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Distinct ITS types of *Symbiodinium* in Clade *C* correlate with cnidarian/dinoflagellate specificity during onset of symbiosis

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ABSTRACT: This study aimed at determining whether fine-scale molecular diversity of *Symbiodinium* corresponds to functional differences in host/symbiont specificity during the onset of symbiosis in cnidarian/alga mutualisms. Infection experiments were conducted in aposymbiotic larvae of the scleractinian *Fungia scutaria* using known algal Internal Transcribed Spacer (ITS)-2 types within Clade *C*. Our results show that algal type is directly related to specificity during the onset of symbiosis in *F. scutaria* larvae. The 3 ITS symbiont types (*C*1f, *C*15 and *C*31) within the major *Symbiodinium* Clade *C* engaged differentially in symbiosis with coral larvae. The homologous symbiont (*C*1f), found in adult *F. scutaria* from the field, showed a significantly better association with the host larvae than the other 2 heterologous symbionts (*C*15 extracted from *Porites compressa*, and *C*31 from *Montipora capitata*). This is the first evidence that fine-scale ITS diversity of *Symbiodinium* confers functionality in the symbiosis.

KEY WORDS: Symbiosis · Specificity · ITS-2 types · *Symbiodinium* · Scleractinia

INTRODUCTION

Symbiotic dinoflagellates, engaged in mutualistic associations with a variety of coral-reef dwellers including protists, cnidarians and molluscs, were once thought to be members of a single species (*Symbiodinium microadriaticum* sensu Freudenthal 1962). Over the last 2 decades, however, *Symbiodinium*-like dinoflagellates have been shown to be a diverse group of endosymbionts (Trench 1993, Rowan 1998, Baker 2003). Evidence of morphological, biochemical and, especially, genetic differences now suggest that this genus is indeed a multi-species complex (Schoenberg & Trench 1980a,b, Trench & Blank 1987, Rowan & Powers 1991a,b, Banaszak et al. 1993, LaJeunesse 2001). The description of symbiont molecular diversity has changed remarkably from a report of 3 small subunit-rDNA-restriction fragment length polymorphism (RFLP) types over a decade ago (Rowan & Powers 1991a,b), to hundreds of ITS rDNA genotypes described recently (see Rodriguez-Lanetty 2003 for review, LaJeunesse et al. 2004). Many of these genotypes group within a single major small subunit (ssu)-rDNA Clade *C*, which to date encompasses numerous closely related ITS types, many of which differ by a single base-pair change (LaJeunesse 2002). However, whether or not these *Symbiodinium* ITS types define distinct ecological niches and represent actual independent evolutionary lineages is still unresolved.

In a recent study using a geographical nested-clade analysis, Rodriguez-Lanetty (2003) examined whether the 174 *Symbiodinium* ITS-1 genotypes within the 7 major clades (*A*–*G*) deposited in GenBank by early 2002 represent different evolutionary lineages. The phylogeographical analysis showed that only 23 appear to be genetically independent evolving lineages, which may represent 'ecological types'. Our understanding of these relationships is constantly changing as new data are reported. New phylogeographical evidence suggests that many ITS-2 *Symbio-*

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dinium types are restricted to certain host taxa, depth ranges, and geographic regions (LaJeunesse 2002, LaJeunesse et al. 2003, Santos et al. 2004).

Few studies have attempted to correlate physiology with molecular diversity in *Symbiodinium* dinoflagellates (LaJeunesse 2001). A number of studies have shown photo-physiological differences between strains (not identified with molecular sequence data) of endosymbionts isolated from distinct cnidarian hosts (Chang et al. 1983, Iglesias-Prieto et al. 1997, Perez et al. 2001). More recently, some evidence suggests that the mycosporine-like amino acid production (Banaszak et al. 2000), and algal growth rate under thermal stress (Kinzie et al. 2001) correlate with genetic types of *Symbiodinium* grown in cultures. However, recent data have shown that photo-physiological responses of *in hospite Symbiodinium* do not map directly onto major algal clades (Savage et al. 2002).

A variety of studies have demonstrated host/symbiont specificity during the onset of symbiosis using experimental infection of both adult and larval hosts with homologous and heterologous algae (Schoenberg & Trench 1980c, Fitt & Trench 1983, Colley & Trench 1985, Davy et al. 1997, Weis et al. 2001). None of these studies, however, examined the molecular identity of the algal types. The only recent works to correlate host/symbiont specificity with symbiont genetic identification have been examinations of the onset of symbiosis in juveniles of the gorgonian *Plexaura kuna* (Coffroth et al. 2001), and in adult anemones (Belda-Baillie et al. 2002, Rodriguez-Lanetty et al. 2003). These studies have shown a limited flexibility by these cnidarian hosts in establishing successful symbioses with different major genetic endosymbiont clades (i.e. *A*–*F*). In a study aimed at examining specificity during the early stages of symbiosis between larvae of the scleractinian *Fungia scutaria* and *Symbiodinium*, Weis et al. (2001) found that homologous algae were better able to colonize larvae than were heterologous algae. This differential ability to establish a partnership suggested that there is a recognition process between the partners, which plays a role in the ultimate establishment of a specific host/symbiont combination. However, because the genetic identity was not determined for the algal strains used in the study, the relationship of the different algal types, and therefore the degree of specificity observed in *F. scutaria* larvae, is unknown.

In the interest of determining whether fine-scale molecular diversity of *Symbiodinium* corresponds to functional differences in host/symbiont specificity, this study replicated our previous infection studies in *Fungia scutaria* using known algal ITS-2 types within Clade *C* (described in LaJeunesse et al. [2004] and confirmed in this study). Our results show that algal type is directly related to degree of infection during the onset of symbiosis with *F. scutaria* larvae. This is the first evidence that fine-scale ITS diversity of *Symbiodinium* confers functionality in the symbiosis.

MATERIALS AND METHODS

Gamete collection and larval cultures of *Fungia scutaria*. Adult *Fungia scutaria* were collected from several different patch reefs in the southern-to-middle end of Kaneohe Bay in July 2003 and placed in seawater tables at the Hawaii Institute of Marine Biology on Coconut Island 4 d before full moon. This sexually dimorphic coral species (Kramarsky-Winter & Loya 1998) generally spawns between 17:00 and 19:00 h, 2 to 4 d after full moon (Krupp 1983). Collection and fertilization of gametes were performed as previously described (Schwarz et al. 1999, Weis et al. 2001), except that the fertilized gametes from 3 female corals were kept and cultured separately in 3 l bowls to estimate the individual effect in the algal infection experiments.

Preparation of *Symbiodinium* isolates, genetic typing and infection experiments. Freshly isolated algae were prepared from single individuals of *Fungia scutaria*, *Porites compressa* and *Montipora capitata* as previously described (Schwarz et al. 1999). Algae from each preparation were used in the infection experiments described below and an aliquot was used to determine genetic type. The genetic typing of the symbiotic dinoflagellates was determined from sequences of ITS-2 rDNA amplified from genomic DNA extracts of algal preparations, using specific *Symbiodinium* primers described in LaJeunesse & Trench (2000). Total DNA extraction, PCR amplification, and sequencing were performed following the protocols described in Rodriguez-Lanetty & Hoegh-Guldberg (2003). Searches of ITS-2 homologues were performed in the GenBank database, and then network phylogenetic trees were constructed to resolve *Symbiodinium* identities. Phylogenetic analysis was performed using TCS α 1.13 software developed by Clement et al. (2000).

For infection experiments, 4 d old larvae (approximately 2000) from each female were transferred to each of nine 20 ml dishes, giving a total of 27 dishes (3 individuals × 9 dishes). These dishes were arranged in 3 experimental groups (each group with larvae from 3 corals × 3 replicates). Each group of dishes was inoculated with 1 of the 3 symbiont types (*C*1f, *C*15 and *C*31 algae, from *Fungia scutaria*, *Porites compressa* and *Montipora capitata* coral colonies, respectively) (see Fig. 1). Each dish was inoculated with approximately the same number of symbionts (~1.6×10^6 cells). Before inoculation, the algae were mixed with a few

drops of homogenized *Artemia* sp. to stimulate a feeding response in the larvae as described by Schwarz et al. (1999). After 4 h, larvae from all the dishes were washed and any remaining freshly isolated algae were removed by concentrating the larvae onto a 60 µm-mesh filter and placing them in clean dishes.

Percentage of larvae infected by *Symbiodinium* isolates and algal density per larva. Twenty-four h after inoculation, each larval culture was gently stirred and 3 ml from each dish was collected and fixed in 4% paraformaldehyde — PBS (48 µM NaH_2PO_4, 0.85 mM Na_2HPO_4, 0.12 M NaCl). From the collected samples, 3 separate counts of approximately 150 larvae were made from each of the 27 dishes. The count procedure was the same as that described in Weis et al. (2001), and larvae containing symbionts were scored as infected. Algal density was also quantified within each infected larva.

Statistical analysis. Two-way analyses of variance (ANOVA) were performed using SSPS 9.0 software. The experimental groups (i.e. symbiont types) were assigned as fixed factors, and individual females as random factors. Data expressed as percentages were arcsine-transformed, and data for algal density were log-transformed as heteroscedasticity was encountered with the original data.

RESULTS

The symbiotic dinoflagellates isolated and genetically typed from *Fungia scutaria*, *Porites compressa* and *Montipora capitata* corresponded to *Symbiodinium* Types *C*1f, *C*15 and *C*31, respectively, as illustrated in the phylogenetic network trees in Fig. 1. Homologous *C*1f and heterologous *C*15 from *P. compressa* were closely related, differing at 4 base pairs, and grouped within the same network tree. In contrast, heterologous *C*31 from *M. capitata* resolved in a separate network tree from *C*1f and *C*15, indicating that within Clade *C* this endosymbiont is more distant from *C*1f and *C*15 than the latter from each other. These 2 network trees are not connected, since no plausible linkage among the genes from these 2 trees (with a 95% confidence level) was resolved.

The highest percentage infection (94.6%) was observed in those larvae infected with the homologous *Symbiodinium C*1f (Fig. 2A); however, no significant difference was observed between larvae inoculated with *C*1f and heterologous *C*15 from *Porites compressa* ($p = 0.191$, Tukey's HSD test; Table 1). In contrast, larvae infected with heterologous *C*31 from *Montipora capitata* showed a significantly lower percentage infection (70.8%) than the groups infected with *C*1f and *C*15 ($p = 0.041$, 2-way ANOVA; Table 2, Fig. 2A). No significant individual effect or interaction with the treatment groups was detected in the analysis (Table 2).

Symbiont density per larva was very different between the treatments ($p = 0.001$, 2-way ANOVA; Table 2, Fig. 2B). As with infection percentage, larvae inoculated with homologous symbionts *C*1f had the highest number of algae per planula (22.2 ± 5.7). This density was significantly higher than densities in larvae infected with either of the heterologous symbiont types *C*15 or *C*31 ($p < 0.001$, Tukey's HSD; Table 1). Further, Treatments *C*15 and *C*31 were significantly different from each other ($p = 0.009$, Tukey's HSD; Table 1), with those larvae with *C*31 having the fewest symbionts (3.34 ± 1.6). Within these data, no individual effect or treatment group interactions were detected (Table 2).

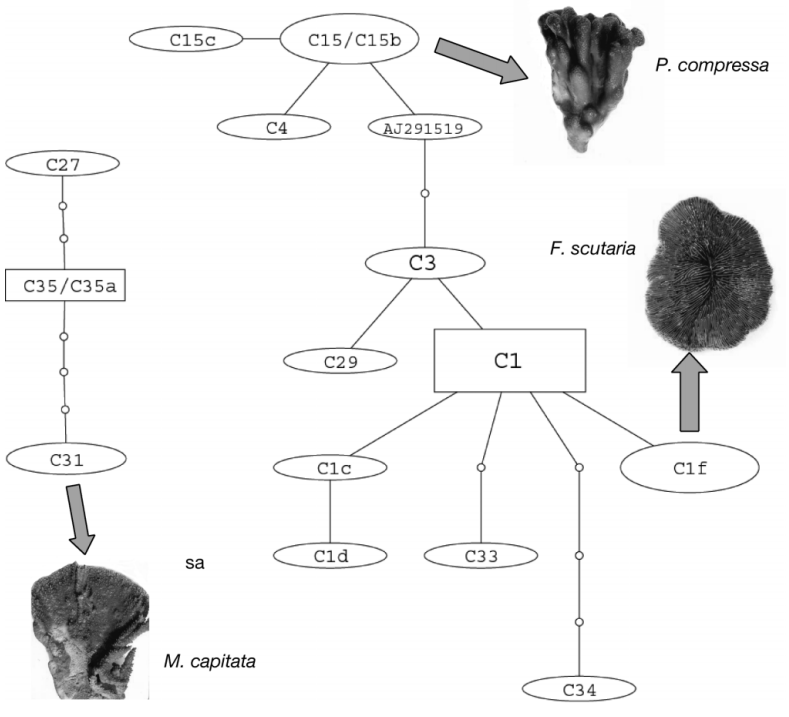

Fig. 1. *Symbiodinium.* Phylogenetic network trees of ITS-2 sequences of isolates from scleractinian corals *Fungia scutaria*, *Porites compressa* and *Montipora capitata*. ITS-2 reference sequences used in the analysis were common *Symbiodinium* members of Clade *C* found in the Hawaiian Archipelago (data from GenBank, submitted by LaJeunesse et al. 2004)

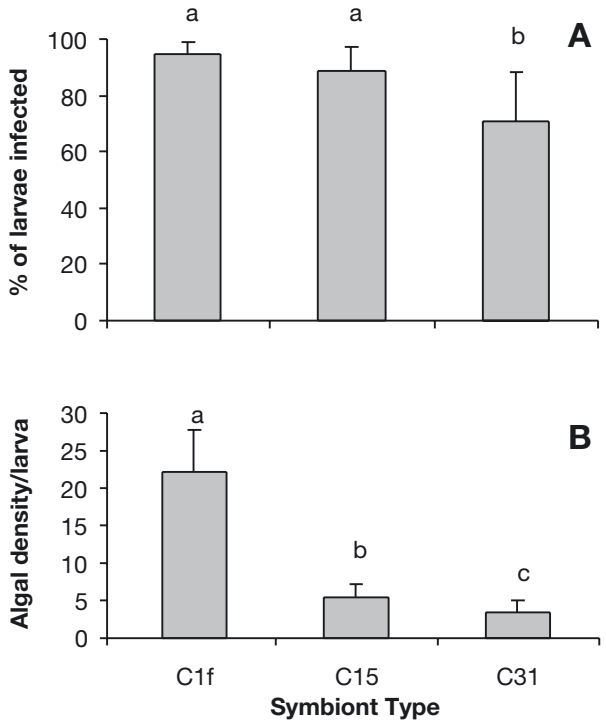

Fig. 2. *Fungia scutaria* larvae infected with different strains of *Symbiodinium*. Infection dynamics. The 3 symbionts used for infection were *Symbiodinium* sp. Clade *C*1f from *F. scutaria*, *C*15 from *Porites compressa* and *C*31 from *Montipora capitata*. Data were collected 24 h after symbiont inoculation. (A) Percentage of larvae infected; (B) algae density per larva. Bars represent means ± SD (n = 27). Different letters above bars indicate significant differences between treatments ($p < 0.05$)

DISCUSSION

This study presents, for the first time, evidence that cnidarian host/*Symbiodinium* specificity during the onset of symbiosis can be correlated to and perhaps determined by genetic differences between endosymbiont types within a major clade. The 3 ITS symbiont types (*C*1f, *C*15 and *C*31) within the major *Symbiodinium* Clade *C* used in our infection experiments engaged differentially in symbiosis with *Fungia scutaria* larvae. The homologous symbiont (*C*1f) showed a significantly better association with host larvae than the other 2 heterologous symbionts (*C*15 and *C*31).

While the dynamics of infection between cnidarian hosts and symbiotic dinoflagellates has been documented in the past (Kinzie & Chee 1979, Schoenberg & Trench 1980c, Fitt & Trench 1983, Colley & Trench 1985, Davy et al. 1997, Weis et al. 2001), none of these studies assessed the actual genetic identity of the endosymbionts. More recently, some genetic studies addressing whether a particular cnidarian host could be manipulated to associate with different types of symbiotic dinoflagellates utilized molecular markers that only discerned the major sub-generic clades of *Symbiodinium*. Coffroth et al. (2001) demonstrated that although aposymbiotic juveniles of the gorgonian *Plexaura kuna* were initially infected in the field by *Symbiodinium* from Clades *A*, *B* and *C*, after 3 mo juveniles harbored exclusively Clade *B* symbionts, the same type found in adult *P. kuna*. Rodriguez-Lanetty et al. (2003) showed, by algal infection experiments with aposymbiotic hosts, that the anemone *Heteractis* sp. established a successful association only with Clade *C Symbiodinium*, at least among the endosymbionts (Clades *C* and *F*) occurring in the study area. Belda-Baillie et al. (2002) demonstrated the presence of a host/symbiont recognition mechanism in the *Aiptasia* sp.-*Symbiodinium* association. Aposymbiotic anemones formed stable associations with freshly isolated or cultured homologous Clade *B* symbionts, but formed less robust associations with cultured strains of Clades *A* and *C* originally isolated from giant clams and corals, respectively.

The preference of *Fungia scutaria* larvae for *C*1f algae is consistent with algal symbiont populations observed in adult *F. scutaria* collected from the field. LaJeunesse et al. (2004) have shown that 7 adults, collected from 2 to 10 m depth, harbored exclusively Type *C*1f. Future studies should examine the infection capability of other symbionts more closely related to *C*1f to further test the idea of fine-scale diversity and its link to specificity. Finally, the expression of specificity between host corals and their horizontally acquired endosymbionts raises doubt about the adaptive bleaching hypothesis (Buddemeier & Fautin 1993, Baker 2001), which is based on the premise of a flexible host/symbiont specificity.

Table 1. *Fungia scutaria* infected with *Symbiodinium*. Tukey's HSD multiple-comparison test among treatments (Symbiont Types *C*1f, *C*15 and *C*31) in both percentage of larvae infected and algal density per larva. *Statistically significant at $p < 0.05$. I–J: mean difference between treatments

Treatment comparison		% larvae infected			Algal density per larva		
I	J	I–J	SE	p	I–J	SE	p
*C*1f	*C*15	0.157	0.0838	0.191	0.616	0.065	<0.0001*
	*C*31	0.466	0.0838	<0.0001*	0.835	0.065	<0.0001*
*C*15	*C*31	0.313	0.0838	0.004*	0.219	0.065	0.009*

Table 2. *Fungia scutaria* infected with *Symbiodinium*. Results of 2-way analysis of variance (ANOVA) for percentage of larvae infected and algal density per larva. Fixed factor = treatment (Symbiont Types: *C*1f, *C*15 and *C*31), random factor = individual female. *Statistically significant at $p < 0.05$

Source	df	% larvae infected				Algal density per larva			
		SS	MS	*F*	p	SS	MS	*F*	p
Intercept	1	31.01	31.010	399.12	0.002	19.44	19.44	446.71	0.002
Error	2	0.155	0.078			0.087	0.0435		
Treatment	2	1.015	0.507	7.85	0.041*	3.37	1.687	50.78	0.001*
Error	4	0.259	0.065			0.133	0.033		
Individual	2	0.155	0.078	1.2	0.390	0.087	0.043	1.31	0.365
Error	4	0.259	0.065			0.133	0.033		
Treat × Ind	4	0.259	0.065	2.044	0.131	0.133	0.033	1.74	0.185
Error	18	0.569	0.031			0.343	0.019		

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