quenching occurred during the period the microscope was being used. Slides were viewed on Nikon Eclipse E800 with a Qimaging Retiga camera under light of wavelength 488 nm (to visualize labeling) and 594 nm (to visualize algal autofluorescence). Micrographs were taken of at least 30 cells with adjacent standard fluorescent beads using NIS Elements Software (Nikon Corporation, 2006). All micrographs were taken using the same settings on the microscope and with all the same software settings.

FLUORESCENCE AND STATISTICAL ANALYSIS. The fluorescence intensities of the algal mucilage layer and of the beads were measured using ImageJ software to calculate the highest brightness value from the histogram. The 0%, 0.3% and 1% InSpeck beads were used to create a standard curve. This standard curve could then be used to estimate the percent of fluorescence measured in the mucilage layer of the algal cell. Fluorescence differences among strains were compared using a one-way ANOVA (SAS Version 9.2) to ascertain if any significant differences in labeling existed amongst labeled *Symbiodinium* strains. Post-hoc multiple comparison tests were run with a Tukey-Kramer adjustment to examine which strains exhibited significant differences in fluorescence (SAS Version 9.2).

EXTRACTION, AMPLIFICATION AND SEQUENCING OF Cp23S rDNA. Based on the labeling results (see below), there was a question about the cp23S-rDNA identity of *Symbiodinium* culture Mf8.5Tb.2. DNA extraction and sequencing were performed on this particular isolate as well as several others (to serve as controls) after the methods of Santos *et al*. (2002). PCR success was confirmed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining and ultraviolet (UV) light. Amplicons were sent to Genewiz, Inc. for sequencing.

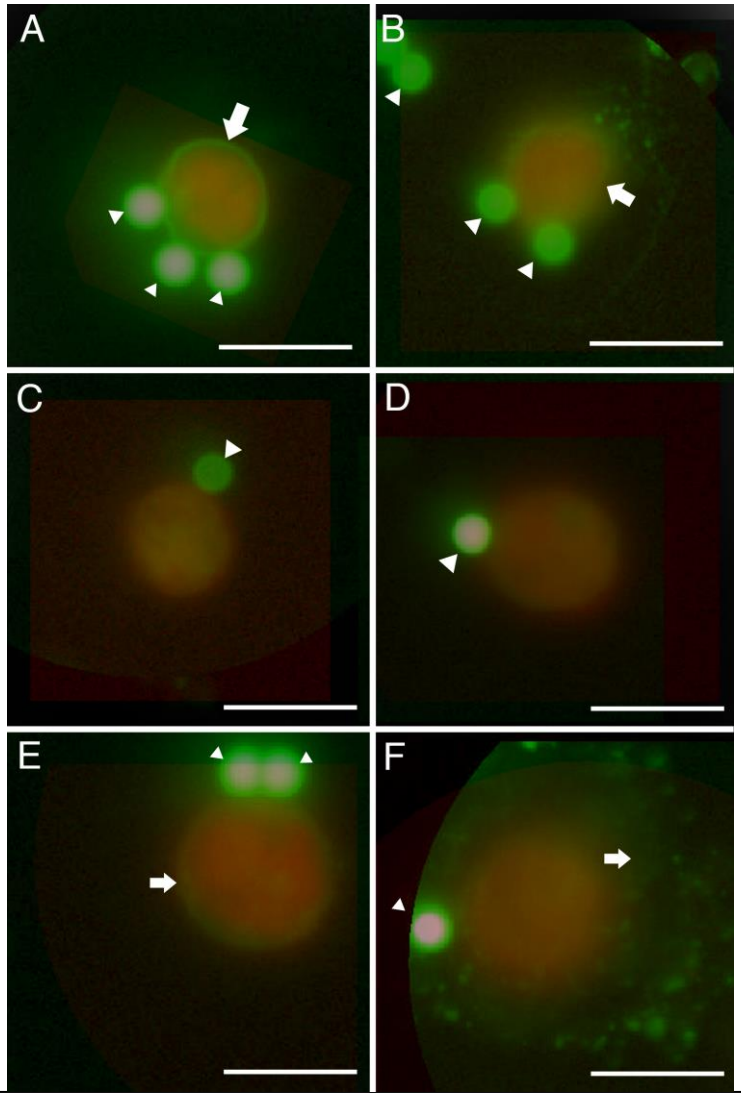

Figure 1. PC3 antibody labeling (Panels A-D) and BF10 antibody labeling (Panels E and F) of *Symbiodinium* strains. A. FLAp4 originally cultured from *Aiptasia pallida* collected from Long Key, FL. The cp23-rDNA genotype of this strain is A193. This strain contains the antigen PC3 antibody was created against and showed labeling. B. 702 originally cultured from *Plexaura kuna* collected in Panama. The cp23S-rDNA genotype is B211. This strain showed faint labeling. C. Ap31 originally cultured from an unknown anemone collected from Sesoko Jima, Okinawa, Japan. The cp23-rDNA genotype of this strain is C180. This strain did not show labeling. D. A002 originally cultured from *Acropora* sp. collected in Okinawa. The cp23S-rDNA genotype is D206. This strain did not show labeling. E. PtBr originally cultured from *Briareum* sp. collected from Long Key, FL. The cp23S-rDNA genotype is F178. This is the strain that contains the antigen that the BF10 antibody was created against and showed labeling. F. Mf8.5Tb2 originally cultured from *Monstastrea faveolata* collected from Florida. The cp23S-rDNA genotype determined in this study is F178. This strain showed very faint and diffuse labeling. All scale bars are 10 µm. Arrows indicate a ring of green labeling around the cell; arrowheads indicate standard florescent beads. All images were adjusted using Photoshop auto levels to better visualize the florescence here. These adjustments were made only for visualizing and not for florescence measurements.

RESULTS

DEVELOPMENT OF MONOCLONAL ANTIBODIES BINDING TO MUCILAGE FROM SYMBIODINIUM *CULTURE*. Two monoclonal antibodies (BA6 and BF10) were developed that labeled mucilage secreted by cultured *Symbiodinium* cp23S-rDNA genotype F178. In early examinations, BF10 labeled some isolates (typically isolates closely related to the isolate the antibody was created against), but did not label other isolates and was chosen for further investigation. In contrast, BA6 labeled mucilage from every *Symbiodinium* isolate tested (31 different cultures representing 18 cp23S-rDNA genotypes).

IMMUNOCYTOCHEMISTRY. Incubation with the PC3 antibody (Wakefield *et al.*, 2000) exhibited labeling only in *Symbiodinium* from clades A and B (Figure 1). In clade A, 1of 5 cp23S-rDNA genotypes (i.e., A191) did not show labeling (Table 2). Likewise for clade B, 2 of 4 cp23S-rDNA genotypes (i.e., B223 and B224) did not exhibit labeling. No labeling with the PC3 antibody was observed in the tested isolates from clades C, D, E and F (Table 2). Incubation with the BF10 antibody exhibited labeling only in 2 isolates, one of cp23S-rDNA genotype F178 and one of unknown cp23S-rDNA genotype (culture ID Mf8.5Tb.2). Analysis of the cp23S-rDNA sequence of culture Mf8.5Tb.2 identified this as cp23S-rDNA genotype F178 (Figure 3).

STATISTICAL ANALYSIS OF ANTIGEN LABELING AMONG SYMBIODINIUM *CLADES AND STRAINS*. There were significant differences between *Symbiodinium* isolates in labeling intensity with the PC3 and BF10 antibodies ($p<0.0001$, one-way ANOVA). Multiple comparison tests found that the isolates could be divided into significantly different categories (Figure 2). For example, non-labeling isolates were significantly different from all those that labeled. Amongst those that labeled with the PC3 antibody, there are several groups composed

Table 2: Labeling fluorescence presence of each *Symbiodinium* culture with each of 2 antibodies.

Host Organism	Culture ID	Cp23S-rDNA	Label PC3	Label BF10
Zoanthus sociatus	Zs	A188	+	-
Unknown Host	Y109	A191	-	-
Aiptasia pallida	FLAp4	A193	+	-
Aiptasia pallida	FLAp3	A193	+	-
Cassiopea sp.	CassKB8	A194	+	-
Cassiopea xamachana	Cx	A194	+	-
Cassiopea xamachana	FLCass	A194	+	-
Pseudoplexaura porosa	719	A194	+	-
Cassiopea sp.	CassMJ300	A198	+	-
Tridacna gigas	T	A198	+	-
Cassiopea sp.	CassEli	A198	+	-
Plexaura kuna	708	Undetermined (A)	+	-
Montastrea faveolata	Mf11.4	Undetermined (A)	-	-
Aiptasia pallida	FLAp210AB	B184	+	-
Plexaura kuna	706	B184	+	-
Zoanthus pacificus	Zp	B184	+	-
Aiptasia pulchella	Ap10	B184	+	-
Porites evermanni	Pe	B184	+	-
Plexaura kuna	13	B184	+	-
Plexaura kuna	702	B211	+	-
Briareum asbestinum	579	B223	-	-
Aiptasia pulchella	Ap	B224	-	-
Plexaura flexuosa	PurPFlex	B224	-	-
Montastrea faveolata	Mf11.5b.1	Undetermined (B)	-	-
Unknown anemone	Ap31	C180	-	-
Montastrea faveolata	Mf8.5Tb.2	F178[a]	-	+
Acropora sp.	A002	D206	-	-
Acropora sp.	A001	D206	-	-
Unknown	CCMP421	E202	-	-
Montipora verrucosa	Mv	F178	-	-
Briareum sp.	PtBr	F178	-	+

[a] This culture was classified as a clade C symbiont using the 18S RFLP (Santos, personal communication, 2012). Here, this strain was retested using the cp23S-rDNA sequence information and was reclassified as clade F178. Clade F and clade C are closely related and using the 18S RFLP they appear the same.

of isolates that are not significantly different from one another, but that were different from other groups of isolates (indicated by lines connecting isolates; Figure 2). Analysis of labeling with BF10 showed that most isolates did not label, and did not exhibit significantly different levels of background fluorescence, from other non-labeling isolates. The two isolates that did label with this antibody were significantly different in their levels of fluorescence, both from each other and from all other isolates (indicated by symbols above the bar; Figure 2).

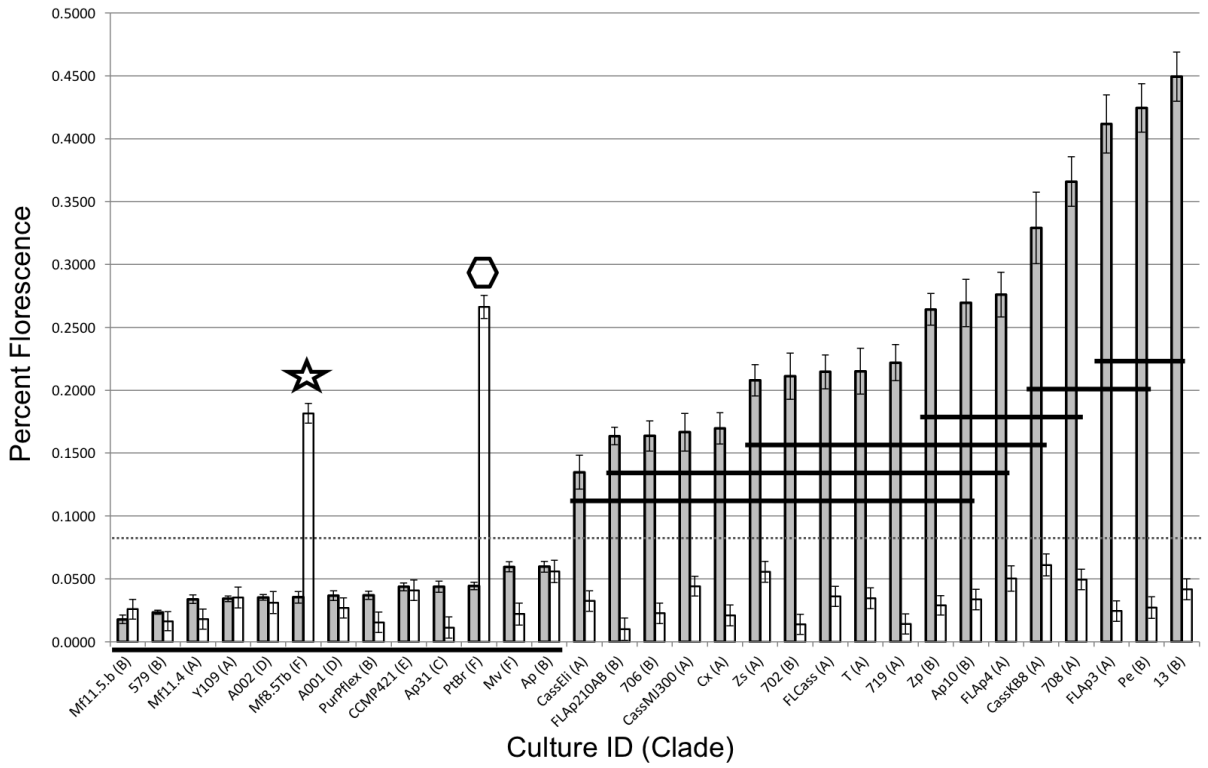

Figure 2: Graph of Percent Fluorescence of PC3 antibody labeling (filled bars) and BF10 antibody labeling (empty bars). Error bars indicate the standard deviation for each sample. Statistical analyses were performed comparing the PC3 labeling amongst all Symbiodinium cultures and another set of analyses were performed comparing the BF10 labeling amongst all Symbiodinium cultures. Solid lines indicate significance when labeling with the PC3 antibody. Cultures grouped under the same black line are not significantly different from each other but are significantly different from all other cultures ($P<0.005$). Symbols are used to indicate significance when labeling with the BF10 antibody. All other open bars did not show labeling with the BF10 antibody and are not significantly different from each other or from background fluorescence. Bars labeled with a star and hexagon are significantly different from all other bars ($P<0.005$). All bars falling below the dotted line do not exhibit specific labeling but are representative of minor differences in background fluorescence.

DISCUSSION

Variation in symbiont strain both genetically, as seen in many studies (Rowan and Powers, 1991b; Carlos *et al.*, 1999; LaJeunesse and Trench, 2000; LaJeunesse, 2001; Pochon *et al.*, 2001; Pochon *et al.*, 2004; Pochon and Gates, 2010), and antigenically as shown here, indicate a basis for specificity between the host and symbiont. The nature of the host and symbiont relationship is quite specific in many cases (LaJeunesse, 2002; Rodriguez-Lanetty *et al.*, 2004; Santos *et al.*, 2003). However, host colonies that are large or shaped in such a way that different areas of the colony have different light regimes may have different symbiont populations in these different habitats (Rowan *et al.*, 1997; LaJeunesse and Trench, 2000; Rodriguez-Lanetty *et al.*, 2001; van Oppen *et al.*, 2001; LaJeunesse *et al.*, 2003; Ulstrup and van Oppen, 2003; Iglesias-Prieto *et al.*, 2004). For instance, in *A. tenuis* colonies at Pioneer Bay, C1 was found on the shaded side of the colony while C2 was found on the side of the colony exposed to light (van Oppen *et al.*, 2001). This is indicative of a relatively complex process occurring between the host and the symbiont.

The process of initiation of the symbiosis between *Symbiodinium* and its cnidarian host contains multiple steps. First the host and symbiont must come into contact with each other. Thus, as the host is sessile in most cases of horizontal transmission, it is likely that the process of encounter is often initiated by the motile stage of the symbiont. *Symbiodinium* have been shown to exhibit chemotaxis towards aposymbiotic host organisms, fed symbiotic hosts, and brine shrimp nauplii. However, no chemotaxis toward starved host organisms has been documented suggesting that at least part of the taxis is directed toward ammonia or nitrate (Fitt, 1984). Further investigation found that the taxis is directed toward a variety of nitrogen compounds such as ammonium, nitrate, urea, and some amino acids, but there was no taxis toward

phosphate, sulfate, vitamins, trace metals, or sugars (Fitt, 1985). These studies indicate that *Symbiodinium* taxes toward either a host or a prey item for the host when there is nitrogenous compound present. Investigations into coral bleaching indicate that bleaching hosts release

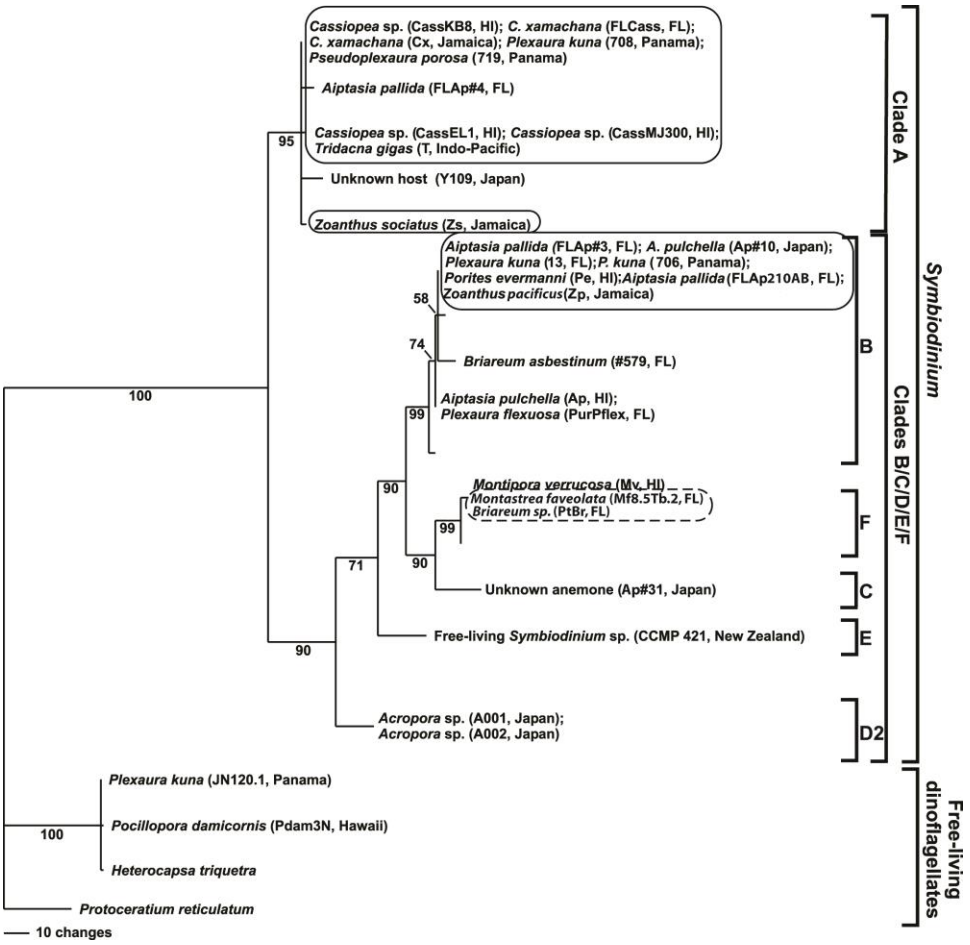

Figure 3. PC3 and BF10 antigen presence in Symbiodinium strains indicated on cp23S-rDNA phylogeny. Strains inside a solid box showed some level of PC3 labeling. Strains inside a dashed box showed labeling with BF10 antibody. Symbiodinium strains without a box were tested here and showed no labeling with either antibody. Strains indicated as free-living dinoflagellates were not tested in this study. This phylogeny is an edited version of one originally presented in Santos et al, 2002. The phylogeny was constructed by maximum-parsimony (MP) of the cp23S-rDNA Domain V sequences. Values at nodes indicate bootstrap support vales (percentages based on 1000 re-samplings). The most parsimonious tree was 436 steps in length with a consistency index of 0.77. The host name a sample was collected from is listed with the culture ID and location of collection in parentheses.

nitrogenous compounds (Perez and Weis, 2006) or show less activity of nitric oxide synthase (Trapido-Rosenthal *et al.*, 2001). Thus, symbionts and hosts may have a mechanism for reinfection *via* symbiont chemotaxis to hosts stressed by episodic bleaching by virtue of higher production of nitrogen compounds.

Once the host and symbiont are in close proximity the symbiont must be ingested by the host. There is some level of recognition that occurs at this point as not all potential symbionts will enter a host coelenteron (Trench *et al.*, 1981). Subsequently, symbionts are phagocytosed into the gastrodermal cells without entering a digestive endocytic pathway. The process of phagocytic uptake has been directly observed in *Chlorohydra* (McNeil, 1981). Once the symbiont is within the host cells, it must survive any attempt to be digested by the host. Any phagocytosed dead *Symbiodinium* will quickly be digested and only live *Symbiodinium* will be maintained in the gastrodermal cells indicating that living cells have some characteristic involved in symbiont uptake (Fitt and Trench, 1983). Examinations of infection in *Fungia scutaria* show that multiple strains of living *Symbiodinium* can initially infect the host, but after a short time only the preferred type of symbiont is present in the larvae (Rodriguez-Lanetty *et al.*, 2006). In some situations, it appears that this early uptake period has few barriers to prevent non-specific associations with non-preferred *Symbiodinium* strains and that more infectious or opportunistic strains, such as clade D strains, can advantageously colonize a host (Abrego *et al.*, 2009a). At some point, either during these initial steps or shortly following them, cell-cell recognition processes establish a more specific relationship between a host and its preferred symbiont. While it is possible that the selection we see is not due to early cell-cell recognition but instead due to which *Symbiodinium* isolate is better adapted to grow in that host environment, the symbiont would presumably still secrete cell-cell recognition molecules to avoid digestion and

bypass any host immune response. Regardless of which type of system is occurring here, the molecules involved in infection and specificity must be important during these early steps in infection.

A number of studies have determined which *Symbiodinium* strains associate with which host cnidarians and the conditions that support such associations. In most cases, infection begins with a limited number of strains entering the host (often only a subset of what is present in the water column) and after a relatively short time it appears that the preferred association will establish itself (Belda-Baillie *et al.*, 2002; Berkelmans and van Oppen, 2006; Thornhill *et al.*, 2006). The preferred associations fall into the full gamut of possibilities. They can be highly specific, where one host is found with only a single symbiont strain on a given reef (LaJeunesse, 2002, Goulet and Coffroth, 2003a, Goulet and Coffroth, 2004, Thornhill *et al.*, 2006). Alternatively, the association my be somewhat flexible where one host has a dominant symbiont but it may be one of a few strains(LaJeunesse *et al.*, 2003, Iglesias-Prieto *et al.*, 2004). In mixed assemblages one host might have a dominant strain with one or more background strains that vary between host animals (Rowan and Knowlton, 1995, Rodriguez-Lanetty *et al.*, 2001, Santos *et al.*, 2003). Hosts may also associate with several strains that occupy specifically low-light, medium light, and high light regions of the host (Rowan and Knowlton, 1995; Secord, 1995; Rowan *et al.*, 1997; LaJeunesse and Trench, 2000; Rodriguez-Lanetty *et al.*, 2001; van Oppen *et al.*, 2001; Diekmann *et al.*, 2003; Goulet and Coffroth 2003a, b; LaJeunesse *et al.*, 2003; Ulstrup and van Oppen, 2003; Goulet and Coffroth, 2004; Iglesias-Prieto *et al.*, 2004). The diversity of associations suggest that processes that establish a particular symbiotic state may involve a combination of cell-cell recognition and differential survival of symbionts. In this scheme, as the

association develops, *Symbiodinium* that are better adapted to the local host microenvironment steadily displace those that are less well adapted.

Work on symbioses involving Chlorella has implicated the algal surface layer in the cell-cell recognition and initiation of the symbiosis (Meints and Pardy, 1980; Reisser *et al.*, 1982). In this study we have investigated the mucilage that comprises the outermost surface of the *Symbiodinium* cell to determine if molecular variation exists within this layer that could be used in a cell-cell recognition process during the initiation of the symbiosis. One of the antibodies, BA6, created against a clade F algal mucilage layer positively labeled all cultures that were tested. Previous work has described similar components among strains in the outer mucilage layer of macromolecules (Markell *et al.*, 1992). While this antigen is not a good candidate for identifying differences amongst *Symbiodinium* strains that may be useful in recognition, it does suggest that the mucilage in all strains has some components in common. These common elements could include compounds that predispose most strains to be taken up during the early stages of infection (Coffroth *et al.*, 2001; Rodriguez-Lanetty *et al.*, 2006; Abrego *et al.*, 2009a).

In order for this mucilage layer to be involved in the establishment of the symbiosis there must be differences in the chemical composition of this layer that could be part of a specific host-symbiont recognition process. Using both the BF10 antibody created here and the PC3 antibody (Wakefield and Kempf, 2001), we detected antigenic variation among *Symbiodinium* strains. BF10 was highly specific, labeling only two cultures, both in clade F. PC3 antigen was found to be present only in clades A and B with some variation within these clades. In clade A, only one known cp23S-rDNA strain did not possess PC3 antigen (another culture did not label but the cp23S-rDNA strain type of this culture is unknown). In clade B, of the 4 distinct cp23S-rDNA types tested, 2 possessed the PC3 antigen while 2 did not. The other clades tested with

this antibody (clade C, D, E, and F) all showed no labeling. This is consistent with the finding that different isolates of *Symbiodinium* possess some of the same, but also many different, cell wall associated macromolecules (Markell *et al.*, 1992). These results indicate that the mucilage layer possesses molecular variation among *Symbiodinium* strains and that the antigenic composition of this layer is, to some extent, correlated to genotype (Figure 3).

The strain that exhibited the weakest specific labeling was a clade A198 culture while other clade A strains (and even another culture from a different host with the same cp23S-rDNA strain, A198) showed stronger labeling. Thus, the cp23S-rDNA type did not correlate with differences in intensity of fluorescent label. For example, the strain A198 cultures from different hosts (see Table 1 for host species and culture identity) show considerable variation in labeling intensity despite having the same cp23S-rDNA genotype (Figure 2). This indicates that while there is significant variation in the amount of antigen present in the mucilage, the variation is not related to the cp23S-rDNA strain identity. Instead the observed variation is almost certainly the result of as yet unknown genetic and/or physiological differences in mucilage secretion between cultures from different hosts instead of the genotype strain as determined by cp23S-rDNA.

We have seen variation in antigenicity in the mucilage that forms the outermost surface layer of material surrounding the *Symbiodinium* cell. Since this layer is the part of the symbiont that actually contacts the host it would have to be the location for any recognition/communication that occurs between the host and the symbiont. While other investigations have recognized that the algal surface would have to perform this function, e.g., the cell wall (Lin *et al*., 2000), exuded macromolecules (Markell *et al.*, 1992; Markell and Trench, 1993; Markell and Wood-Charlson, 2010) or simply cell-surface glycans (Logan *et al.*, 2010), little is known about the molecular processes that function in cell-cell recognition

between host and *Symbiodinium* sp. Our study further confirms variation in cell surface molecules and demonstrates that there is variation within the mucilage component that demonstrates a correlation with symbiont clade. This is a first step in identifying components of the algal cell that may act in recognition/communication between host and symbiont.

Lectin-carbohydrate reactions have been implicated in a number of cell-cell recognition processes (Weis and Drickamer, 1996), including those involving organisms from very different groups (such as the Rhizobium bacteria symbiosis with legumes and the immune system in animals; Bhuvaneswari *et al.*, 1977). Due to the nature of cnidarian *Symbiodinium* symbioses it is very likely that a similar process occurs in these associations. A mannose-binding lectin has been found in *Acropora millepora* that binds to both bacterial pathogens as well as *Symbiodinium* (Kvennefors *et al.*, 2008). Another group of studies investigated the presence of lectins in *Sinularia lochmodes* and discovered a D-galactose-binding lectin (SLL-2; Jimbo *et al.*, 2000). Subsequent studies revealed that this lectin 1) localized to the cell surface, 2) arrested motility in *Symbiodinium* motile stages, and 3) bound D-galactose within the symbiont glycoconjugate (Koike *et al.*, 2004; Jimbo *et al.*, 2013). The presence of these types of molecules and their localization to the *Symbiodinium* cell surface suggest that this type of response may be involved in establishing and maintaining the association between *Symbiodinium* and their cnidarian hosts.

It has been demonstrated that the *Symbiodinium* outer covering (sometimes referred to as exudate or cell wall, but here referred to as the mucilage) contains macromolecules with carbohydrate components (Markell and Trench, 1993; Lin *et al.*, 2000). *Symbiodinium* strains have been shown to have reduced infectivity if surface glycoproteins either embedded in the cell wall or external to the cell wall are masked or digested (Lin *et al.*, 2000; Wood-Charlson *et al.*,

2006). Here we have tested a wider variety of *Symbiodinium* strains and determined that there is widespread variation in antigenicity that is consistent with previous studies (Markell *et al.*, 1992; Markell and Trench, 1993; Logan *et al.*, 2010) that examined usually only a few strains. This study gives support to previous work investigating the cell surface of *Symbiodinium* by using 2 antibodies to reveal widespread variation in the outermost layer of the *Symbiodinium* cell correlated to strain. Our results strengthen the argument that the mucilage layer has the required variation to establish specificity between host and symbiont, as well as function in host - symbiont communication in the intact symbiosis. While the exact process of recognition and maintenance of *Symbiodinium* symbioses and the molecules involved are still unknown, this study supports the hypothesis that the potential for cell-cell recognition between the host and symbiont is present in the outermost layer of the symbiont cell. Future studies will hopefully reveal the molecules that drive initiation and maintenance of *Symbiodinium* symbioses.

III. ULTRASTRUCTURAL VARIATION AMONGST *SYMBIODINIUM* ISOLATES

ABSTRACT

Symbiodinium are unicellular dinoflagellates that reside intracellularly in a variety of invertebrate hosts, including cnidarians. There is great diversity amongst isolates of this genus; however, very few ultrastructural studies have examined differences among them. Here, ultrastructural examinations using High Pressure Rapid Freezing were performed on 5 isolates (2 from clade A, and 1 from clade B, 1 from clade F, and a free-living isolate from clade E) to examine variation amongst these strains that could affect the ability of a particular symbiont to reside within a particular host. All 5 isolates studied exhibited the standard dinokaryon nucleus, a peripheral multi-lobed chloroplast with a single stalked pyrenoid surrounded by a starch cap, and dinoflagellate-type mitochondrion with tubular cristae. Electron dense inclusions were typically positioned near the cell periphery and may be related to mucocysts sometimes seen in dinoflagellates. Some isolates (CCMP421 and Y109) had a few cells that exhibited large electron dense structures containing calcium oxalate crystals. Interestingly, the cell size amongst different isolates was significantly different and consistent with previous studies on the same or similar isolates. Examination of the ultrastructure of 5 isolates of *Symbiodinium* revealed that there is very little variation in morphological characters. Therefore, it seems unlikely that isolates or species of *Symbiodinium* can be usefully differentiated on the basis of morphology alone. However, characterization of the morphology of the genus *Symbiodinium* as it relates to its symbiosis with the host may lead to a better understanding of how the association between host and symbiont is established and maintained.

INTRODUCTION

Symbiodinium microadriaticum was originally described using cultured cells collected from *Cassiopeia* sp. (Freudenthal, 1962). Using light microscopy, the life cycle of *Symbiodinium microadriaticum* was found to begin with a vegetative cell which appears to contain numerous irregular shaped chloroplasts, an inconspicuous nucleus, and, as the cell ages, an increasingly large "assimilation product". This vegetative cell can divide forming two nearly identical daughter cells or thicken its cell wall and encyst. The vegetative cyst can then form a zoospore motile form or divide within the cyst. At the time it was thought possible that gametes could form from the cyst stage, but this was not actually observed (Freudenthal, 1962). An initial ultrastructural investigation described the periplast (cell wall) as a thick amorphous layer between 2 membranes (Kevin *et al.*, 1969). The chloroplast was described as a single, multi-lobed, peripheral structure surrounded by an envelope of 3 membranes and having repetitive lamellae each consisting of 3 closely stacked thylakoids. All cells had a large nucleus enclosed by an envelope with pores and containing chromosomes having a typical dinoflagellate appearance, i.e., condensed and with a tightly coiled structure (Kevin *et al.*, 1969). Additional identified structures included mitochondria, calcium oxalate crystals inside large vacuoles, and lipid droplets (Kevin *et al.*, 1969).

This description was done at a time when *Symbiodinium microadriaticum* was thought to be a single pandemic species (Taylor, 1974). However, further descriptive studies found significant variation between cultured *Symbiodinium* isolates from a wide variety of hosts. Differences included variation in isoenzyme and soluble protein patterns (Schoenberg and Trench, 1980a), morphology (Schoenberg and Trench, 1980b), specificity as measured by infectivity in hosts (Schoenberg and Trench, 1980c), motility rhythm (Fitt *et al.*, 1981), and

chromosome volume (Blank and Trench 1985). These studies led to the understanding that there were a number of distinct species that were erroneously grouped into the single species *Symbiodinium microadriaticum.*

With the understanding that there were many different isolates within the genus *Symbiodinium* new species began to be named based on biochemical, physiological, morphological, and behavioral differences (Trench and Blank, 1987). Three of the earliest novel species named after *S. microadriaticum* include *S. goreauii*, *S. kawagutii*, and *S. pilosum* (Trench and Blank, 1987). Despite the vast variety that was seen in the earliest genetic studies (Rowan and Powers, 1991a; Rowan and Powers, 1992), further formal species descriptions were very few (2) and initially still based on ultrastructure. The next formal species description was *Gymnodinium linucheae* (later reclassified as *Symbiodinium linucheae*; Trench and Thinh, 1995). Finally, *Symbiodinium natans* was described using a combination of light and electron microscopy combined with nuclear-encoded partial LSU rDNA sequence information (Hansen and Daugberg, 2009).

While the naming of species may prove informative, what has been consistently used to understand the phylogeny of *Symbiodinium* is identification of type using a variety of genetic markers. Currently there are 9 clades into which isolates of this genus can be placed. Clades A, B and C were the first identified using restriction fragment length polymorphisms (RFLPs) (Rowan and Powers, 1991b). Later, clades D (Carlos *et al.*, 1999), E (LaJeunesse and Trench, 2000; LaJeunesse, 2001), F (LaJeunesse, 2001), G (Pochon *et al.*, 2001), H (Pochon *et al.*, 2004), and I (Pochon and Gates, 2010) were added based on variation in the internal transcribed spacer (ITS) sequence (LaJeunesse, 2001; Pochon *et al.*, 2004), 18S rDNA sequence (LaJeunesse, 2001), 28S rDNA sequence (Pochon *et al.*, 2006), and cp23S rDNA sequence (Santos *et al.*,

2001; Santos *et al.*, 2002). All these studies and many more have come to the same conclusion, namely that there is a huge amount of variation between isolates within genus *Symbiodinium* (Coffroth and Santos, 2005).

Along with the investigation into *Symbiodinium* genetics, further ultrastructural examinations were undertaken to examine morphological variation amongst isolates. Examination of *Symbiodinium* from *Zoanthus* sp. confirmed the presence of a lobed chloroplast and stalked pyrenoid (Van Thinh *et al.*, 1986). Symbionts from *Montipora verrucosa* were found *in situ* to occur as a coccoid cell with an amphiesma of 5 membranous layers; a single, peripheral lobed chloroplast with parallel thylakoids and a stalked pyrenoid; and a dinokaryon nucleus with 26 chromosomes (Blank, 1987). Symbionts similar to *Symbiodinium microadriaticum* from 3 foraminiferans (Leutengger, 1977) and *Tridacna maxima* (Bishop *et al.*, 1976) were examined ultrastructurally and were similar to previous descriptions of *Symbiodinium*. As further ultrastructural examinations of isolates symbiotic with cnidarians were performed, refined descriptions of the ultrastructure of some cell structures, such as the chloroplast (Hannack *et al.*, 1997) and cell covering including thecal vesicles were made (Wakefield *et al.*, 2000).

Table 1: Genetic characterization of the *Symbiodinium* isolates in the current study.

Species	Culture	Host	Clade	Cp23S	Nr28S	Nr18S	ITS1/5.8S/ ITS2
Symbiodinium sp.	Y109	Unknown host	A191	AY035413			
Symbiodinium sp.	FLAp4	*Aiptasia pallida*	A193	AY035404	AF427453	AF427441	AF427465
Symbiodinium pulchrorum	HIAp or Ap	*Aiptasia pulchella*	B224	AY035421	AF427457	AF427445	AF360564
Symbiodinium sp.	CCMP 421	None	E202	AY055240			
Symbiodinium kawagutii	Mv	*Montipora verrucosa*	F178	AY035422	AF427462	AF427450, AY035413	AF360577

Since ultrastructural studies have all found similar cell structure while the genetic studies have all found a huge variety of molecular differences within the genus, it is difficult to determine if there are morphological differences that would be informative in regard to the great genetic variety that exists amongst isolates of the genus *Symbiodinium.* Along with all the genetic information and a variety of physiological studies examining cultured *Symbiodinium* it is important to begin parsing through the information and relating ultrastructural, genetic, and physiological traits to give a thorough understanding of the biology of the symbiont. Here we provide ultrastructural details of genetically well-characterized isolates in 4 clades in order to provide a basis for variations between previously undescribed isolates and to compare ultrastructure between clades. This work is meant to provide a baseline against which future ultrastructural analyses can be compared.

METHODS

METHODS FOR ASSESSMENT OF ULTRASTRUCTURAL DIFFERENCES IN SYMBIODINIUM ISOLATES. In looking for ultrastructural differences between *Symbiodinium* isolates, it is critical that all isolates be cultured under identical conditions and that they be sampled at the same time in the cell cycle. Similarly, it is also critical that all samples be processed in the same manner using the same fixation and embedding techniques. To that end, we suggest the following approach as a standardized method that can be followed by investigators doing future comparisons of these and other isolates. The culture methods are chosen because they are relatively standard among labs working with *Symbiodinium*. The high pressure rapid freezing technique for electron microscopy is chosen because it offers the best fixation with the fewest artifacts.

CULTURE GROWTH. Cultures were grown in F/2 media (Guillard and Ryther, 1962) in 125mL erlenmeyer flasks under 12/12 light dark cycle at about 24.5°C (± 0.5°) at Auburn University for 3 weeks. Cultures were transported to the culture room of Dr. Fitt at the University of Georgia and allowed to grow for 2 weeks under a 12/12 light dark cycle at 26.5°C (± 0.5°). Isolates subjected to ultrastructural analyses are listed in Table 1.

HIGH PRESSURE RAPID FREEZING. Fixative solutions were prepared several days prior to fixation. 0.1% uranyl acetate in HPLC grade acetone was mixed at room temperature 2 days prior to freezing and the day before freezing was transferred to a -80°C freezer. A vial of 25 mL of HPLC grade acetone was pre-chilled in the -80°C freezer overnight and then 1 g of OsO_4 was added (making a 4% OsO_4 solution) while the solution was kept on dry ice for 10 minutes before storage in a -80ºC freezer the day before freezing. Isolated samples were collected at virtual 'dawn' as defined by the light/dark cycle by pipetting 100µL of cells+media using a Gilson pipetman starting at the bottom of the flask (where the cells accumulate) and slowly drawing the pipette up about 1 cm from the bottom. The pipette was then removed from the flask and the sample was transferred to a 1.5 ml microcentrifuge tube and centrifuged briefly (MyFuge at 2000g) to produce a loose pellet. Most of the remaining F/2 media was removed (leaving the cell pellet in about 50 µL of F/2 media) and 50 µL of 20% dextran in F/2 media was added and the cells were re-suspended in a final concentration of 10% dextran in 100 µL of F/2 medium. The 10% dextran encouraged the cells to remain clumped together after freezing. Cells were transferred to planchettes using a 2-10µL Gilson pipetman and then rapidly frozen in a High Pressure Freezing Machine (HPM 010 ABRA Fluid AG). After freezing, pellets were transferred to cryotubes submerged in liquid nitrogen and then transferred to a pre-cooled dewer and maintained in liquid nitrogen until transfer into fixative. The 0.1% uranyl acetate in acetone and

4% OsO_4 in acetone were then mixed (all vials kept on dry ice) in a 1:1 ratio and aliquoted into enough vials for the frozen samples. After 2 hours in the -80°C freezer, the frozen samples in planchettes were quickly added to the vials of 1:1, 0.1% uranyl acetate: 4% OsO_4 in acetone, all vials and cryotubes being kept on dry ice. Samples in fixative were then placed in a - 80°C freezer for 4 days. Samples were next moved to a -20°C freezer for 3 hours, then to a 4°C refrigerator for 2 hours, and finally into a fume hood at room temperature for 30 minutes. After this gradual return to room temperature the fixative was removed and samples were rinsed 3 times in 100% HPLC grade acetone for 15 minutes each. Samples were then removed from planchettes and infiltrated with Low viscosity Embedding Media Spurr's Kit (hard recipe; Electron Microscopy Sciences) by first adding the embedding resin as a 1:2 mix in acetone and leaving it overnight at room temperature. The next day the samples were changed to 2 parts embedding resin to 1 part acetone for 8 hours and then into 100% resin overnight. The next morning samples were placed in fresh 100% resin for 8 hours. Near the end of the day the samples were transferred to catalyzed resin in Beem capsules. After 1 hour at room temperature in the catalyzed resin the samples were placed in an oven at 60°C and left for 24-48 hours to ensure hardening.

TRANSMISSION ELECTRON MICROSCOPY. Samples were sectioned using a Reichert-Jung Ultracut E Microtome and placed on copper 75 mesh grids (EMS). Samples were stained using 4% aqueous solution of uranyl acetate for 10 to 20 minutes and then stained with 0.1% aqueous solution of lead citrate for 5 minutes. Samples were viewed on a Zeiss EM 10C 10CR Transmission Electron Microscope and digital images were taken using MaxIm DL5 software and SIA-L3C 4.3 megapixel (Scientific Instruments & Applications, Duluth, GA 30096).

MEASUREMENTS AND STATISTICS. In order to obtain accurate measurements of cell

size, only cells larger than 4 µm in diameter were measured and added to a list to obtain 100 measured cells from each isolate. Since cells were often oblong in shape, the diameter was measured twice, at the largest point and the smallest; only one of those measurements had to be greater than 4 µm. These cells were then sorted by size (based on the greatest of 2 diameter measurements) and only the largest 30% (n =30) were used to determine average cell size. Measurements were made first using MaxIMDL5 software and then confirmed using Image-Pro Plus (Version 7.0; Media Cybernetics, 2009). The first measurement made on each cell was a cross-sectional area measurement. This was obtained by setting the scale of the image and then manually encircling the entire cell to get an area. Cell size differences (both of the greatest diameter and of a cross-sectional area measurement) among isolates were compared using a one-way ANOVA run on SAS (Version 9.2) to ascertain any significant differences. Post-hoc multiple comparison tests were run with a Tukey-Kramer adjustment to examine which strains exhibited significant differences in size. Additionally, thylakoid size, cell membrane size, cell wall size, and outer membrane size were analyzed using a one-way ANOVA run on SAS (Version 9.2) followed by a Tukey-Kramer adjustment.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING OF Cp23S rDNA. DNA extraction and sequencing were performed on all 5 isolates according to Santos *et al.* (2002). PCR success was confirmed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining and ultraviolet (UV) light. Amplicons were sent to Genewiz, Inc. for sequencing.

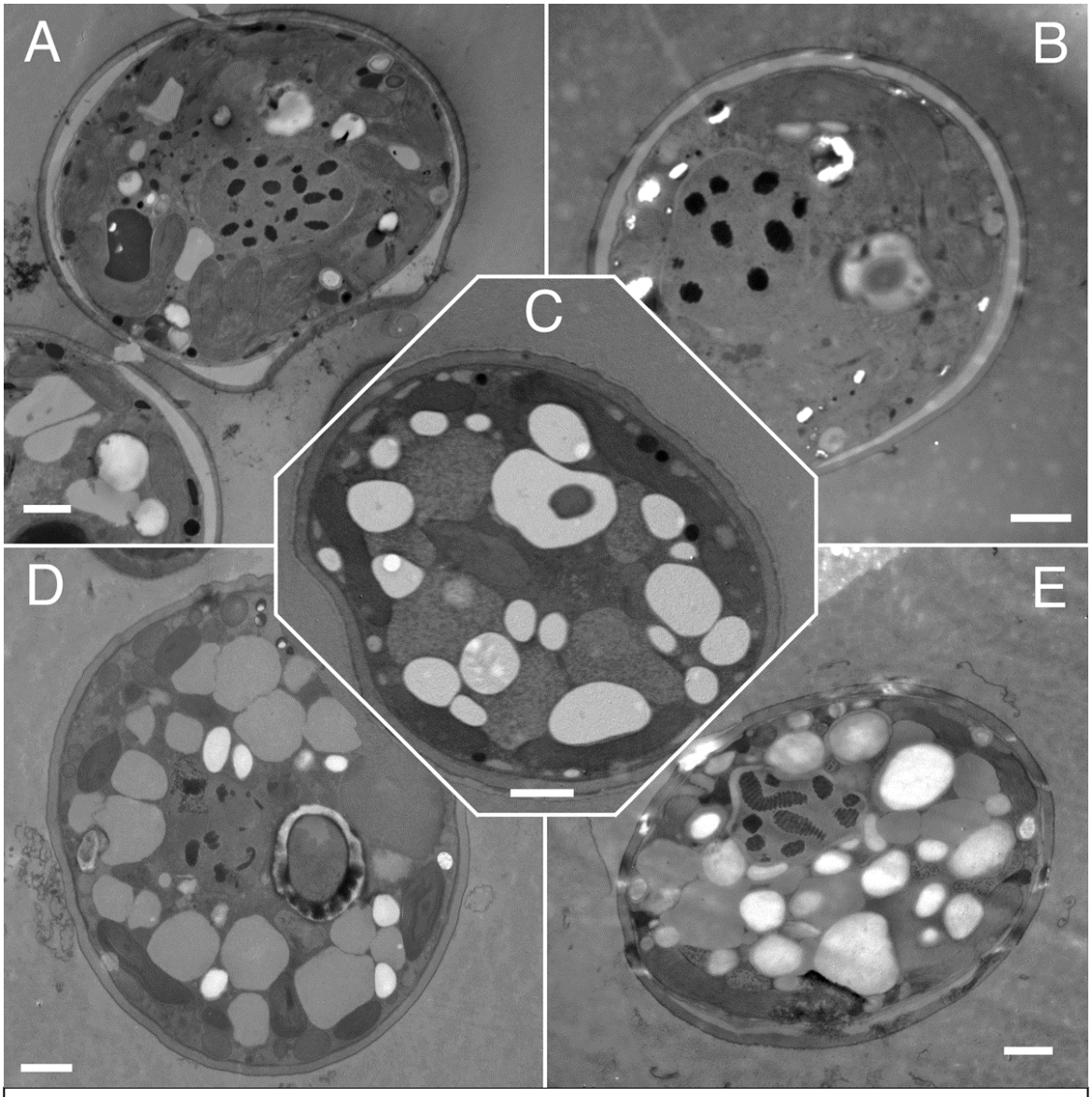

Figure 1: Representative examples of 5 isolates of *Symbiodinium* showing characteristic ultrastructure. All diameters listed are of the greatest distance across the cell in the case of oblong cells. A. An average cell of Y109 with a diameter of 8.5 µm. The peripheral chloroplast and central dinokaryon nucleus are visible along with some inclusions. B. An average cell of FLAp4 with a diameter of 7.1 µm. Here you can see the peripheral lobed chloroplast and the starch cap around the pyrenoid is visible. The section also shows the dinokaryon nucleus near the center of the cell. C. An average cell of Ap with a diameter of 7.9 µm. This cell is filled with starch granules and lipid droplets. The peripheral chloroplasts are visible as is the pyrenoid though the stalk of the pyrenoid is not included in this section. D. An average cell of CCMP421 with a diameter of 10.1 µm. The cell has peripheral chloroplast lobes, a dinokaryon nucleus, starch granules and lipid droplets throughout. E. An average cell of Mv with a diameter of 9.1 µm. Here the dinokaryon nucleus is clearly visible pushed a bit to the edge of center by the presence of a number of starch granules and lipid droplets. The peripheral chloroplast lobes are visible as well. All scale bars are 1µm.

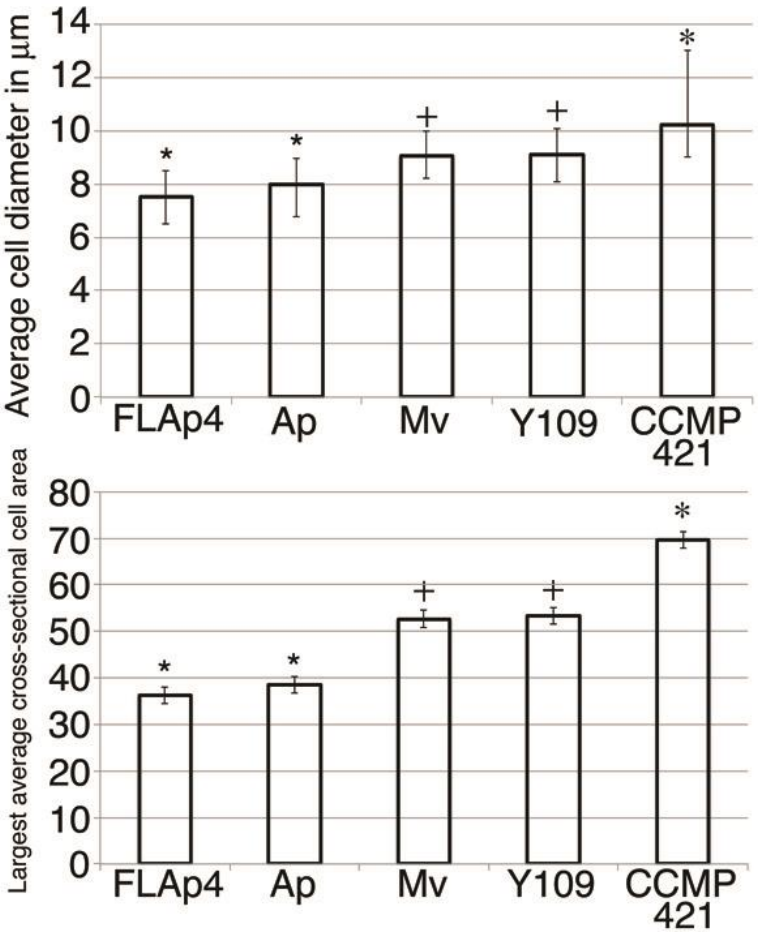

Figure 2: Cell size of coccoid cells grown in culture during log phase growth. Symbols indicate which isolates are not significantly different from each other ($p<0.05$). Error bars indicate the standard error. Cell diameters are of the greatest straight-line distance across a cell; cell cross-sectional area was measured in Image-Pro Plus which allowed the area to be measured very accurately.

RESULTS

SYMBIODINIUM *ISOLATE Y109*. Y109 was originally cultured by M. Hidaka from an unknown host in Okinawa, Japan. Coccoid cells measured during the log phase of growth range in average size from 8µm to 10µm in diameter (Average 9.0 ±0.83 µm; n=30; Figures1A and 2). The nucleotide sequence of the cp23S-rDNA was previously reported (AY035413; Santos *et al.*,

2002) and confirmed here. The chloroplast is located peripherally and a single stalked pyrenoid with a starch cap was observed (Figure 3A). Thylakoids are arranged in groups of 3, each 9.4 ± 1.2 nm (n=10; Figure 4A and 5). The lamellae of 3 thylakoids are closely pressed together as is common in dinoflagellates and in *Symbiodinium*. The accumulation body is circular and centrally located (Figure 6A). During log phase growth, cells contain many medium to large lipid and starch granules (Figure 6A). The cell is bordered by an outer membranous layer that averages 10.5 ±1.3 nm (n=10, Figure 7A and 8). Inside the outer membranous layer, the cell wall averages 134.6 ±47.9 nm (n=10). The plasma membrane (7.3 ± 1.1 nm; n=6) is just outside other membranous or thecal vesicle structures that are sometimes seen just below the plasma membrane (Figure 7A). Some cells contain a large dark central structure that contains plentiful calcium oxalate crystals (Figure 9A, C, and E). At the periphery of the cell, usually between the cell membrane and chloroplast, there are many small dark staining granules (Figure10A and D). The nucleus has the standard dinokaryon nuclear structure and is located centrally (Figure11A). The mitochondria also have the tubular cristae typical in dinoflagellates (Figure 12A).

ULTRASTRUCTURAL DESCRIPTION OF SYMBIODINIUM *ISOLATE FLAP4.* FLAp4 was originally isolated by S.R. Santos from *Aiptasia pallida* in the Florida Keys. Coccoid cells of isolate FLAp4 during log phase growth averaged 6.5µm to 8.5µm in diameter with an average diameter of 7.5 ±0.6 µm (n=30) (Figures 1B and 2). The cells have a peripheral chloroplast attached to a single stalked pyrenoid with a starch cap (Figure 3B). Within the chloroplast, thylakoids are arranged in groups of 3, each approximately 10.5 ±1.8 nm (n=10; Figure 4B and 5). The lamellae of 3 thylakoids are stacked closely as is common in dinoflagellates and in *Symbiodinium*. The accumulation body is circular and centrally located (Figure 6B). The cell wall (122 ±51.7 nm) is bordered by an outer membranous layer 9.1 ± 0.9 nm across (n=10,

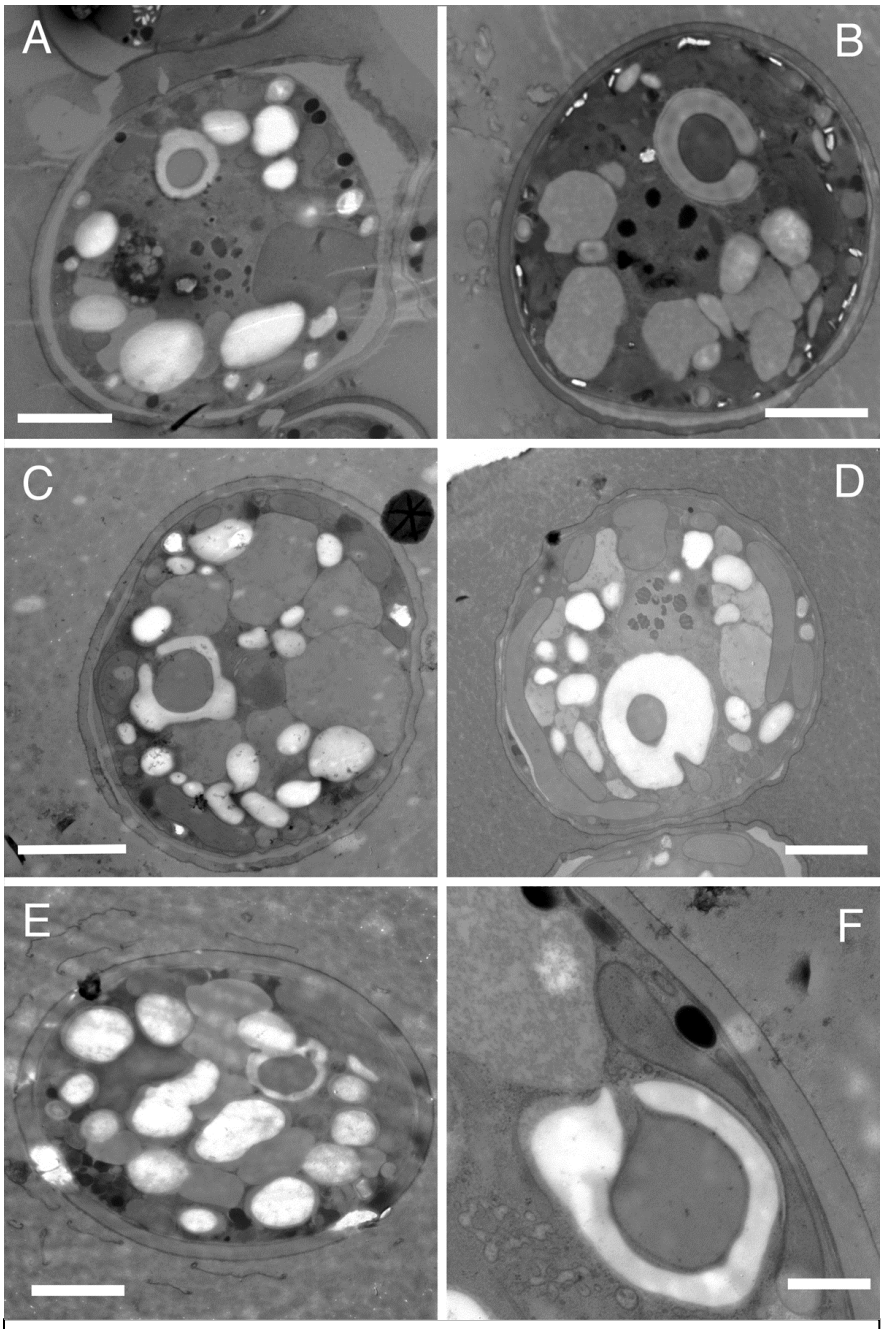

Figure 3: Examples of the pyrenoid with starch cap in 5 isolates of *Symbiodinium.* A. A cell of Y109 with the pyrenoid clearly indicated by the starch cap surrounding. B. An example of the pyrenoid in FLAp4 with the stalk visible. C. A cell of Ap with the classic pyrenoid and starch cap. D. A cell of CCMP421 the stalk only partially visible. E. An example from Mv with no visible stalk of the pyrenoid. F. A high magnification image of the pyrenoid from Ap; notice that there are no lamellae present within the pyrenoid. A-E have scale bars of 2µm. The scale bar in F is 500nm.

Figure 7B and 8). Inside the cell wall lies a plasma membrane (7.0±0.9 nm; n=8) and below that membrane other membranous structures are often seen (Figure 7B and F). Cells often have electron-dense structures near the periphery which may be related to mucocyts seen in other dinoflagellates (Figure 10 B and E; Hoppenrath and Leander, 2008). The nucleus has the standard dinokaryon structure and is usually central or close to central (Figure 11B). The mitochondrion also has the typical dinoflagellate appearance with tubular cristae (Figure 12B).

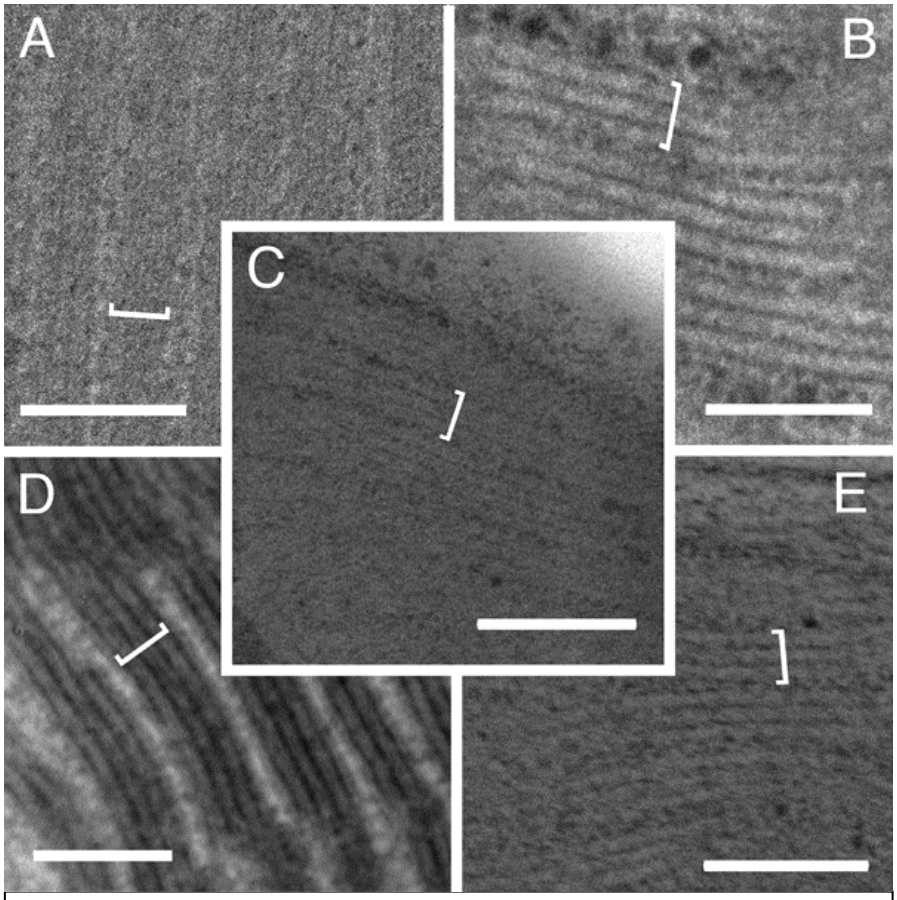

Figure 4: An example of thylakoids in 5 *Symbiodinium* isolates. A. Isolate Y109 thylakoids. B. An example of the thylakoid in FLAp4. C. A stack of 3 thylakoids in isolate Ap. D. A cell of CCMP421 with they stack of 3 thylakoids clearly visible. E. An example from Mv of the thylakoid arrangement. Scale bars are 100nm. All grana were composed of 3 thylakoids. One stack of 3 thylakoids is indicated by brackets.

ULTRASTRUCTURAL DESCRIPTION OF SYMBIODINIUM ISOLATE AP. Isolate Ap was originally cultured by R. A. Kinzie from the host *Aiptasia pulchella* from Kaneohe Bay, Hawaii. Subsequently, this isolate was named as *S. pulchrorum* by R. K. Trench in Banaszak *et al*. (1993). During log phase growth, coccoid cells of *S. pulchrorum* vary in diameter from 6.8µm to 9µm in diameter with an average of 8 ± 0.86 µm (n=30; Figures 1C and 2). The chloroplast is located peripherally with a single stalked pyrenoid with a starch cap (Figure 3C and F). Thylakoids are arranged in stacks of 3 with the average thickness of each thylakoid of 9.4 ±1.1 nm (n =10; Figure 4C and 5). In the interior of the cell, starch granules are relatively large while lipid droplets are smaller (Figure 6C). The cell wall (127.7 ±32.4 nm) is bordered by an outer membranous layer 10.0 ±2.0 nm (n=10, Figure 7C and 8). Interior to the cell wall is a plasma

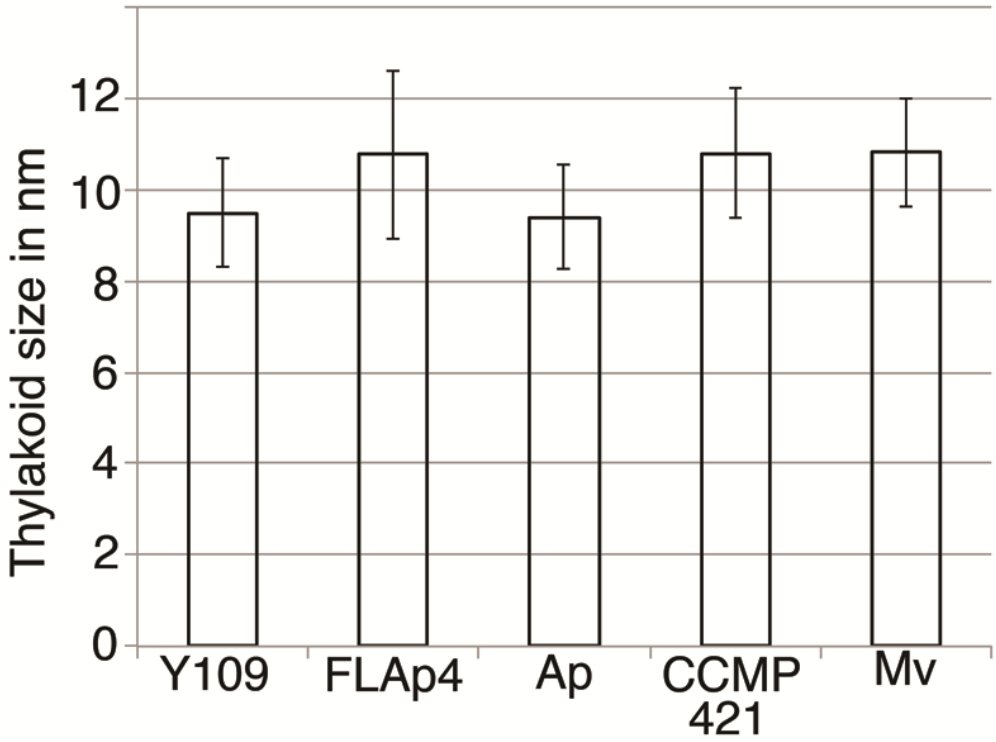

Figure 5: Thylakoid membrane size in 5 isolates of *Symbiodinium* sp. There was no significant difference in thylakoid size or in the organization of the stacks of thylakoids. All thylakoid membranes averaged between 8.5 and 10.5 nm.

membrane (7.4±1.4 nm; n=9) often bordering other membranous or thecal vesicle-like structures (Figure 7C). Some cells have small dark vesicles that may be related to mucocysts (Figure 9C; Hoppenrath and Leander, 2008). The nucleus has the standard dinokaryon appearance and is located centrally (Figure 11C) while the mitochondria also has the standard dinoflagellate appearance with tubular cristae (Figure 12C).

ULTRASTRUCTURAL DESCRIPTION OF SYMBIODINIUM *ISOLATE CCMP421*. CCMP421 was originally cultured from water samples collected from Wellington Harbor, New Zealand and was assumed to be a free-living isolate of *Symbiodinium*. Coccoid cells measured during the log phase of growth range in size from 9 to 13µm in diameter (Average 10.2 ± 1.01 µm, n=30; Figure 1D and 2). The nucleotide sequence of the cp23S-rDNA was previously reported (AY055240; Santos *et al.*, 2002) and confirmed here. The cp23S-rDNA sequence indicates that this species of *Symbiodinium* is in clade E. The chloroplast has a single stalked pyrenoid with a starch cap (Figure 3D). The chloroplast is located peripherally and appears branched so it may be a single, large branched chloroplast. Thylakoids are in groups of 3 with the standard width of 10.7 ±1.4 nm per each thylakoid (n=10; Figure 4D and 5). There are many small to medium starch granules and lipid droplets located within cells in the log phase stage of growth (Figure 6D). The accumulation body is always circular and stains darkly (Figure 6D). The cell wall is bordered by an outer membranous layer 10.5 ± 1.9 nm across (n=10, Figure 7D and 8). The cell wall averages 117.5±37.7 nm (n=10). Inside the cell wall lies a plasma membrane (7.5±1.4nm; n=8; Figure 7D and 8). Cells often contain a large, amorphous, darkly staining, non-membrane bound structure within which calcium oxalate crystals are present (Figure 9B, D, and F; Franceschi and Nakata, 2005). The dinokaryon nucleus is located centrally (Figure 11D) and the mitochondria have tubular cristae common in dinoflagellates (Figure 12D).

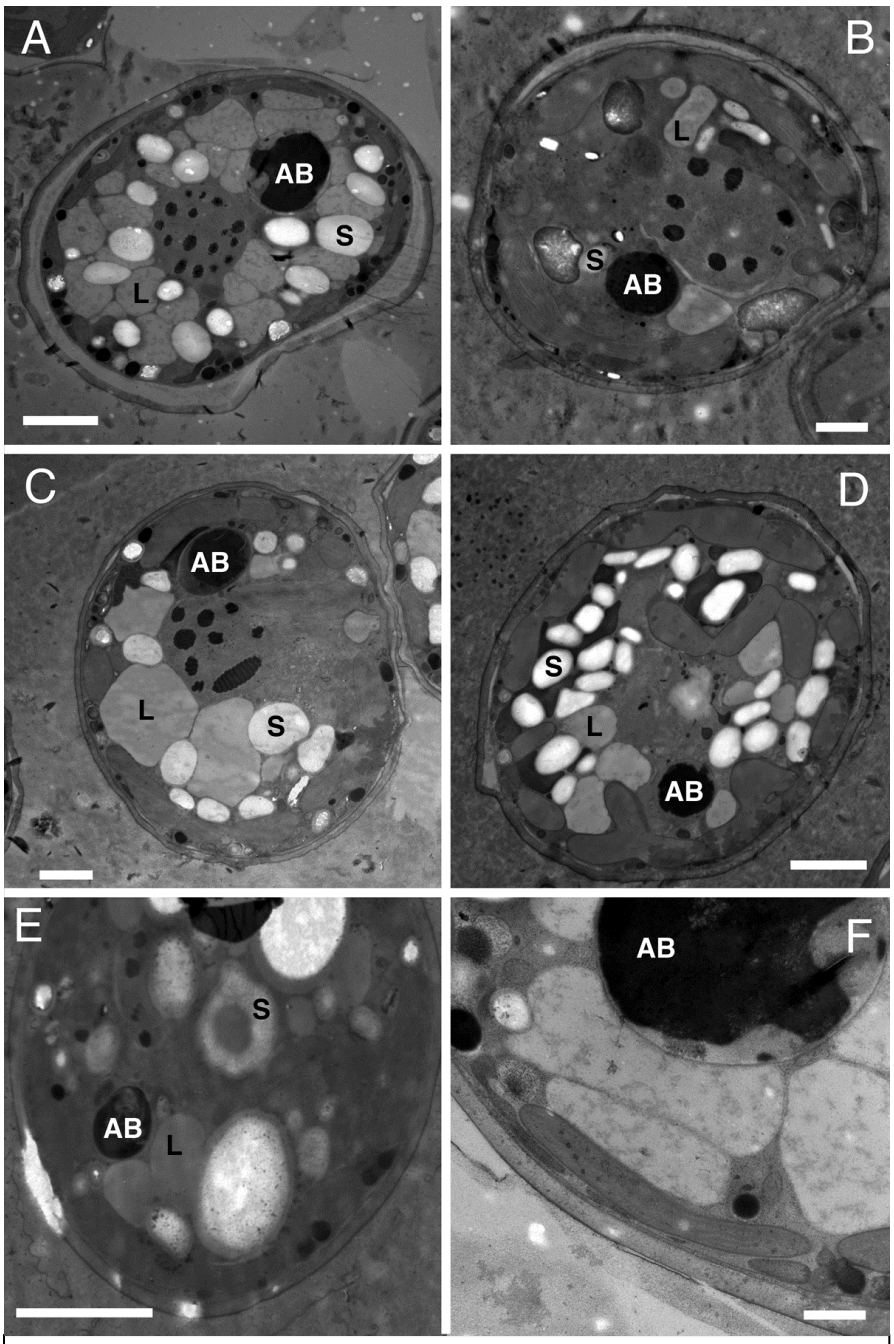

Figure 6: Comparison of accumulation body size inclusions (starch granules and lipid droplets) amongst 5 species of *Symbiodinium*. Accumulation bodies indicated with AB, starch by S, and lipid droplets by L. A. Y109, B. FLAp4, C. Ap, D. CCMP421, E. Mv, F. High magnification of the accumulation body fromY109. Scale bars 1µm in B and C; 2 µm in A, D, and E; and 500nm in F.

ULTRASTRUCTURAL DESCRIPTION OF SYMBIODINIUM *ISOLATE MV.* Mv was originally isolated by R. A. Kinzie from *Montipora verrucosa* in Hawaii. Subsequently, this isolate was described as *S. kawaguti* by Trench and Blank (1987). Coccoid cells of *S. kawagutii* are large with a diameter ranging from 8 µm to 10.6µm during log phase growth for an average of 9.05 ± 0.74 µm (n=30; Figures 1E and 2). The chloroplast is located peripherally and connected to a stalked pyrenoid with a starch cap (Figure 3E). Within the chloroplast, thylakoids are arranged in groups of 3, each approximately 10.8±1.2 nm (n=10; Figure 4E and 5). Lamellae of 3 thylakoids are stacked closely as is common in dinoflagellates and in *Symbiodinium*. The interior of the cell has many starch granules and lipid droplets. Calcium oxalate crystals are rare. Accumulation bodies were rare at the early log phase of growth, but were similar in structure to the other isolates (circular, darkly staining; Figure 6E). The cell wall is bordered by an outer membranous layer 10.5 ±0.9 nm across (n=10, Figure 6). The cell wall averages 169.7 ±41.7 nm (n=10) and just inside the cell wall lies a plasma membrane (7.2±0.9 nm; n=7; Figure 7E and 8). The nucleus has the standard dinokaryon appearance and is located centrally (Figure 11E). The mitochondria contain tubular cristae (Figure 12E).

DISCUSSION

The general ultrastructure for *Symbiodinium* isolates appears to be consistent. All isolates studied here and all previous ultrastructural studies of *Symbiodinium* note a peripheral chloroplast with a stalked pyrenoid surrounded by a starch cap. Likewise, these chloroplasts all contain thylakoids in stacks of 3 and the size of the thylakoids does not vary amongst isolates or from what is typically seen in dinoflagellates (Dodge and Greuet, 1987). *Symbiodinium* also possesses a standard dinokaryon nucleus with permanently condensed chromosomes (Dodge and

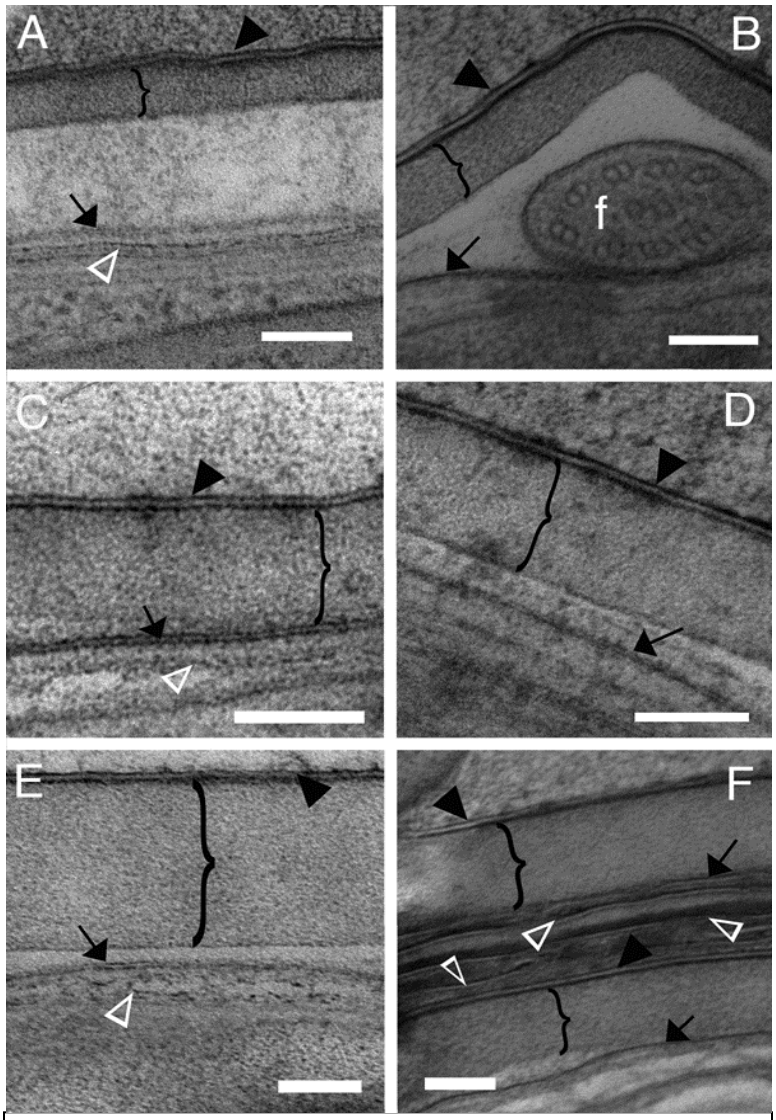

Figure 7: Images of the *Symbiodinium* cell periphery in 5 isolates. A. Y109, B. FLAp4 cell periphery which also contains a cross-section of a flegellum, C. Ap, D. CCMP421, E. Mv. F. FLAp4 example of a recently divided cell that currently has 2 cell walls. In the cyst stage cells can divide within the cell wall and remain 2 cells (each with their own cell wall) inside the parent cell wall for some time. Scale bars in A, B, D, E, and F are 100nm. Scale bar in C is 200nm. The outer double membrane is indicated by an arrowhead. The plasma membrane is indicated by the arrow. The bracket indicates the layer typically referred to as the cell wall or amorphous layer. The open arrowheads indicate membranous layers below the plasma membrane. These are best seen in panel F between the 2 cell walls.

Greuet, 1987). The cell periphery of *Symbiodinium* is interesting, the cell periphery is bounded by an outer membranous layer that is slightly wider than the plasma membrane (Average 10.2 ±0.78 nm, n=50; consistent with previous studies on *Symbiodinium bermudense*, Wakefield *et al.*, 2000). The plasma membrane is the standard 7nm across. The cell wall varies greatly in thickness, probably due to the age of the individual cells as there is no significant difference between *Symbiodinium* isolates investigated here or any *Symbiodinium* species investigated previously (*Symbiodinium* sp., *S. bermudense*, and *S. microadriaticum*; Wakefield *et al.*, 2000) .

Isolate Y109 was previously used to elucidate the variation within the genus *Symbiodinium* using the cp23S-rDNA (Santos *et al.*, 2002). Isolate Y109 is a clade A alga using the cp23S-rDNA method, but it has not previously been tested using other genetic methods or examined ultrastructurally or physiologically. From this study we note that while the

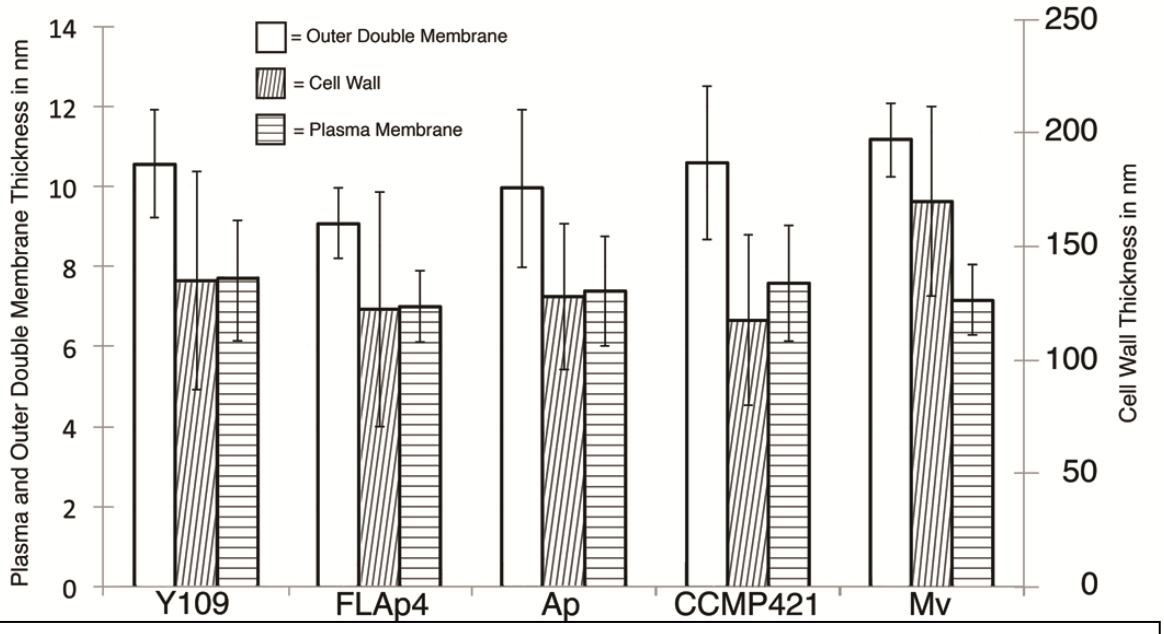

Figure 8: Statistical analysis of the *Symbiodinium* cell periphery. The outer membrane size is indicated by the open bar using the left axis, there was no significant difference between isolates. The plasma membrane is indicated by bars with horizontal lines using the left axis. There was no significant difference between isolates. The size of the cell wall is indicated by the diagonal striped bars using the axis on the right, there was no significant difference.

ultrastructure is similar to other *Symbiodinium* isolates, there are some notable differences such as cell size. Isolate Y109 is significantly smaller than several of the others tested.

Isolate FLAp4 was described using sequences from the *cp23S*-rDNA, *nr*28S, *nr*18S, and *nrITS1/5.8S/ITS2* rDNA (Santos *et al.*, 2002). Since there is very little ultrastructural or physiological data on this isolate, here we presented an ultrastructural description of the same isolate using the *cp23S*-rDNA sequence to confirm the culture identity. The cell size found in this study was consistent with the formal description of another species from the same host, *Symbiodinium minutum* (LaJeunesse *et al.*, 2012). Here we saw that the ultrastructure of this isolate was very similar to other *Symbiodinium* isolates, but was significantly smaller in cell size and contained dark granules near the periphery. These electron dense structures are not membrane bound and are unidentified, but may have some relationship to mucocysts (Hoppenrath and Leander, 2008).

Isolate Ap, *Symbiodinium pulchrorum*, was named based on a culture (Ap, the culture used here) isolated from *Aiptasia pulchella* in Hawaii (Kinzie and Chee, 1979). *S. pulchrorum* has not been formally described, however, genetic testing has revealed that this culture is a clade B1 *Symbiodinium* based on ITS2 sequencing (LaJeunesse, 2001). Sequencing of cp23S-rDNA, 18S-rDNA, n28S-rDNA, and ITS-rDNA supported the placement in clade B and identified the culture as a B224 (Santos *et al.*, 2002; Santos *et al.*, 2003; Pochon *et al.*, 2006). The original isolate of Kinzie and Chee (1979) was also used to examine thermal tolerance and it was found that this isolate showed decreased growth rates at 29° and 31° C as compared to 27° C (Kinzie *et al.*, 2001). Since this isolate has been well studied genetically it can be compared to many other new isolates. Interestingly, a different new species, *Symbiodinium psygmophilum* (LaJeunesse *et al.*, 2012), is genetically identical to *S. pulchrorum* (isolate Ap) at the cp23S-rDNA sequence

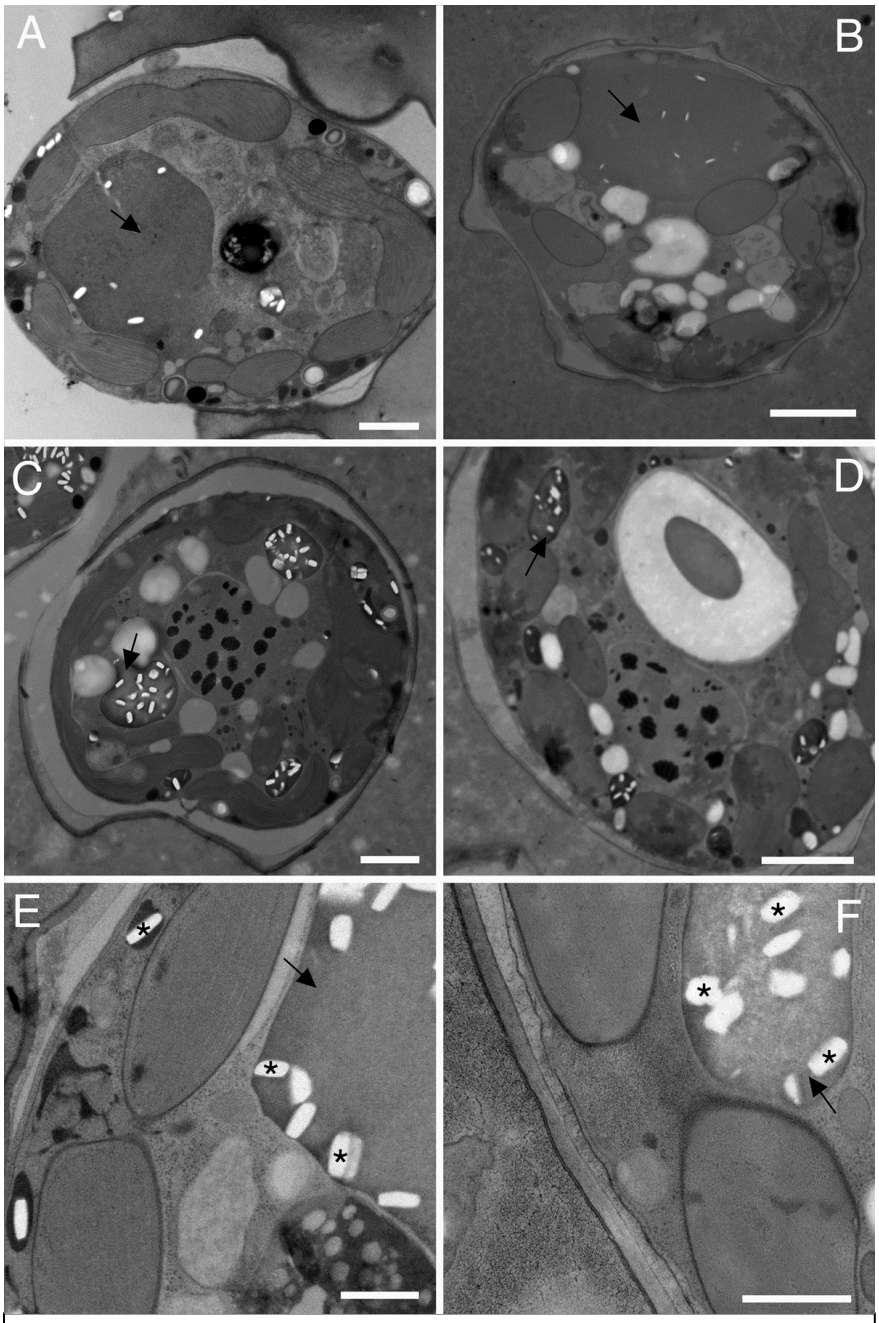

Figure 9: A. Depictions of large amorphous dark structures (arrows) containing calcium oxalate crystals (asterisks) in cells of 2 isolates of *Symbiodinium*. A. C. and E. are isolate Y109. B. D. and F. are isolate CCMP421. A. and B. depict large, central electron dense structures. C and D depict smaller more peripheral examples of what appear to be similar electron dense structures. E. and F. are high magnification images of the calcium oxalate crystals contained in these structures. Scale bars in A, and C are 1µm. Scale bars in B and D. are 2µm. Scale bars in E and F are 500nm.

level, but different using the ITS2 sequence (LaJeunesse, 2001; Santos *et al.*, 2002; Santos *et al.*, 2003). The cell size is also similar between *S. pulchrorum* and *S. psygmophilum* possibly indicating that in some cases morphological characters are consistent with the genetic relationship between isolates. The ultrastructural description here finds that the isolate Ap is very similar to other *Symbiodinium* isolates, but is significantly smaller than some isolates and contains small electron dense structures at the periphery (similar to the structures identified in isolate FLAp4).

Isolate CCMP421 is only the second free-living isolate of *Symbiodinium* described (the first is *Symbiodinium natans;* Hansen and Daugbjerg, 2009). This is also the only ultrastructural

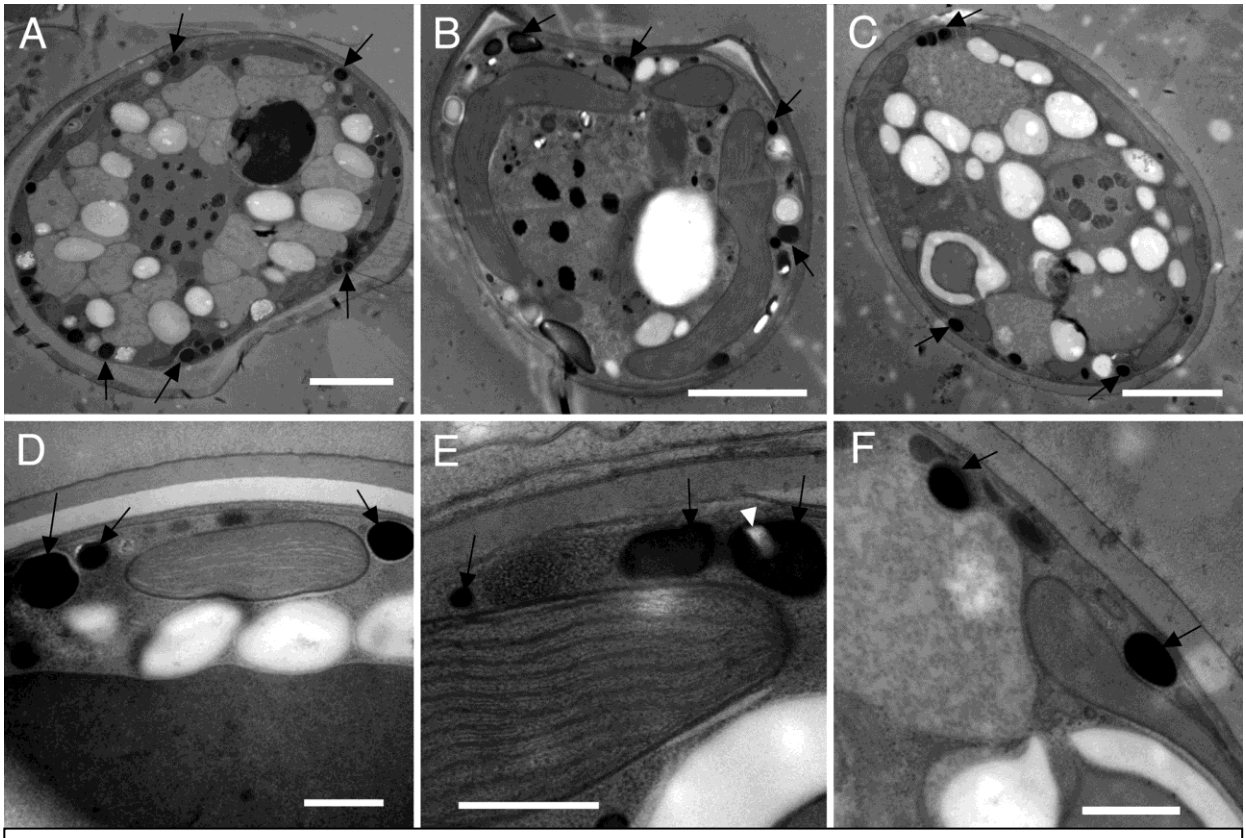

Figure10. Examples of peripheral electron dense structures. These structures are not present in all isolates and may be related to mucocysts. A and D. are isolate Y109. B. and E. are isolate FLAp4. C and F. are isolate Ap. Scale bars for A-C are 2µm. Scale bars for D-F are 500 nm, These putative mucocysts are indicated by black arrows. A white arrowhead was used to indicate a calcium oxalate crystal in E.

description of an isolate within the previously identified clade E. Like *S. natans,* CCMP421 is larger than the symbiotic species described and contains many storage products including lipid droplets and starch granules (Hansen and Daugbjerg, 2009). While *S. natans* and isolate CCMP421 are from two different clades within the genus *Symbiodinium* their shared life history pattern seems to have conferred upon them these similar morphological traits. If these two species are permanently free-living it is interesting that they seem to contain more storage products than symbiotic species and are significantly larger. Interestingly, when isolate CCMP421 and other *Symbiodinium* cultures were tested with a variety of lectin probes CCMP421 labeled less strongly with most of the lectin probes than the symbiotic cultures (Logan *et al.*, 2010). This could indicate that lectins are involved in aspects of the symbiosis and such interactions are unnecessary for the free-living dinoflagellate.

Isolate CCMP421 has been well studied genetically, beginning with an examination of the small ribosomal subunit (18S) RNA where it was found that the difference within the genus *Symbiodinium* was similar to other dinoflagellate orders and the first 3 clades, A, B, and C, were identified (Rowan and Powers, 1992). CCMP421, referred to as *Gymnodinium varians* in that study, was identified within clade B (though it was an outgroup from most of the clade - Rowan and Powers, 1992). Again, as *G. varians*, this culture was used to examine the LSU rRNA and grouped near clades B and C but not as a member of either (Wilcox, 1998). Subsequent work looking at the SSU rRNA found that this culture grouped with other *Symbiodinium* strains (Saldarriaga *et al.*, 2001). Examinations of the chloroplast 23S-rDNA grouped this culture (now referred to simply by the culture identification code at the Provasoli-Guillard National Center for Marine Algae and Microbiota, CCMP421) in clade E (Santos *et al.*, 2002; Santos *et al.*, 2003). The cp 23S-rDNA and nr28S-rDNA sequences from this culture were used as the

only representative from clade E to examine evolutionary rates in *Symbiodinium* (Pochon *et al.*, 2006). The cp 23S-rDNA sequence was then retested in 2012 to ensure that the culture housed at Auburn University had not been contaminated.

Isolate Mv, *Symbiodinium kawagutii*, was originally described in 1987 (Trench and Blank). The size description given here for isolate Mv is consistent with the findings of Trench and Blank. While the algae isolated for that study were isolated from planulae of *Montipora*

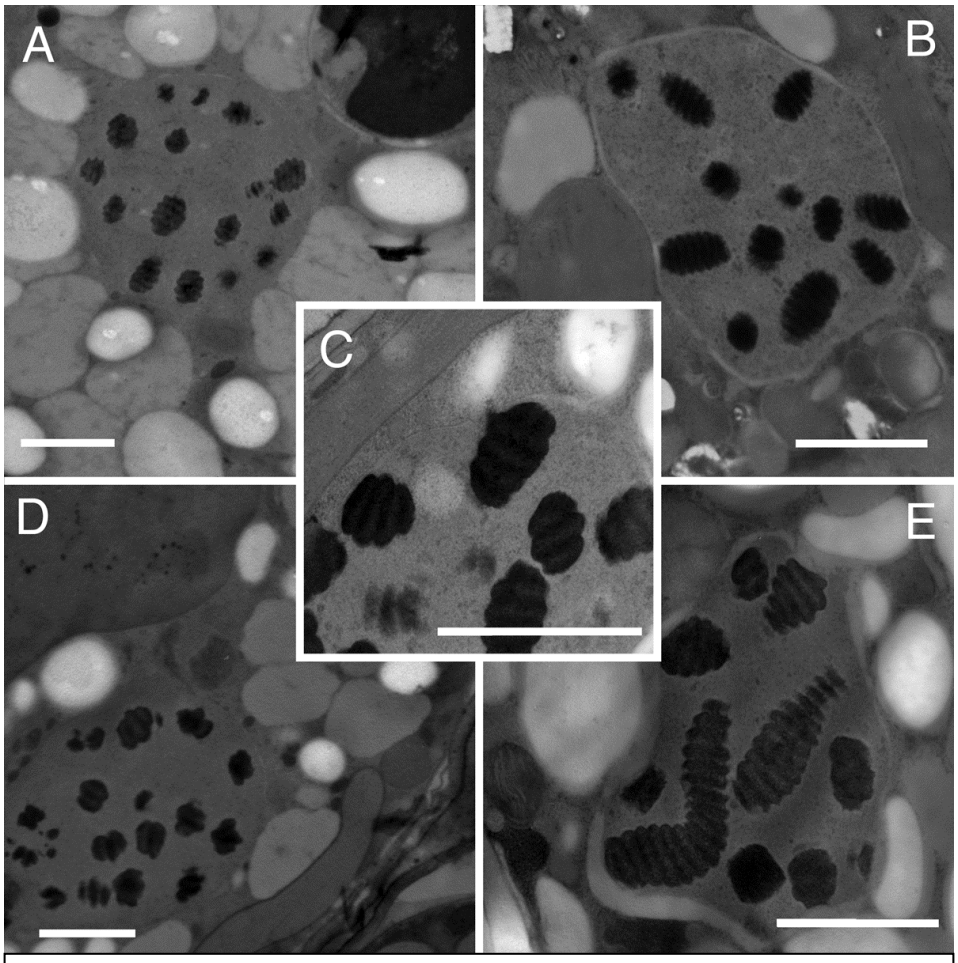

Figure 11: Examples of the dinokaryon nucleus in 5 isolates of *Symbiodinium*. A. The dinokaryon nucleus of isolate Y109 located centrally near an accumulation body. B. An example of the nucleus from FLAp4. C. An image of the nucleus from Ap highlighting the structure of the chromosomes. D. The dinokaryon nucleus from CCMP421 is also centrally located. E. The nucleus in isolate Mv is likewise centrally located and surrounded here by starch granules. All scale bars are 1µm.

verrucosa and the algae originally isolated and in culture for this and all other studies were isolated from a branch of the adult; it seems likely that *Symbiodinium kawagutii* described by Trench and Blank is the same type as those identified as *S. kawagutii* (Kinzie *et al.*, 1984; Kinzie *et al.*, 200) or Mv (Santos *et al.*, 2001; LaJeunesse, 2001; Santos *et al.*, 2002; Pochon *et al.*, 2006) in subsequent studies. The cellular contents described here are also consistent with the original formal species description of *Symbiodinium kawagutii*. *S. kawagutii* has been examined for its growth properties under different wavelengths of light, growing better under blue or white light than under green or red lights (Kinzie *et al.*, 1984). This species was also identified as a thermally tolerant *Symbiodinium* species exhibiting better growth at 29° and 31° C than other *Symbiodinium* strains in culture (Kinzie *et al.*, 2001). Genetic studies have also investigated isolate Mv, this isolate was used as an outgroup from clade C in a study examining clade B strains cultivated from a variety of different host animals (Santos *et al.*, 2001). This isolate was then identified as a strain in clade F1 using the ITS sequences (LaJeunesse, 2001). Using the cp23S-rDNA sequence along with sequences from the 18S-rDNA, n28S-rDNA, and ITS-rDNA genes this isolate was placed in Clade F (Santos *et al.*, 2002). Later genetic examination of more cultures from clade F using the n28S-rDNA and cp23S-rDNA sequences has determined that this isolate is within clade F5 (Pochon *et al.*, 2006).

While most of the cellular structures are similar between different isolates of *Symbiodinium*, understanding the differences can help us both in understanding phylogenies and in understanding the ecology of these dinoflagellates. It is noteworthy that the size of the different isolates determined by our work is consistent with previous studies, some done many years ago on the same cultured isolate. This indicates that while we cannot state that the isolates do not change in culture we can state that cell size is a consistent characteristic that can be useful

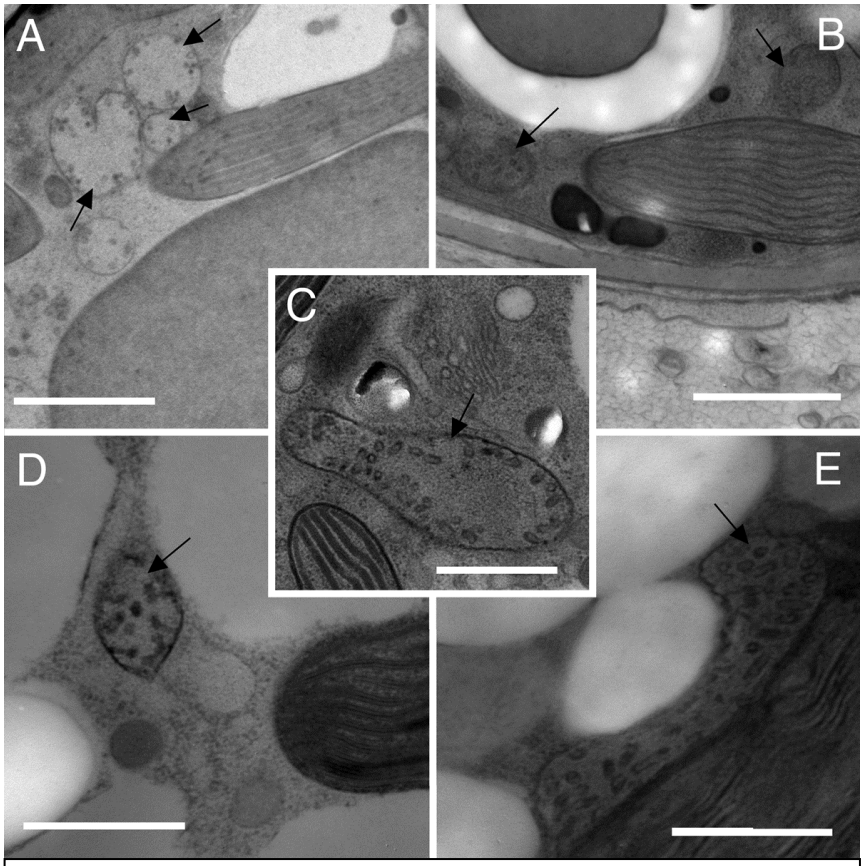

Figure 12: Examples of the mitochondrion structure in 5 isolates of *Symbiodinium*. A. The mitochondrion in Y109 are clustered and tubular cristae are located in the periphery of each. B. Isolate FLAp4 also contains mitochondria with tubular cristae. C. The mitochondria seen here in Ap is elongated with the characteristic peripheral tubular cristae seen in dinoflagellates. D. An example of a mitochondrion from CCMP421 with tubular cristae. E. Mv contains this elongated mitochondria with tubular cristae. Scale bars for panels A, B, D, and E are 1µm. Scale bar in panel C is 500nm.

in identification. It is also interesting that here and in the one previous description of a free-living *Symbiodinium* isolate the cell size is typically larger than symbiotic *Symbiodinium* isolates (Hansen and Daugbjerg, 2009). While the size and spacing of thylakoid membranes and the cell membrane and cell wall do not appear to be useful characters for identification, in this study we have provided an analysis of these characteristics for 5 isolates that any future work can use for comparison. The evidence presented here strongly suggests that there are not sufficient

ultrastructural differences amongst isolates to allow for identification of species or clade. Identification of isolates as members of a clade or species will have to rely solely on molecular identification methods. Despite the inability to use ultrastructural features for taxonomic identification, understanding of the ultrastructure of the symbiotic partners may suggest directions for a better understanding of the physiology of these symbioses as it relates to this extremely diverse genus of symbionts.

IV. ULTRASTRUCTURAL AND BIOCHEMICAL VARIATION AMONGST *SYMBIODINIUM* ISOLATES DEMONSTRATES VARIATION IN STORAGE MOLECULES

ABSTRACT

Symbiodinium is a diverse genus of unicellular dinoflagellates that often reside intracellularly in a variety of invertebrate hosts. Despite what is known about the genetic diversity of the genus, the physiological, morphological, and biochemical variation among members of this diverse group is poorly understood. One important area of research is determining how the symbiont establishes and maintains an association with a cnidarian host. Here we have looked at storage products within 5 isolates to determine any variation in the amount of lipid droplets and starch granules. Amongst the 5 isolates examined, the two smaller isolates from clade A contained the least amount of both starch and lipid. However, another small isolate from clade B contained some of the largest amounts of both starch and lipid. A clade F isolate also contained many storage products while the only free-living isolate examined, a clade E isolate, contained more lipid and starch than the clade A symbionts but less than the clade B and F isolates. Examination of exudate for the presence of carbohydrate and protein components revealed that in all 5 isolates, more carbohydrate than protein was secreted into the culture medium. Examining the cell wall associated compounds that may be involved in initiation and/or maintenance of the symbiosis, it was determined that a previously investigated antigen, PC3, is composed of carbohydrate structures and a probable protein component. This is consistent with the idea that a glycan/lectin interaction may be involved in the initiation and perhaps maintenance of this symbiosis.

INTRODUCTION

Symbiodinium was originally described as a single pandemic species (Freudenthal, 1962), but has since been found to be very diverse based upon molecular homology. Through genetic techniques this genuswas separated into 9 clades (Rowan and Powers, 1991a; Carlos *et al.*, 1999; LaJeunesse and Trench, 2000; LaJeunesse, 2001; Pochon *et al.*, 2001; Santos *et al.*, 2002; Pochon *et al.*, 2004; Pochon and Gates, 2010). Additionally, ultrastructural (Taylor, 1968; Kevin *et al.*, 1969; Bishop *et al.*, 1976; Leutenegger, 1977; Schoenberg and Trench, 1980b; Van Thinh *et al.*, 1986; Blank, 1987; Ambariyanto and Hoegh-Guldberg, 1996; Muller-Parker *et al.*, 1996; Wakefield *et al.*, 2000), physiological (Warner *et al.*, 1996; Iglesias-Prieto and Trench, 1997; Rowan *et al.*, 1997; Kinzie *et al.*, 2001; Perez *et al.*, 2001; Baker *et al.*, 2004; Bhagooli and Hidaka, 2004; Rowan, 2004), and biochemical (Schoenberg and Trench, 1980a; Markell *et al.*, 1992; Markell and Trench, 1993; Markell and Wood-Charlson, 2010) studies revealed differences amongst isolates within this genus. This diversity is important because it may allow for many potential combinations of host species and symbionts. Thus, some combinations of host and symbiont may be better adapted for specific environmental conditions, such as increases in sea surface temperature that can cause detrimental bleaching events, than others (Buddemeier and Fautin, 1993; Hoegh-Guldberg, 1999; Fitt *et al.*, 2001; Rowan, 2004; Weis, 2010; Stat and Gates, 2011).

The basis for this symbiosis is a nutritional one, meaning that the symbiont donates photosynthetically fixed carbon as a nutrient for the host while the host donates its waste products (such as CO_2 and nitrogenous compounds) which are nutrients for the symbiont (Muscatine and Porter, 1977; Muscatine, 1990; Verde and McCloskey, 1998; Yellowlees *et al.*, 2008). Both the type and the amount of translocated photosynthetically fixed carbon

compound(s) are important in determining whether the symbiont is mutualistic or veering into a parasitic lifestyle. Previous studies have shown that *Symbiodinium* isolates can contribute between 20% (*Paylthoa townsleyi*; Trench, 1971a) and 169% (*Cassiopea xamachana*; Verde and McCloskey, 1998) of carbon-based host metabolic needs. Thus, in many associations studied, the symbiont can transfer sufficient photosynthetically fixed carbon to provide for the hosts metabolic needs and often an excess above this level that can be used by the host for growth or reproductive efforts (Muscatine and McCloskey, 1981; Muscatine *et al.*, 1984; Stambler and Dubinsky, 1987; Davy and Cook, 2001; Grottoli *et al.*, 2006; Tanaka *et al.*, 2006; Loram *et al.*, 2007; Gordan and Leggat, 2010). While there are many studies looking at the amount of photosynthetically fixed carbon, fewer studies have examined the specific molecules translocated. Initial work indicated that the primary carbon compound produced is glycerol (Muscatine, 1967), but later investigations revealed that while glycerol was the major translocation product, other compounds such as alanine, glucose, fumaric acid, succinic acid, and glycolyic acid were also translocated(Trench, 1971b). Another study confirmed that glucose and succinate/fumarate were major transfer products in *Anemonia viridis* (Whitehead and Douglas, 2003).

While there is some information about the compounds and amount of photosynthetically fixed carbon transferred to the host, there is less known about the pathway(s) by which the photosynthate moves between symbiont and host. *Symbiodinium* in culture produces a mucilaginous exudate (Markell *et al.*, 1992; Markell and Trench, 1993) that *in situ* appears as a matrix that contains multiple layers of membranous material derived from the symbiont (Wakefield and Kempf, 2001). In culture the exudate is at least in part water soluble (and therefore present in the culture medium) and analysis of the culture medium and of extracted cell

wall indicate that it is composed of glycosylated polypeptides (Markell *et al.*, 1992); it is usually composed of more carbohydrate than protein (Markell and Trench, 1993). These substances could be involved in recognition between host and symbiont, consistent with lectin-glycan recognition systems in other organisms and with lectin-like compounds found in cnidarian hosts (Kvennefors *et al.*, 2008; Jimbo *et al.*, 2013). Some of these carbohydrate containing compounds may be involved in recognition (Logan *et al.*, 2010; Wood-Charlson *et al.*, 2006) and/or host settlement cues (Markell and Wood-Charlson, 2010). Additionally, these compounds may be involved in translocation of photosynthetically fixed carbon from the symbiont to the host since some of the same compounds identified as components of the carbon translocated to the host are present in the exudate (Trench, 1971b; Markell and Trench, 1993).

While it seems that much of the photosynthate is translocated to the host, storage products have been seen in ultrastructural studies of *Symbiodinium*. In the initial ultrastructural description, starch granules were not mentioned, but lipid droplets and vacuoles containing calcium oxalate were mentioned as inclusions (Kevin *et al.*, 1969). In a 1968 study of symbiotic dinoflagellates the accumulation body containing mucopolysaccharide was identified along with the presence of starch grains throughout the cytoplasm and surrounding the pyrenoid (Taylor, 1968). Lipid droplets, also present in the symbiont's cytoplasm, have been well studied (Bishop *et al.*, 1976) and found to mostly be galactosyldiacylglycerols (50%). Several later studies described symbionts from different hosts with the same inclusions as these earlier studies (Schoenberg and Trench, 1980; Van Thinh *et al.*, 1986; Blank, 1987; Wakefield *et al.*, 2000). In an ultrastructural examination of starch inclusions in normal and well-fed (N-enriched) symbioses between *Tridacna maxima* and its symbiont there was an increase in starch under normal conditions possibly due to N limitation on the reef (Ambariyanto and Hoegh-Guldberg,

1996). This is probably due to the carbon stores being utilized for greater amino acid synthesis and therefore, protein synthesis, under nitrogen enriched conditions (Ambariyanto and Hoegh-Guldberg, 1996). Likewise, an examination of symbionts within the host compared to those in culture revealed an increase in starch in cultured symbionts, again possibly due to N limitation in culture compared to within the host (Schoenberg and Trench, 1980). Lipid droplets behaved similarly and in well-fed anemones, *Symbiodinium* lipid content decreased from 30% to 15% (Muller-Parket *et al.*, 1996). While there is some information about the starch grains and lipid droplets, there have been no comparisons of this content amongst different *Symbiodinium* isolates under the same environmental conditions.

It is important to understand how the *Symbiodinium*-cnidarian association is initiated and maintained in order to recognize whether, during bleaching episodes, the host may be able to acquire new symbiont strains or switch to different symbiont strains that may be better suited for the new stress-inducing environmental conditions (the "adaptive bleaching hypothesis"; Buddemeier and Fautin, 1993). We can then better understand the possibility for survival of some associations as sea surface temperatures increase (Baker *et al.*, 2004; Stat and Gates, 2011). The fixation and donation to the host of photosynthetic carbon is vital to the maintenance of the association and the coral reef ecosystem. Here we examine the presence of storage molecules of five *Symbiodinium* isolates to see if there is variation between isolates that may correspond to physiological differences that could alter the efficiency of a particular host-symbiont association. Additionally, we examine the mucilaginous exudate for protein and carbohydrate composition to see if there is a correlation between the amount of stored starch and the compounds secreted to the exterior of the cell.

METHODS

CULTURE OF SYMBIODIMIUM *ISOLATES.* Cultures were grown in F/2 media (Guillard and Ryther, 1962) in 125mL Erlenmeyer flasks under 12/12 light dark cycle at 24.5°C ± 0.5° at Auburn University for 2.5 weeks. Cultures were then transported to the culture room of Dr. William Fitt at the University of Georgia and allowed to grow for 2 weeks under a 12/12 light dark cycle at 26.5°C ± 0.5°. Cultures examined ultrastructurally in this investigation are listed in Table 1.

HIGH PRESSURE RAPID FREEZING. Fixative solutions were made up several days prior to the freezing event. 0.1% Uranyl acetate in HPLC grade acetone was mixed at room temperature 2 days prior to freezing and the day before freezing was transferred to a -80°C freezer. A vial of 25 mL of HPLC grade acetone was pre-chilled in the -80°C freezer overnight and then 1 g of OsO_4 was added (making a 4% OsO_4 solution) while the solution was kept on dry ice for 10 minutes before being replaced in the -80ºC freezer the day before freezing. Isolate samples were collected around 'dawn' by pipetting 100µL of cells and media using a Gilson pipetman starting at the bottom of the flask (where the cells accumulate) and slowly drawing the pipette up about 1 cm from the bottom. The sample was then transferred to a 1.5 ml microcentrifuge tube and centrifuged briefly to produce a loose pellet. Most of the remaining F/2 media was removed (leaving the cell pellet in about 50 µL of F/2 media) and then 50 µL of 20% dextran in F/2 media was added and the cells were re-suspended in a final concentration of 10% dextran in 100 µL of F/2 medium. The 10% dextran encouraged the cells to remain clumped together after freezing. 5µL of cells were transferred to planchettes using a Gilson pipetman and then rapidly frozen in a High Pressure Freezing Machine (HPM 010 ABRA Fluid AG). After freezing, pellets were transferred to cryotubes submerged in liquid nitrogen and then

transferred to a pre-cooled dewer and maintained in liquid nitrogen until transfer into fixative. The 0.1% Uranyl acetate in acetone and 4% OsO4 in acetone were then mixed (all vials kept on dry ice) in a 1:1 ratio and aliquoted into enough vials for the frozen samples. After 2 hours in the -80°C freezer, the frozen samples in planchettes were quickly added to the vials of 1:1, 0.1% Uranyl acetate: 4% OsO4 in acetone, all vials and cryotubes being kept on dry ice. Samples in fixative were then placed in the - 80°C freezer for 4 days. Samples were next moved to the -20°C freezer for 3 hours, then to a 4°C refrigerator for 2 hours, and finally into the fume hood at room temperature for 30 minutes. After this gradual return to room temperature the fixative was removed and samples were rinsed 3 times in 100% HPLC grade acetone for 15 minutes each. Samples were then removed from planchettes and embedded using a Low viscosity Embedding Media Spurr's Kit (hard recipe; EMS) by first adding the embedding resin as a 1:2 mix in acetone and leaving it overnight at room temperature. The next day the samples were changed to 2 parts embedding resin to 1 part acetone for 8 hours and then into 100% resin overnight. The next morning samples were placed in fresh 100% resin for 8 hours. Near the end of the day the samples were transferred to catalyzed resin in Beem capsules. After 1 hour at room temperature in the catalyzed resin the samples were placed in an oven at 60°C and left for 24-48 hours to ensure hardening.

Table 1: Information about the isolates of *Symbiodinium* studied here.

Species	Culture ID	Host	Clade (Cp23S rDNA)	Avg Diameter
Symbiodinium sp.	Y109	Unknown host	A191	9.0 ±0.83 µm, n=30
Symbiodinium sp.	FLAp4	*Aiptasia pallida*	A193	7.5 ±0.6 µm, n=30
Symbiodinium pulchrorum	HIAp or Ap	*Aiptasia pulchella*	B224	8.0 ± 0.86 µm, n=30
Symbiodinium sp.	CCMP 421	None	E202	10.2 ± 1.01 □m, n=30
Symbiodinium kawagutii	Mv	*Montipora verrucosa*	F178	9.1 ± 0.74 µm, n=30

TRANSMISSION ELECTRON MICROSCOPY. Sections were cut using a Reichert-Jung Ultracut E Microtome and placed on copper 75 mesh grids (EMS). They were then stained using 4% aqueous solution of uranyl acetate for 10 to 20 minutes, rinsed and then secondarily stained with 0.1% aqueous solution of lead citrate for 5 minutes. Samples were viewed on a Zeiss EM 10C 10CR Transmission Electron Microscope and digital images were taken using MaxIm DL5 software and an SIA-L3C 4.3 megapixel camera (Scientific Instruments & Applications, Duluth, GA 30096).

ULTRASTRUCTURAL MEASUREMENTS AND STATISTICS. In order to obtain accurate measurements of cell size, only cells larger than 4 µm in diameter were measured and added to a list to obtain 100 measured cells from each isolate. These cells were then sorted by size and only the largest 30% (n=30) were used to determine percent coverage of lipid and starch inclusions. This was done to ensure that comparisons were always made between largest cross sections and therefore approximately in the middle of each cell. Measurements of cell diameter were made first using the MaxIMDL5 software and then confirmed using Image-Pro Plus (Version 7.0; Media Cybernetics, 2009). Measurements of cell cross-sectional area and starch/lipid cross-sectional area were made using Image Pro Plus (Version 7.0; Media Cybernetics, 2009). The percent of lipid and starch cross-sectional area was determined by dividing the total cross-sectional area covered by either starch or lipid by the entire cell cross-sectional area and multiplying by 100. Lipid and starch inclusion differences among isolates were transformed using an arcsin transformation and then compared using a one-way ANOVA run on SAS (Version 9.2) to ascertain any significant differences. Post-hoc multiple comparison tests were run with a Tukey-Kramer adjustment to examine which strains exhibited significant differences.

COLLECTION OF EXUDATE FROM SYMBIODINIUM *CULTURES. Symbiodinium* isolates for exudate analysis were grown as described above at Auburn University for 4-5 weeks (until they were in the log phase of growth). 300µL of culture medium plus cells were then collected and placed in a microcentrifuge tube along with 300µL of a 45ppt salt solution. The samples were next centrifuged at high speed in a clinical centrifuge for 10 minutes. After centrifugation, the supernatant was collected and used for further analysis. Cell numbers were determined using a hemacytometer.

BIOCHEMICAL ANALYSIS OF EXUDATE. 200µL of supernatant containing exudate was transferred to a clean microcentrifuge tube (Tube 1). To separate the carbohydrate and protein components, 100µL of 15% Trichloracetic acid (TCA) was added and the microcentrifuge tube was vortexed for 5 minutes followed by 10 minute incubation at 4º C and then centrifugation at 5000g for 10min. The supernatant was then transferred to another clean tube (Tube 2). 200µL of 5% TCA was then added to Tube 1 from which the supernatant had just been removed. This was vortexed as above, centrifuged for 5 minutes, and then the resulting supernatant was added to Tube 2 (now containing approximately 500µL of carbohydrate containing supernatant). 700µL of 1.0N NaOH in 1% Na-deoxycholate was added to Tube 1 which now had the carbohydrate containing supernatant removed and a protein pellet remaining. The tube was vortexed for 4 minutes, heated to 56º C for 30min, and centrifuged for 10 minutes. During this time BSA standards were prepared. The rest of the protein determination was carried out according to the Lowry method (Lowry *et al.*, 1951).

In order to determine the amount of carbohydrate present in the exudate, 200 µL of supernatant from Tube 2 (see separation of carbohydrate and protein above) was transferred, to each of 2 new 1.5 ml microcentrifuge tubes. Next 20µL of 6.0N HCl was added to each

microcentrifuge tube. The tubes were then vortexed followed by heating for 2 hours at 95ºC. After the 2 hours at 95ºC, the following was used to determine the amount of carbohydrate in each sample (modified from Holland and Gabbot, 1971).

1. The tube was cooled for 5 minutes and centrifuged at 5000g for 2 minutes.
2. 20µL of 6.0N NaOH and 60µL of 5% TCA were added followed by additional vortexing.
3. 100µL of this solution was transferred to each of two glass reaction tubes along with 100µL of 5% TCA.
4. At this point glucose standard tubes were prepared containing 0, 2.5, 5, 10, 12, and 16 µg of glucose.
5. To both the reaction tube and the glucose standard tubes, 500µL of 0.625% NaOH, 100µL of 0.2% potassium ferricyanide, and 200µL of the carbonate-cyanide reagent (500µL 16% NaCO3 + 30µL 5%KCN + 9.5 nanopureH2O) were added, vortexing in between each addition of a solution.
6. The reaction and standards tubes were heated at 95ºC for 10 minutes and then cooled for 10 minutes.
7. 500µL of npH2O was added to each tube and vortexed.
8. The absorbance was read at 420nm on a Genesys 2 Spectrophotometer.

The absorbance values for the glucose standards were used to prepare a standard absorbance versus total mass curve. The amount of carbohydrate in each reaction tube was determined from the standard glucose curve and the average value for the two reaction tubes analyzed was used to determine the amount of carbohydrate present in the volume of the original sample.

ENZYMATIC EXAMINATION OF EXUDATE. 200µL of cells of isolate FLAp4 were collected at dawn, centrifuged, media removed, and then dehydrated and rehydrated through PBS+, 30% ethanol, 50% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, PBS+ centrifuged between each step to remove chlorophyll autofluorescence. Then each of 9 pellets was treated in

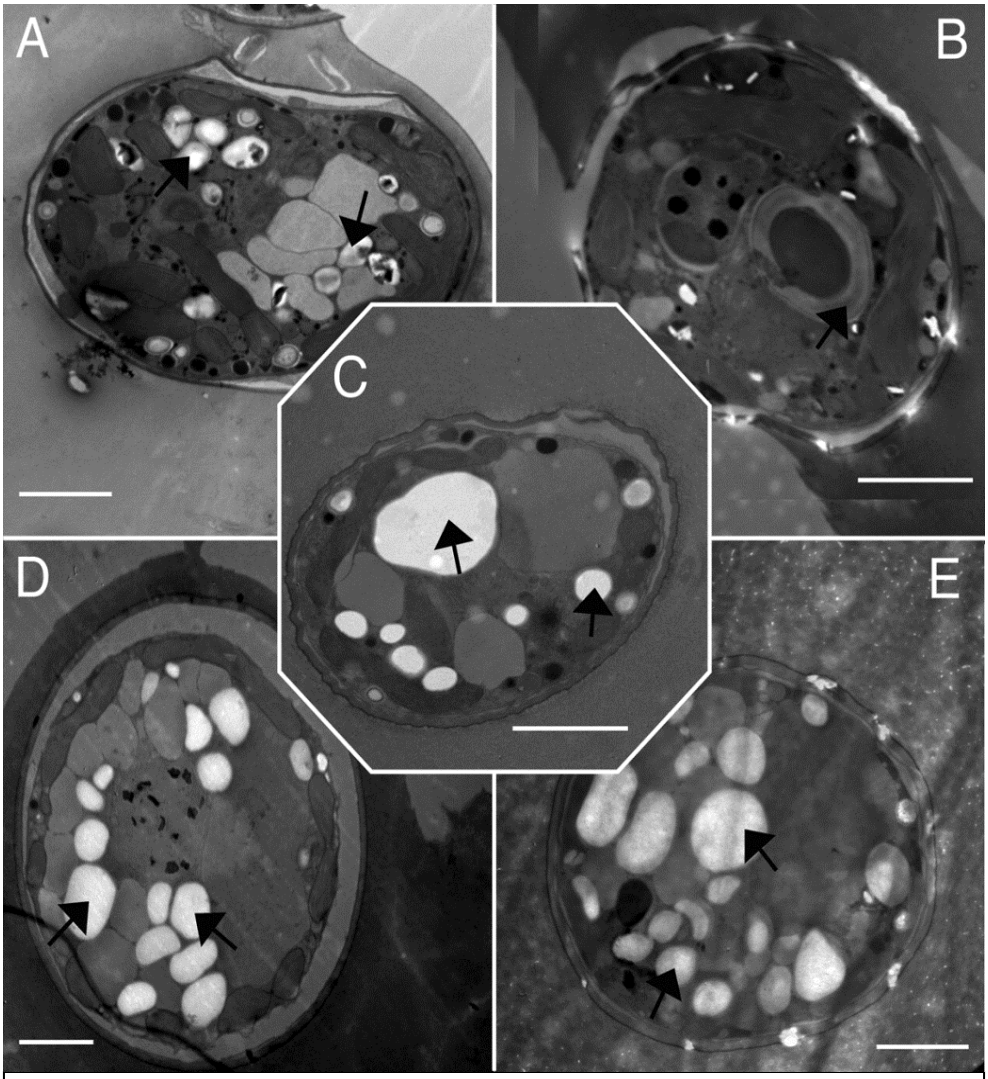

Figure 1: Examples of cells from 5 *Symbiodinium* isolates containing the average cross-sectional percent area starch granules present in that isolate. A. Isolate Y109 contains 9.45% ± 5.5% cross-sectional area composed of starch. B. 8.82% ± 6.3% of cross-sectional area is starch in isolate FLAp4. C. Isolate Ap is composed of 16.84% ± 4.6% cross-sectional area of starch. D. 12.05% ± 5.2% cross-sectional area is composed of starch in isolate CCMP421. E. Isolate Mv contains 19.99% ± 6.7% cross-sectional area composed of starch. Scale bars = 2µm.

a different way. Treatments were always suspended in 200µL of the solution mentioned and included (1) incubation with 25 units of trypsin in 20mM phosphate buffered saline containing 0.1% sodium azide and 0.1% Triton X100 (PBS+) for 1 hour at 37°C; (2) incubation with 25 units of neuraminidase in PBS+ for 1 hour at 37°C; (3) incubation with 25 units of hyaluronidase in PBS+ for 1 hour at 37°C; (4) incubation with 25 units of chymotrypsin in PBS+ for 1 hour at 37°C; (5) media removal *via* centrifugation, resuspension in PBS+ and homogenization using the VirTis hand-held homogenizer; (6) incubation with 1% periodic acid in nanopure water for 1 hour; (7) incubation in nanopure water and boiled for 5 minutes; (8) incubated at 37ºC for 1 hour in PBS+ (control + heat); (9) kept at 4°C for 1 hour (control). All treatments were then divided into 2 1.5mLmicrocentrifuge tubes, centrifuged at 2000g, supernatant removed and incubated 5% heat-inactivated goat serum in PBS+ (blocking medium) for 10 minutes at room temperature. Blocking medium was removed by centrifugation and 1 tube of each treatment was incubated with spent medium containing primary antibody PC3 (an antibody specific to the outer coat of *Symbiodinium* of isolate FLAp4) diluted 1:1 in blocking medium for 1 hour at room temperature (the second tube of each strain was incubated with only blocking medium for this hour as a negative control). Samples were then washed in PBS+ 3 times for 5 minutes each, followed by incubation in secondary antibody (Alexa fluor 488 Goat anti-mouse IgM (µ chain) - Invitrogen, A-21042) diluted 1:300 in blocking medium for 30 minutes at room temperature in darkness (to prevent quenching). Samples were then rinsed 3 times in PBS+ for 5 minutes each and resuspended in a small amount of Nanopure-H_2O (np-H_2O). Cells were then put on slides and viewed on a Nikon Optiphot fluorescence microscope and imaged with a Canon Eos Rebel T2i camera. A minimum of 150 cells of each treatment were examined for the presence of fluorescence indicating that the antigen remained intact. Percent fluorescence data was

transformed using an arcsin transformation and then examined with an ANOVA using SAS 9.2 for statistical significance. Post-hoc multiple comparison tests were run with a Tukey-Kramer adjustment to examine which strains exhibited significant differences.

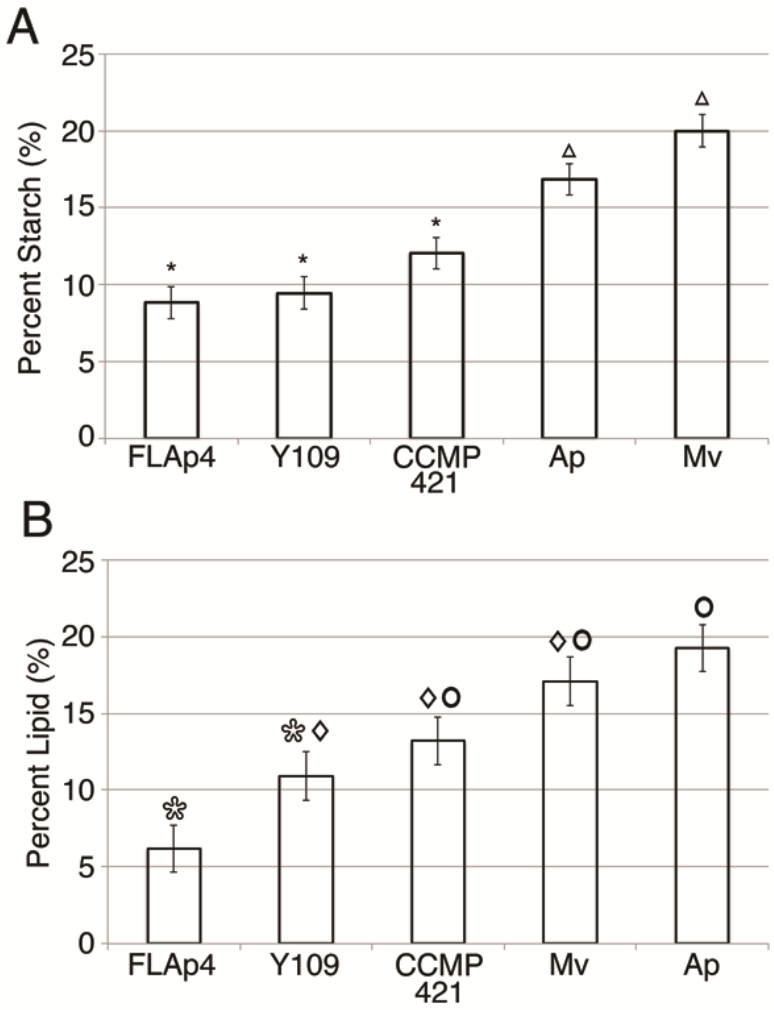

Figure 2: Graph of statistical analysis of the percent cross-sectional area of starch granules (A) and lipid droplets (B). Error bars indicate the standard error. Symbols above bars indicate isolates that are not significantly different from each other. $P<0.0001$

RESULTS

ULTRASTRUCTURAL STARCH VARIATION. Starch content varied dramatically and significantly amongst 5 different *Symbiodinium* isolates (Figure 1, 2). Isolates FLAp4 (8.82% ± 6.3%, n=30 cells, p <.0001), Y109 (9.45% ± 5.5%, n=30 cells, p <.0001 and CCMP421 (12.05% ± 5.2%, n=30 cells, p <.0001) were not significantly different from each other but were significantly different from isolates Ap (16.84% ± 4.6%, n=30 cells, p <.0001) and Mv (20.0% ± 6.7%, n=30 cells, p <.0001).

ULTRASTRUCTURAL LIPID VARIATION. Lipid content varied amongst the 5 *Symbiodinium* isolates examined. The lipid droplets are identified as gray inclusions (Dodge and Greuet, 1987) within the cell cytoplasm (Figure 3). The average percent of lipid varied significantly amongst isolates (Figure 2). Isolate FLAp4 (6.20% ± 6.7%, n=30 cells, p < .0001) was not significantly different from Y109 (10.93% ± 7.13%, n=30 cells, p < .0001), but was significantly different from the other 3 isolates. Isolate CCMP421 (13.20% ± 9.8%, n=30 cells, p < .0001) was not significantly different from Y109 or Mv (17.07 ± 8.32%, n=30 cells, p < .0001), but was significantly different from both FLAp4 and Ap (19.24 ± 8.55%, n=30 cells, p < .0001).

BIOCHEMICAL ANALYSIS OF EXUDATE. Examination of the exudate from isolates of *Symbiodinium* used in this study revealed that in all cases the exudate contained both protein and carbohydrate components (Table 2). In every isolate, cells secreted more carbohydrate than protein. There was no significant difference between isolates in the amount of either protein or carbohydrate present in the exudate.

ENZYMATIC DIGESTION OF EXUDATE. Incubating cells of isolate FLAp4 with various enzymes showed a slight (but not significant) decrease in labeling with the PC3 antibody

for the enzymes trypsin, and hyaluronidase (Figure 4). Neuraminidase showed a significant decrease in number of cells that labeled with PC3 antibody (Figure 4; $p=0.03$) indicating the presence of glycosidic linkages. There was also a significant decrease in antibody labeling in treatments with 1% periodic acid and boiling (Figure 4; $p<0.0001$).

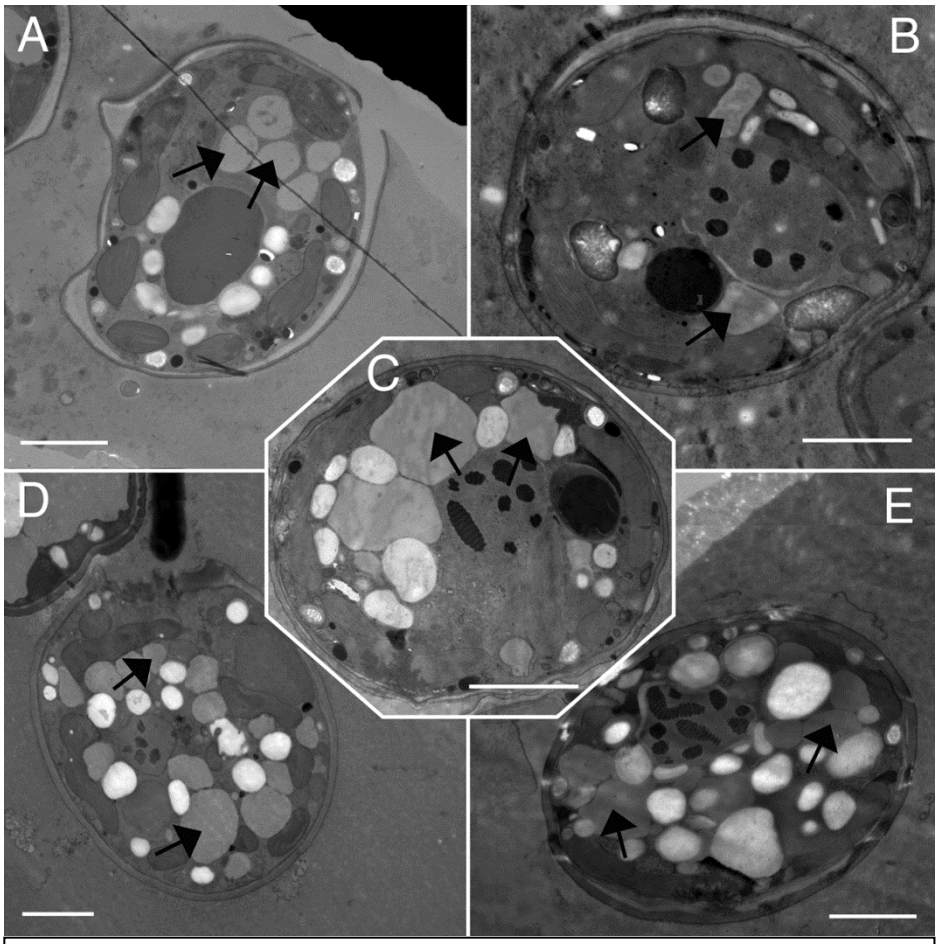

Figure 3: Examples of cells from 5 *Symbiodinium* isolates containing the average percent cross-sectional percent coverage of lipid droplets. A. Isolate Y109 contains 10.93 ± 7.13% cross-sectional area of lipid. B. Isolate FLAp4 contains an average of 6.20 ± 6.7% cross-sectional area composed of lipid. C. 19.24 ± 8.55% of cross-sectional area is composed of lipid in isolate Ap. D. Isolate CCMP421 contains 13.20 ± 9.8% cross-sectional area of lipid. E. Isolate Mv contains 17.07 ± 8.32% cross-sectional area composed of lipid. Scale bars are all 2µm.

DISCUSSION

Since the symbiosis between *Symbiodinium* and its cnidarian hosts is nutritionally based, the production, storage, and excretion of photosynthetically fixed carbon by the symbiont is vital to the understanding of the association. Any variation in production, storage or excretion of photosynthetically fixed carbon could lead to differences in the potential for a successful association between a specific host and a specific *Symbiodinium* isolates. Isolates that can either photosynthesize more efficiently or translocate a higher percentage of their photosynthetically fixed carbon to the host should be better partners for the host (Muscatine *et al.*, 1984). Here we have examined the presence of storage products in 5 isolates of *Symbiodinium.* Isolates Ap and Mv contain significantly more starch granules than other isolates. Interestingly, isolate Mv (a clade F5 isolate; Pochon *et al.*, 2006) was identified as a thermally tolerant strain with increased growth rates at 29º and 31ºC (Kinzie *et al.*, 2001). Conversely, isolate Ap showed decreased thermal tolerance (Kinzie *et al.*, 2001), but under the culture conditions here stored similar amounts of starch granules.

Table 2: Biochemical Analysis of Exudate.

Isolates	Protein (per 1,000,000 cells)	Carbohydrate (per 1,000,000 cells)
Y109	0.133 ± 0.056 µg n=14	1.632 ± 0.775 µg n=8
FLAp4	0.149 ± 0.086 µg n=14	3.785 ± 1.532 µg n=8
Ap	0.087 ± 0.032 µg n=10	2.123 ± 1.030 µg n=8
CCMP421	0.093 ± 0.042 µg n=8	2.691 ± 1.173 µg n=10
Mv	0.339 ± 0.083 µg n=14	2.405 ± 1.142 µg n=10

Isolates FLAp4, Y109 and CCMP421 all contained less starch. Interestingly, both FLAp4 and Y109 are isolates from clade A that tend to be somewhat smaller (especially FLAp4) than all of the other studied isolates (Chapter 3, pg 66). Since statistics were performed on a percent of total cross-sectional area, the small cell size should not impact this conclusion. While there is little known about these isolates physiologically, a variety of different isolates within clade A have exhibited traits of both thermal tolerance and thermal susceptibility (Robison and Warner, 2006; Diaz-Almeyeda *et al.*, 2011; Steinke *et al.*, 2011). Without knowing about the thermal tolerance of isolates Y109 and FLAp4 we cannot make any suggestions about the connection between lower amounts of starch and thermal conditions. Isolate CCMP421 is a free-living isolate of *Symbiodinium* and the only other free-living isolate described is *Symbiodinium natans* (Hansen and Daugbjerg, 2009). *S. natans* has been reported to contain lipid and starch inclusions, but, unfortunately, only a qualitative description was given.

Storage of lipid droplets also showed significant variation amongst the *Symbiodinium* isolates investigated. Based on our % area calculations, isolate Ap contained the greatest amount of lipid (significantly different from clade A isolates FLAp4 and Y109). This isolate is a thermally intolerant member of clade B. In previous studies, *Symbiodinium* in nitrogen starved conditions tended to store more lipid droplets and starch granules (Ambariyanto and Hoegh-Guldberg, 1996; Muller-Parker *et al.*, 1996). The differences seen here occur in identical, healthy, nutrient rich cultures which suggest that they are due to physiological differences among the isolates tested. Thus, it is likely that isolate Ap has different lipid metabolism requirements than isolates FLAp4 and Y109. Isolate Mv also contained a large amount of lipid (17%) that was significantly different from the amount present in FLAp4, as well as a large amount of starch. Since Mv is known to be thermally tolerant (Pochon *et al.*, 2006) it would be interesting to

examine whether the large amounts of storage product accumulated relate to its ability to withstand higher temperatures.

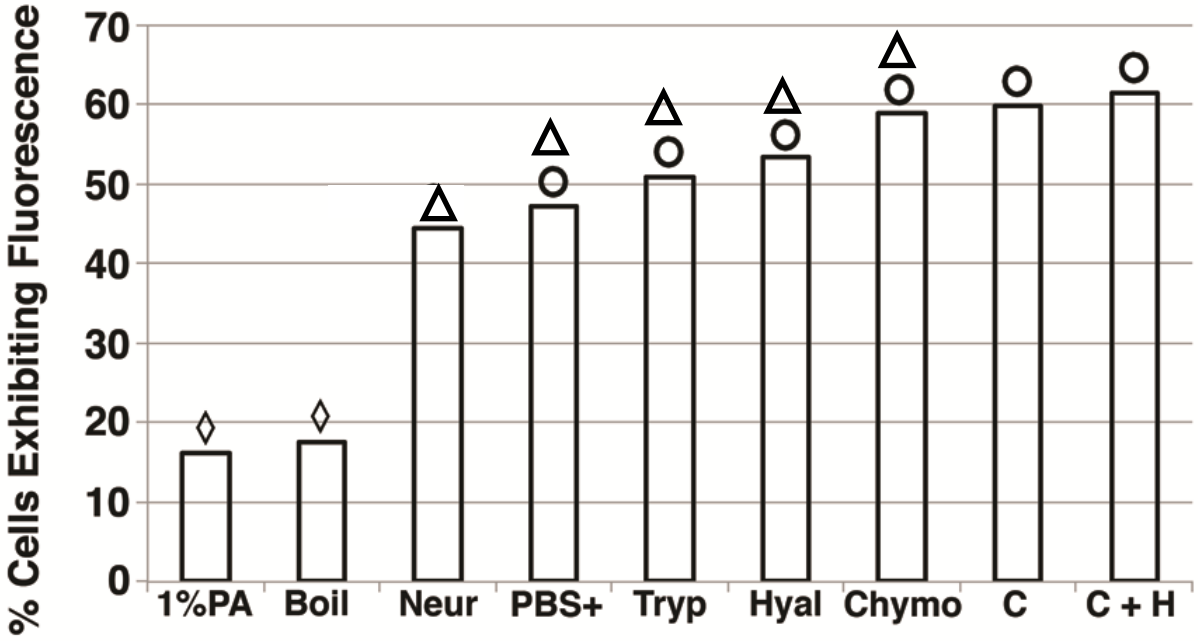

Figure 4: Number of cells of isolate FLAp4 exhibiting fluorescence after a variety of different treatments. 1%PA = 1% Periodic acid, boil was boiled for 5 minutes, neur = neuraminidase treatment, PBS+ = homogenized in PBS+, Tryp = trypsin treatment, Chymo = chymotrypsin treatment, C = Control, C + H = Control with the same heat treatment as the enzymes. Identical symbols above the bars indicate treatments that are not significantly different from one another.

Isolate CCMP421 contained 13.2 ± 9.8% lipid and 12.1 ± 5.2% starch. Perhaps the relatively high amount of both types of storage products seen in the free-living strain indicates that it would not be as efficient at translocation of photosynthate under symbiotic conditions. As a free-living isolate there is essentially no need to translocate photosynthate and thus such carbon may be channeled into storage products. This would be consistent with the life history of this isolate and with previous descriptions of free-living isolates that report a number of inclusions (Hansen and Daugbjerg, 2009), but very little in the way of excreted carbon compounds (as evidenced by a lack of labeling with lectin probes; Logan *et al.*, 2010). Clade A isolates FLAp4

and Y109 again had fewer lipid droplets (FLAp4 significantly fewer than CCMP421, Mv and Ap; Y109 significantly fewer than Ap). Isolates FLAp4 and Y109 are both physically smaller (FLAp4 especially) however, little is known of their physiology. Clade A contains members that are both thermally tolerant and intolerant, however, there is no information about the thermal tolerance of these two isolates in particular. The fewer inclusions in these strains could mean that they have different nutrient requirements than the other isolates or that they do not store food reserves as efficiently. As above, the differences in amount of stored lipid probably reflect differences in the physiology of these isolates. Such differences could have important consequences for the efficiency of translocation and possibly thermal and light tolerance as well as for the suitability of specific isolates as symbionts for specific hosts. While more needs to be understood about the physiology of these isolates, differences in the production and storage of lipid and/or starch could be important factors in maintaining a successful association with specific hosts or under specific environmental conditions.

While the storage of products derived from photosynthetically fixed carbon may have implications for the health of the association between *Symbiodinium* and their host cnidarian, it is also likely that any compounds exuded by the symbiont are important in the establishment and/or the maintenance of the association. Here we saw that all 5 isolates exuded some protein and carbohydrate into the culture media. This is consistent with previous work that found a variety of compounds exuded into the media, including protein, neutral sugars, glucosamine, galactosamine, and uronic acid (Markell and Trench, 1993). There could be differences in the amounts of secreted compounds and it is likely that the components in the exudate would vary amongst *Symbiodinium* isolates.

These secreted compounds are similar to the compounds others have noted as being associated with the cell wall of *Symbiodinium* (Markell *et al.*, 1992; Lin *et al.*, 2000). It has been suggested that cell wall associated glycoproteins may be involved in cell-cell recognition during the establishment of the symbiosis (Lin *et al.*, 2000). Such being the case, the glycans that are associated with the symbiont cell wall would interact with lectins associated with the cnidarian host cell to initiate the symbiosis (Wood-Charlson *et al.*, 2006; Jimbo *et al.*, 2012). Some lectins have been identified in cnidarian hosts including a mannose-binding lectin in *Acropora millepora* (Kvennefors *et al.*, 2008) and a D-galactose-binding lectin from *Sinularia lochmodes* (Jimbo *et al.*, 2000). The glycans identified thus far have shown no pattern relative to clade, but regardless of *Symbiodinium* clade, isolates from the same host have shown similar profiles (isolates from clade A and B from the same host had similar profiles; Logan *et al.*, 2010). Logan found that all 8 isolates tested had exudates containing glycans with mannose residues while 6 out of 8 isolates contained glycans with N-acetyl groups (2010). Other glycans were present in smaller concentrations and associated with fewer isolates and it is possible that these play an important role in specificity (Logan *et al.*, 2010). Here examination of an antigen present in the exudate of *Symbiodinium* isolate FLAp4 (as well as some other isolates; Chapter 2, pg 47) that is recognized by the PC3 antibody (Wakefield and Kempf, 2001) also showed that binding of the antibody is lessened when chemicals that damage the structure of glycans are added (such as periodic acid, which showed a very strong significant effect; and neuraminidase which also showed significant effects).

The variation seen here in the storage of lipid and starch is likely due to physiological differences amongst isolates that could impact the transfer of photosynthetically fixed carbon and therefore the host-symbiont association. The biochemical analysis of exudate from a number of

solates is consistent with previous work (Markell and Trench, 1993; Markell and Wood-Charlson, 2010). Also, our examination of the cell wall associated antigen PC3 (Wakefield and Kempf, 2001) indicated that it is composed of both carbohydrate and protein components which agrees with cell wall associated compounds examined in previous studies (Markell *et al.*, 1992; Lin *et al.*, 2000; Wood-Charlson *et al.*, 2006; Kvennefors *et al.*, 2008; Logan *et al.*, 2010; Jimbo *et al.*, 2012). These results are consistent with the idea that cell surface associated compounds are exuded by isolates of *Symbiodinium* and could be involved in both the initiation and the maintenance of the *Symbiodinium*-cnidarian association.

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